Cell

# Live-Cell Imaging Reveals Replication of Individual Replicons in Eukaryotic Replication Factories

Etsushi Kitamura,[1] J. Julian Blow,[1] and Tomoyuki U. Tanaka[1,*]
[1] School of Life Sciences, University of Dundee, Wellcome Trust Biocentre, Dow Street, Dundee, UK
*Contact: t.tanaka@dundee.ac.uk
DOI 10.1016/j.cell.2006.04.041

## SUMMARY

Faithful DNA replication ensures genetic integrity in eukaryotic cells, but it is still obscure how replication is organized in space and time within the nucleus. Using timelapse microscopy, we have developed a new assay to analyze the dynamics of DNA replication both spatially and temporally in individual *Saccharomyces cerevisiae* cells. This allowed us to visualize replication factories, nuclear foci consisting of replication proteins where the bulk of DNA synthesis occurs. We show that the formation of replication factories is a consequence of DNA replication itself. Our analyses of replication at specific DNA sequences support a long-standing hypothesis that sister replication forks generated from the same origin stay associated with each other within a replication factory while the entire replicon is replicated. This assay system allows replication to be studied at extremely high temporal resolution in individual cells, thereby opening a window into how replication dynamics vary from cell to cell.

## INTRODUCTION

Faithful DNA replication is essential for all prokaryotic and eukaryotic cells to maintain their genetic integrity. DNA replication is initiated at replication origins and proceeds as sister forks from the same origin move along parental DNA in a bidirectional manner. DNA polymerases involved in replicating both leading and lagging strands, together with their accessory proteins, such as replication factor C (RFC) and proliferating cell nuclear antigen (PCNA), are thought to form a large complex (called the "replisome") that moves along with each replication fork (Baker and Bell, 1998; Waga and Stillman, 1998; Johnson and O'Donnell, 2005).

It was originally thought that the two replisomes at sister forks (i.e., initiated from the same origin) would behave independently since they travel in opposite directions along parental DNA. However, it was found that on bacterial circular chromosomes where DNA replication starts from a single defined origin, sister forks move along DNA and normally complete DNA replication with similar timing at a defined region on the chromosome (studied in *Escherichia coli*, *Bacillus subtilis*, etc.; Bussiere and Bastia, 1999). To explain this coordinated termination of DNA replication, it was proposed that two replisomes at sister forks (sister replisomes) remain attached during DNA replication (Dingman, 1974; Falaschi, 2000). This model predicts that template DNA moves into two associated replisomes and newly replicated sister DNA strands are extruded as replication proceeds. Such DNA motion relative to centrally located stationary replisomes (Lemon and Grossman, 1998) was indeed recently confirmed in bacteria (Lemon and Grossman, 2000; Jensen et al., 2001; Lau et al., 2003; Migocki et al., 2004).

In simian virus 40, the large tumor antigen (T-antigen) forms a hexamer that works as a DNA helicase at replication forks (Herendeen and Kelly, 1996). An electron microscopy study revealed that unwound DNA from the viral replication origin forms two single-strand loops, both of which were pinched by the same pair of associated T-antigen hexamers (Wessel et al., 1992). This is also consistent with the model of associated sister replisomes.

In contrast to viruses and bacteria, DNA replication in eukaryotes initiates at multiple replication origins along linear chromosomes. The DNA region replicated by sister forks from a single origin is called a replicon. In contrast to bacterial circular chromosomes, sister forks terminate at two different loci along eukaryotic linear chromosomes, meeting the replication forks of adjacent replicons. In such circumstances, two sister replisomes may operate independently of each other. So, it is sill unclear whether the two sister replisomes are associated with each other in eukaryotic cells.

How is DNA replication spatially organized in the eukaryotic cell nucleus? During DNA replication in vertebrate cells, replisome components such as PCNA and DNA polymerases assemble into dozens of globular foci called replication factories in the nucleus, and it has been shown that new DNA replication takes place within these replication factories (Nakamura et al., 1986; Hozak et al., 1993; Newport and Yan, 1996; Berezney et al., 2000; Frouin et al.,

2003). To account for the number of DNA replication forks generated during S phase, a single replication factory must contain 20–200 DNA replication forks (Berezney et al., 2000). A simple model is that two sister replisomes localize inside the same factory during replication. However, if this is the case, it is still unclear whether sister replisomes are closely associated with each other or stay at a distance within a replication factory, which may have a diameter of up to 1 μm in vertebrate cells (Leonhardt et al., 2000; Somanathan et al., 2001; Sporbert et al., 2002). Moreover, recent data indicate that replication factories continuously assemble and disassemble during S phase (Leonhardt et al., 2000; Somanathan et al., 2001; Sporbert et al., 2002); thus, sister replisomes may sometimes if not always be redistributed between different replication factories.

Here we describe dynamics of both replication factories and individual replicons in budding yeast *S. cerevisiae*, using timelapse microscopy. Replication factories are much smaller in yeast cells than in vertebrate cells and show dynamic behavior during S phase. Crucially, we show that the formation of replication factories is a consequence of DNA replication itself. We have established an assay system to analyze DNA replication of chromosomal loci both spatially and temporally in individual live cells. Using this system, we find evidence that while a replicon is in the process of DNA replication, sister replication forks remain closely associated with each other within a replication factory.

## RESULTS

### Visualizing Replication Factories during S Phase of Budding Yeast

Polymerases α, ε, and PCNA are components of replisomes (Baker and Bell, 1998; Waga and Stillman, 1998; Johnson and O'Donnell, 2005). To visualize replisomes in budding yeast, we tagged *POL1*, *POL2* (the catalytic subunits of polymerases α, ε, respectively) with four tandem copies of green fluorescent protein (GFP) and *PCNA* with yellow fluorescent protein (YFP). All tagging was done at their original gene loci, and all tagged genes were expressed with their authentic promoters.

When diploid cells were not in S phase, only weak globular *POL1-4GFP*, *POL2-4GFP*, and *YFP-PCNA* signals were found in the nucleus (Figure 1A; unbudded cells or cells with medium or large buds). In S phase (small-budded) cells, however, each construct showed up to 12 bright globular signals in the diploid nucleus. The size of each globular signal was 180–280 nm in diameter (230 ± 50 nm; mean ± SD), much smaller than the replication factories of vertebrate cells (Leonhardt et al., 2000; Somanathan et al., 2001; Sporbert et al., 2002). After DNA replication (cells with large buds), Pol1-4GFP, but not Pol2-4GFP or YFP-PCNA, relocalized along the nuclear envelope (see Note S1 in Supplemental Data available with this article online). We obtained similar results using haploid cells with *POL1-4GFP* and *POL2-4GFP*, but the number of bright globular signals was up to eight per nucleus. We demonstrate below that these bright globules of replication proteins represent replication factories where the bulk of DNA synthesis takes place.

### Formation of Replication Factories Is a Consequence of DNA Replication

We next addressed whether formation of the clusters of replication proteins is directly related to DNA replication. When B-type cyclins *CLB5* and *6* are deleted, DNA replication is significantly delayed relative to bud emergence (Schwob and Nasmyth, 1993). In *clb5,6*-deleted cells, the formation of bright globular signals of Pol1-4GFP was also delayed relative to bud emergence (Figure 1B), suggesting that they are formed as a consequence of B type cyclin/cyclin-dependent kinase (CDK) activation in S phase. This notion is reminiscent of observations in vertebrate cells showing that cyclin E-CDK is required for the globular signals of replication protein A (RPA) becoming tightly bound to DNA during S phase (Jackson et al., 1995; Dimitrova et al., 1999).

We next studied Pol1-4GFP and YFP-PCNA localization in Cdc6-depleted cells. Cdc6 associates with DNA replication origins (Tanaka et al., 1997) and is required for replication initiation (Blow and Tanaka, 2005). Cdc6-depleted cells do not replicate DNA but still undergo other cell-cycle events, such as bud emergence and CDK activation (Piatti et al., 1995). We inhibited *CDC6* expression in cells where the only functional *CDC6* was under control of a galactose-inducible promoter (Piatti et al., 1996). After *CDC6* expression was suppressed, small-budded cells never showed bright globular signals of Pol1-4GFP (Figures 1C and S1). Similar results were obtained with YFP-PCNA (data not shown). Taken together, our data indicate that bright globular foci of replication proteins are formed as a consequence of DNA replication.

We then treated cells with the DNA synthesis inhibitor hydroxyurea (HU) from G1 phase (Figure S2). When HU-treated cells started budding (S phase), globular Pol1-4GFP signals became brighter than in G1 phase but remained dimmer than those in small-budded cells in the absence of HU (Figure S2; pink bars). The number of such Pol1-4GFP globules was generally not more than two to three in HU-treated cells. The results are consistent with HU limiting DNA replication to the vicinity of early firing origins (Santocanale and Diffley, 1998).

Subsequently, we followed the behavior of bright globular signals of Pol1-4GFP with timelapse microscopy. During observation over 30 s, the globular signals showed significant changes in their shape and location, indicating dynamic behavior of replication factories (Figures S3 and S4; see Note S2).

### DNA Replication of Chromosomal Loci Observed in Individual Live Cells

We next established an assay system for the replication of specific chromosomal loci in individual live cells. In budding yeast, a genome-wide study previously revealed the replication timing of all chromosomal loci (Raghuraman et al., 2001). We chose for further study a single large

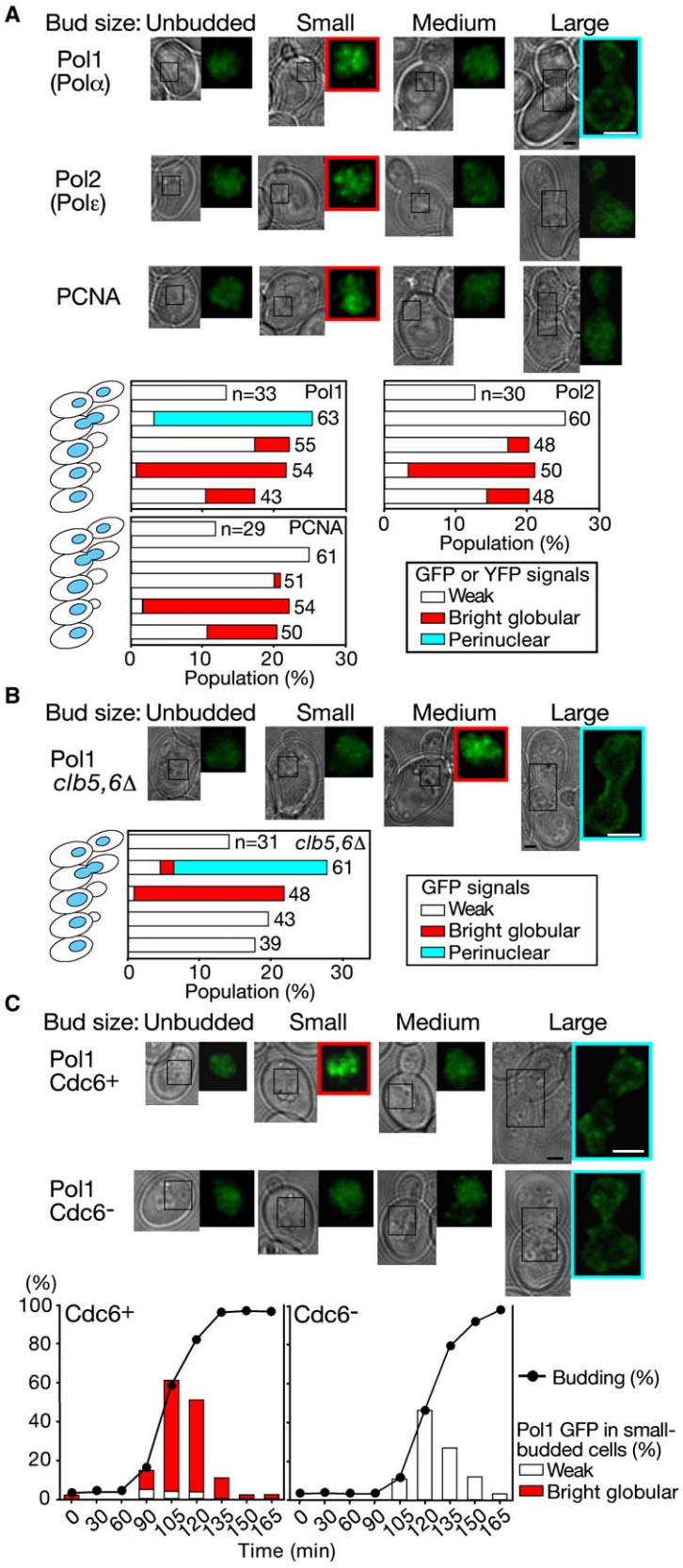


**Figure 1. Replication Factories in Budding Yeast**

(A) Replication factories are found specifically during S phase. Homozygous *POL1-4GFP* (T3030), homozygous *POL2-4GFP* (T3031), and heterozygous *YFP-PCNA/PCNA*$^{+}$ (T3060) diploid cells were cultured asynchronously. Top: representative images of bright-field and fluorescence, classified by the bud size. Scale bar: 2 μm. Bottom: percentages of cells with classified bud sizes, showing the fraction of fluorescence with a bright globular (red) or perinuclear (pale blue) pattern. n: numbers of observed cells with each classified bud size.

(B) Formation of replication factories is dependent on B type cyclin-CDKs. *POL1-4GFP clb5Δ clb6Δ* (T3262) homozygous diploid cells were cultured asynchronously and observed as in (A).

(C) Formation of replication factories is dependent on DNA replication. *POL1-4GFP GAL-CDC6 cdc6Δ* (T3264) homozygous diploid cells were grown in galactose plus raffinose-containing medium. After culture in raffinose-containing medium lacking galactose for 2.5 hr, small unbudded cells were collected by elutriation and incubated (start of incubation: 0 min) in either galactose/raffinose- (Cdc6$^{+}$) or glucose (Cdc6$^{-}$) containing medium. Top: representative images of bright-field and fluorescence, classified by the bud size. Bottom: percentages of cells with buds (black line) and of small-budded cells with weak (white bar) or bright globular (red bar) Pol1 GFP signals. See Figure S1.

replicon on chromosome IV. We selected two loci, 60 kb apart and on opposite sides of the replication origin, which are replicated at similar times (Figure 2A). We then marked one of these loci with an array of 224 *tet* operators (*tetOs-1*) and marked the other with an array of 256 *lac* operators (*lacOs*) (Straight et al., 1996; Michaelis et al., 1997). To minimize potential perturbation of the replication profile, only the *tet* and *lac* operator arrays, each ~11 kb in size, were inserted, but no other DNA sequences, such as plasmid backbones, were inserted. The *tetO* array can be bound by a fusion protein of the Tet repressor plus three tandem copies of cyan fluorescent protein (TetR-3CFP; Bressan et al., 2004), while the *lacO* array can be associated with a fusion protein of the *lac* repressor plus GFP (GFP-lacI, Straight et al., 1996). Using strains expressing TetR-3CFP and GFP-lacI, we could visualize these chromosomal loci as small CFP and GFP dots.

When these dots were observed in asynchronous cell cultures, their fluorescence intensity was approximately doubled in cells with medium-size buds (before sister-chromatid separation in anaphase) when compared to unbudded cells (Figure S5). The dot intensity did not increase if DNA replication of these loci was inhibited by HU (Figure S6). We reasoned that DNA replication doubled the number of *tet* and *lac* operators, which led to recruitment of more TetR-3CFP and GFP-lacI fusion proteins. Consistent with this idea, both loci in individual cells doubled in intensity at approximately the same time during the cell cycle (Figure 2B). A slight difference in replication timing of the two loci, which was seen in a few cells, is expected to occur due to differences in fork velocity or if the replication origin between the two loci failed to fire.

How long does it take for each dot to become brighter after the locus replicates? To address this, we compared the kinetics of changes in the dot intensity (Figure 2B) with incorporation of the thymidine analog 5-bromodeoxyuridine (BrdU), into DNA at each locus, during the cell cycle (Figures 2C and 2D). BrdU incorporation was analyzed by chromatin immunoprecipitation of sheared and denatured DNA, using an antibody against BrdU (Pichler et al., 1997), followed by PCR amplification of the two loci plus the replication origin lying between them (Figures 2C and 2D).

As expected from the previously published timing data (Raghuraman et al., 2001), the *tetOs-1* and *lacOs* sites showed BrdU incorporation in late S phase about 20 min later than the replication origin (Figures 2D and 2E). Notably, BrdU incorporation and the increase in dot intensity showed almost identical kinetics for both *tetOs-1* and *lacOs* integration sites (Figures 2B and 2D), suggesting that *tetOs-1*-CFP and *lacOs*-GFP dots became brighter very soon after they were replicated. We can therefore use the intensity of *tetOs-1*-CFP and *lacOs*-GFP dots as a readout of replication of these loci.

## DNA Replication of Chromosomal Loci Observed at Replication Factories

Having established techniques for visualizing replication factories and for visualization of the replication of specific

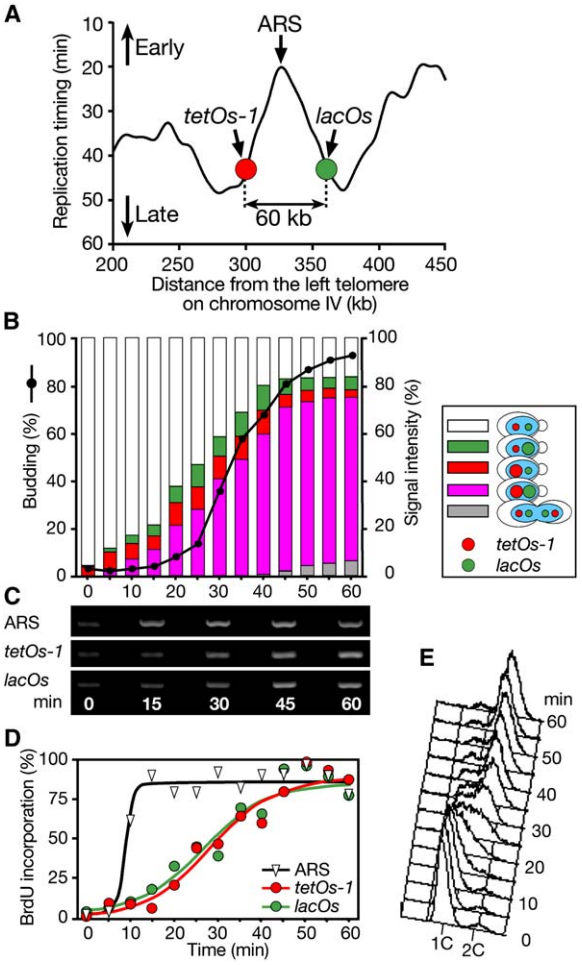


**Figure 2. Determining Replication Timing by Timelapse Microscopy**

(A) Map of the integration sites of *tet* and *lac* operators. The replication timing profile of the chromosome region was taken from Raghuraman et al. (2001).

(B–E) *MATa* $P_{GPD1}$*-thymidine kinase* (five copies) $P_{ADH1}$*-ENT1 tetR-3CFP GFP-lacI* haploid cells with *tetO×224* and *lacO×256* (as indicated in A: T3765) were arrested by α factor treatment, washed, and released in BrdU-containing medium. After 20 min, samples were collected for time point 0 min, and other time-point samples were subsequently collected. (B) The budding index and the intensity of each *tetOs-1* (red) and *lacOs* (green) dot of individual cells at different times are shown. Key: dimmer and brighter dots are schematically shown by small and large dots, respectively. The intensity of the dots was investigated in 128–300 cells at each time point. (C) BrdU-labeled DNA was amplified by PCR using primers for the ARS (replication origin) and *tetOs-1*/*lacOs* integration sites. Throughout the time course, DNA template derived from the same number of cells was used for PCR amplification. If no BrdU was added to the culture, there was no increase observed in the PCR products while the budding index increased (data not shown). If cells were treated with HU, there was an increase observed in PCR products for the ARS, but not for the *tetOs-1* or *lacOs* site, while the budding index increased (data not shown). (D) Quantification of the intensity of the bands in (C). The intensity at time point 0 and the maximum intensity were set to 0 and 100%, respectively. (E) Cellular DNA content was measured by FACS.

chromosomal loci, we were in a position to test whether replication actually occurs within the factories. To this end, we created a strain combining Pol1-4GFP, TetR-3CFP, and the *tetO* array (without the *lacO*s dot). If DNA at the *tetO* array replicates in one of the replication factories, the intensity of the dot would be expected to increase when it colocalizes with a bright globular Pol1-4GFP signal (Figure 3A). To test this hypothesis, we measured the intensity of the *tetOs-1*-CFP dot and its three-dimensional position relative to the Pol1-4GFP signal in cells undergoing replication of this locus.

We observed 11 cells in which their *tetOs-1*-CFP dot increased in its intensity during observation, and in all these cells the dot colocalized with one of the bright globular Pol1-4GFP signals for a period of 2–7 min when the dot intensity started to increase (Figures 3B and S7). The peak of colocalization frequency was found 1 min prior to replication time, defined as the midpoint of the increase in the dot intensity on the regression curve (Figure 3C). The number and size of replication factories did not increase when the *tetO* array was replicated (Figure S8; the gradual decrease of their signals is probably due to photo bleaching during timelapse microscopy), suggesting a specific colocalization of the two structures. Colocalization was occasionally observed at times when the *tetO* array was not in the process of replication (Figures 3C and S7), but this occurred at a frequency below that expected by chance, given that the globular Pol1-4GFP signal occupies about a third of the nuclear volume (32.8% ± 3.8%). This suggests that when the *tetO* array was not actually in the process of DNA replication, it was excluded from replication factories. Similarly, when replication of the *tetO* array was not observed during the timelapse experiment, the *tetO* array only occasionally colocalized with bright globular Pol1-4GFP signals (Figure S9).

These results suggest that bright globular Pol1-4GFP signals, found specifically during S phase, are the sites of DNA replication and indeed represent replication factories. We can therefore visualize replication of chromosomal loci at replication factories by time-lapse microscopy.

## Evidence that Sister Replication Forks are Closely Associated during Replication

The method we describe here allows us to determine which chromosomal loci are replicated in close proximity to one another at particular replication factories. In particular, we wanted to test the hypothesis that sister replication forks, generated from the same origin, are associated with each other during replication (Dingman, 1974; Falaschi, 2000). This hypothesis predicts that, as replication proceeds, template DNA enters an associated double replisome located within a replication factory, and newly synthesized DNA is then extruded from it (Figure 4A). To address this point we used the strain described above, where the *tetO* and *lacO* arrays were integrated on either side of a replication origin in a single large replicon (Figure 4A; *tetOs-1* and *lacOs*). If the two sister forks from the central origin stay together at an associated double

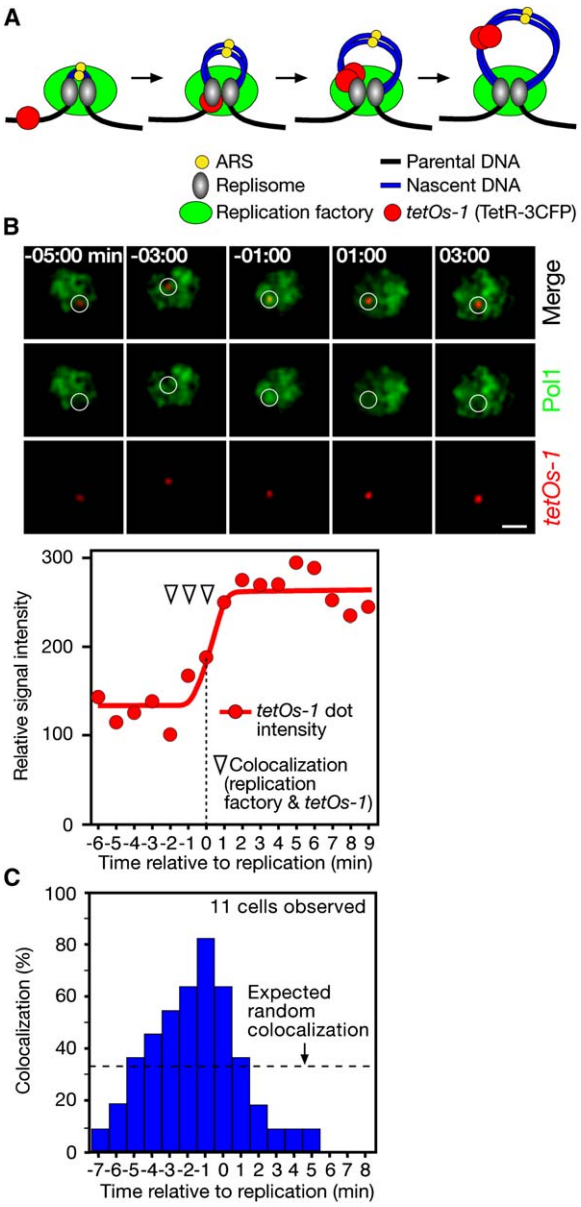


**Figure 3. DNA Replication of Chromosomal Loci in Replication Factories**

(A) Model of a closely associated double replisome at a replication factory, and the expected behaviors of *tetOs-1*-CFP dots.

(B and C) *MATa POL1-4GFP tetR-3CFP* haploid cells with *tetO×224* (*tetOs-1*: as indicated in Figure 2A, but without the *lacOs* dot: T3639) were arrested by α factor treatment, washed, released into fresh medium, and observed by timelapse. Representative images (B, top) of Pol1-4GFP signals and *tetOs-1*-CFP dots, and fluorescence intensity of the dot during observation (B, bottom). Time is shown relative to replication (0 min, midpoint of the increase in the dot intensity on the regression curve). Inverted triangles indicate time points when colocalization between the *tetOs-1*-CFP dot and Pol1-4GFP bright globular signals (replication factories) was found. Shown is the percentage of cells (out of all observed cells) showing such colocalization at each time point (relative to replication; C). Scale bar: 1 μm. For individual data of all observed cells, see Figure S7.

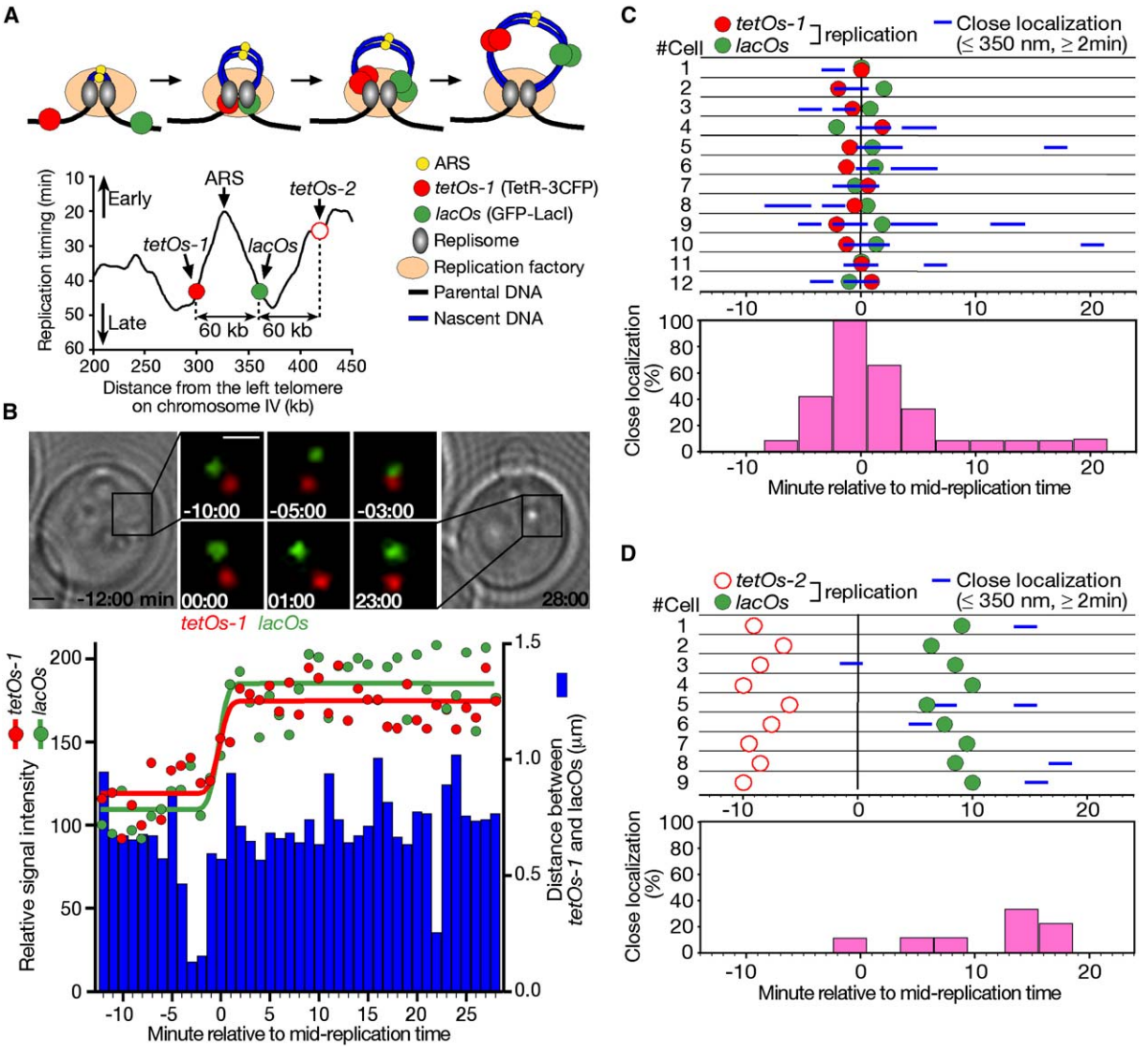


**Figure 4. Behavior of Two Chromosomal Loci on the Opposite Sides of a Replicon with Similar Replication Timing**

(A) Model of a closely associated double replisome at a replication factory, and expected behavior of two chromosomal loci (*tetOs-1* and *lacOs*; top) whose positions are shown below. The position of a control locus *tetOs-2* is also indicated.

(B) *MATa tetR-3CFP GFP-lacI* haploid cells with *tetO×224* (*tetOs-1*) and *lacO×256* (*lacOs*: as indicated in A: T3580) were arrested by α factor treatment, released into fresh medium, and observed by time-lapse microscopy. CFP (red), GFP (green), and bright field images of a representative cell (top) are shown. Also displayed are signal intensity of *tetOs-1* (red) and *lacOs* (green) dots and distance between the two dots (blue bars at the bottom). Time is shown relative to midreplication time (see text). Scale bar: 1 μm.

(C) Replication time of *tetOs-1* and *lacOs* dots was measured as in (B) and shown in closed red and green circles, respectively, for each cell (top). Time is shown relative to midreplication (see text). Times of close localization of the two dots (≤ 350 nm, ≥ 2 min) are indicated by a blue line (top). The percentage of cells with close localization of the two dots is shown for each time window by a pink bar (bottom). Images of cell 1 were shown in (B). For individual data of all observed cells, see Figure S10.

(D) *MATa tetR-3CFP GFP-lacI* haploid cells with *tetO×224* (*tetOs-2*) and *lacO×256* (*lacOs*: as indicated in A: T3959) were treated as in (B). Replication time of the two dots is shown in open red (*tetOs-2*) and closed green (*lacOs*) circles for each cell (top). Percentage of the cells showing close localization of the two dots is shown as in the bottom of (C). For individual data of all observed cells, see Figure S12.

replisome during replication, then *tetO* and *lacO* arrays would be brought together when they are replicated and would then separate as they are extruded from the replisomes (Figure 4A).

With time-lapse microscopy, we observed 12 yeast cells in which the *tetOs-1* and *lacOs* dots increased their intensity during the observation period (Figures 4B, 4C, and S10). As expected from Figure 2, the two loci

replicated at very similar times, with a difference of only 2.0 ± 1.4 min (mean ± SD). We standardized the observation times to the midreplication time, defined as the mean replication time of the two dots. An example is shown in Figure 4B; 2–3 min before the midreplication time, the two loci moved very close together (120–150 nm) for two consecutive time points just before the dot intensity started to increase.

All cells observed showed a close localization of the two dots (separation ≤ 350 nm) for 2 min or longer, 0–3 min before the midreplication time (Figure 4C). Although close localization of the two dots was occasionally found at other times, a peak of frequency in close localization of the two dots was evidently at −2 to 0 min relative to midreplication time of the two dots (Figure 4C).

When more stringent thresholds were set to judge close localization, the peak of frequency in close localization of the two dots was still found at -2 to 0 min, but the height of the peak became lower (Figure S11A). We assume that the *lacOs*-GFP and *tetOs-1*-CFP dots did not always come very close (e.g., < 200 nm) around midreplication time because their replication time was never exactly the same, and it was more different in some cells than in others. In fact, there was a correlation between the synchrony of replication and the proximity of the loci (95% confidence, r = 0.58), so that if two dots showed larger differences in replication time, they tended to show larger minimum distances around their midreplication time (see Note S3 for more details).

To confirm further the close localization of the two dots upon replication, we also measured their median distance at each time point (relative to midreplication time) among the 12 cells (Figure 5A). Although taking median distance among different cells significantly masked the minimum separation at any particular time, the median distance was indeed smaller, especially at −2 to +1 min relative to midreplication time. These data suggest that the *tetOs-1* and *lacOs* dots are in closer proximity when DNA replication takes place at these loci and that they move apart from each other after replication.

To address whether close localization of the two loci upon replication is dependent on their particular positions within the replicon, we made a control strain where the array of *tetOs* was at a different site, 60 kb from the array of *lacOs*, but in a direction away from the replication origin (*tetOs-2*; Figure 4A). From the previously published genome-wide replication study (Raghuraman et al., 2001), we would expect *tetOs-2* to replicate significantly earlier than *lacOs*. We observed nine cells in which the *tetOs-2* and *lacOs* dots increased their intensity during the observation period (Figure S12). As expected, the *tetOs-2* dot showed an increase in its intensity 14–20 min earlier than the *lacOs* dot. In contrast to the *tetOs-1* dot, the *tetOs-2* dot did not show frequent close localization with the *lacOs* dot around the midreplication time (Figures 4D and S11B). The median distance between the two dots was not significantly smaller around the midreplication time (Figure 5B).

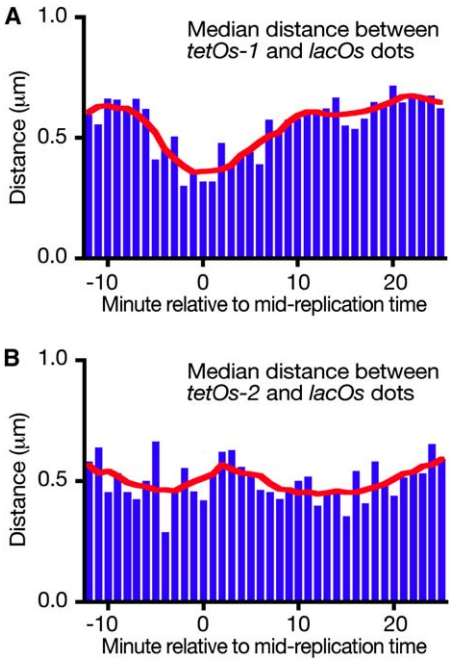


**Figure 5. Median Distance between *tetOs-1* and *lacOs* Dots Is Reduced around Midreplication Time**

(A) Median distance (blue bar) between *tetOs-1* and *lacOs* dots at each time point among 12 cells shown in Figures 4C and S10.

(B) Median distance (blue bar) between *tetOs-2* and *lacOs* dots at each time point among nine cells shown in Figures 4D and S12. The red lines show smoothed curves. Time is shown relative to midreplication time.

## Localization and Replication of Two Chromosomal Loci on the Same or on Separate Homologous Chromosomes In Diploid Cells

The above experiments revealed that two chromosomal loci on opposite sides of a single replicon with similar replication timing show close localization as they replicate. We next addressed whether such close localization was found even if the two loci were on separate homologous chromosomes. For this purpose, we made two kinds of diploid yeast strains. In diploid strain A, the *tetOs-1* and *lacOs* dots were on separate homologous chromosomes, while in diploid strain B they were on the same chromosome (Figure 6A). We analyzed seven diploid A cells and five diploid B cells where the two dots increased in intensity during the observation period (Figures 6B and S13).

The *tetOs-1* and *lacOs* loci showed similar replication timing in diploid A cells (difference: 3.8 ± 2.8 min) and in diploid B cells (difference: 2.4 ± 1.0 min). However, in three diploid A cells (cells 2, 3, and 5), the difference in replication timing was 5 min or larger, which seldom happened in either diploid B cells or in haploid cells (Figures 4C and 6B). This was probably due to differences in the timing of replication initiation from the two relevant origins on the homologous chromosomes.

In all diploid B cells, as in the haploid cells, the *tetOs-1* and *lacOs* loci closely localized at about the time both loci replicated (Figure 6B). By contrast, in none of diploid A

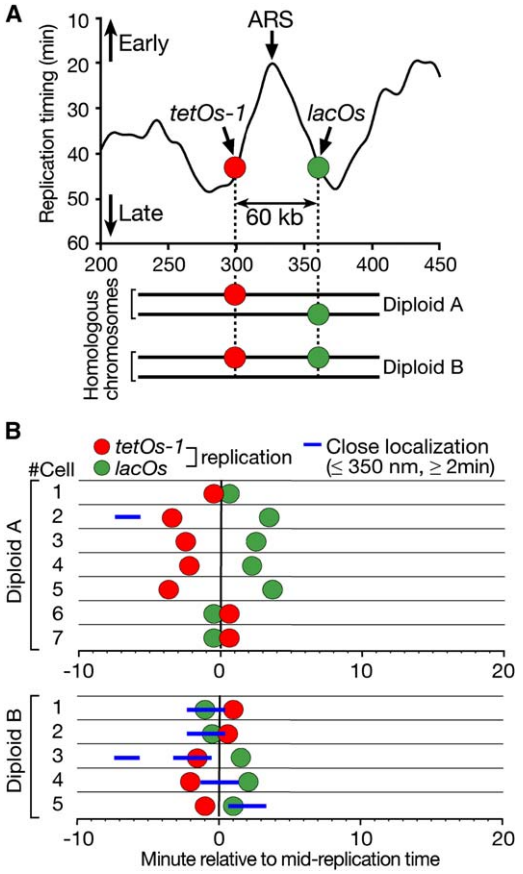


**Figure 6. Behavior of Two Loci on the Same or on Homologous Chromosomes in Diploids**

(A) *MATa/a* diploid A (T4275) and B (T4277) cells harboured *tetO×224* (*tetOs-1*) and *lacO×256* (*lacOs*) on homologous chromosomes and on the same chromosome, respectively, as indicated schematically. Both strains also had one copy of *tetR-3CFP* and *GFP-lacI*.

(B) The *MATa/a* diploid A and B cells were arrested by α factor treatment, released to a fresh medium, and observed by timelapse microscopy, as in Figure 4B. Time of replication at *tetOs-1* (red circles) and *lacOs* (green circles) and times of close localization (≤ 350 nm, ≥ 2 min) between the two dots (blue lines) are shown for each diploid A and B cell. For individual data of all observed cells, see Figure S13.

cells was close localization of the two dots observed around the midreplication time of the two dots. Even when the two loci replicated at a very similar time in the diploid A cells (cells 1, 6, and 7 with a replication timing difference of 1–2 min), their close localization was not observed. Although some diploid A cells had a larger distance between the *tetOs-1* and *lacOs* dots before DNA replication than did diploid B cells, this was not the sole reason for the lack of close localization upon replication of the two dots in diploid A. In fact, a couple of diploid A cells (Figure S13; diploid A cells 2 and 3) had a separation of the two dots as small as diploid B cells (400–800 nm) before DNA replication, but they still did not show close localization around midreplication time.

These results show that only when the two loci are on the same chromosome (and thus within the same replicon) do they consistently show close localization as they replicate. Replication of two chromosomal loci with a similar timing does not necessarily lead to their close localization upon replication.

### Global Sites of DNA Replication Associated with Replication Factories

We then studied how newly synthesized DNA globally localizes in the nucleus relative to replication factories. Previous studies showed that new DNA synthesis, visualized by incorporation of BrdU, etc., takes place at multiple foci in the budding yeast nucleus (Pasero et al., 1997; Lengronne et al., 2001; Hiraga et al., 2005). We incubated *POL1-4GFP* cells with BrdU for 3 min, the minimum incubation time after which BrdU incorporation into chromosomes can be detected. After the lysis of cells, chromosomes were fixed on glass slides, and Pol1-4GFP and incorporated BrdU were visualized by immunostaining. BrdU incorporation was observed in a subset of nuclei with bright globular Pol1-4GFP signals, which correspond to S phase nuclei. In such nuclei, most, if not all, of the BrdU signals were found colocalized or closely associated with bright (and some weak) globular Pol1 signals (Figure S14). The BrdU signals, which were adjacent to, rather than colocalized at, globular Pol1 signals, may be a consequence of newly synthesized DNA spooling out of replication factories. If so, newly synthesized DNA must have moved up to 350 nm or even more in 3 min after replication. This is consistent with our estimation, based on time-lapse analyses of the two chromosomal loci, that replicated DNA can move away from a replication factory at a maximum speed of 250 nm/min (Figure 4B and Note S4).

## DISCUSSION

By marking individual chromosomal loci with bacteria-derived operators, we have developed a new assay to study the dynamics of DNA replication with timelapse microscopy. For temporal studies of DNA replication, conventional methods (e.g., FACS DNA-content analyses [Amberg et al. 2005]; density-shift analyses of isotope-labeled DNA [Raghuraman et al., 2001]; and immunoprecipitation of BrdU-incorporated DNA [Pichler et al., 1997]) have used cells cultured synchronously in the cell cycle. However, it is not technically possible to obtain cells in perfect synchrony. Further, even in perfectly synchronized cells, many replication processes may have a significant stochastic element. For example, because not all origins fire in every cell cycle, some origins may fire in some cells but not others; similarly, particular origins may fire at different times in S phase in different cell cycles (Schwob, 2004; Machida et al., 2005). This intercell variation is a major obstacle to understanding the dynamics of DNA replication using population-based assays. Because our assay analyzes timing of DNA replication in individual cells, much greater temporal resolution can be obtained than

by the conventional methods, and intercell variation can be directly monitored. Moreover, by simultaneously following intensity and localization of marked chromosomal loci, chromosome duplication can be studied both spatially and temporally in individual live cells.

Using this novel assay, we have studied how individual replicons are processed for DNA replication. We discovered that two chromosomal loci, having the same replication timing but located at the opposite sides of the same replicon, move close together upon DNA replication and separate from each other afterwards. Our discovery strongly supports a long-standing hypothesis, which has however lacked firm evidence in eukaryotic cells, that sister replication forks generated from the same origin stay associated with each other during replication of the relevant replicon (Dingman, 1974; Falaschi, 2000). In prokaryotic cells, recent evidence also supports the idea that the two replisomes stay together in the middle of the cell during chromosomal DNA replication (Lemon and Grossman, 2000; Jensen et al., 2001; Lau et al., 2003; Migocki et al., 2004).

The second advance demonstrated in our study is visualization of replication factories in *S. cerevisiae*. Previous studies using fixed nuclei have suggested that replication proteins in budding yeast are clustered together at potential sites of DNA synthesis (Ohya et al., 2002; Hiraga et al., 2005). Similarly, observations using a cell-free replication system suggested that DNA synthesis occurs in small globular foci within yeast nuclei (Pasero et al., 1997). We show here that replication factories are formed in globular patterns specifically during S phase of the budding yeast, and they colocalize with sites of DNA synthesis. This is similar to factories observed in vertebrate cells (Hozak et al., 1993; Frouin et al., 2003), though the yeast foci have a smaller size. A recent report also suggested that fission yeast GFP-PCNA shows bright globular signals specifically in S phase nuclei (Meister et al., 2005).

We show that factory formation in yeast is dependent on S phase-cyclin CDKs (S-CDKs) as it is in vertebrate cells (Jackson et al., 1995; Dimitrova et al., 1999). We also addressed whether replication factory formation is dependent on DNA replication itself, rather than being independent events both regulated by S-CDKs. We found that factory formation was dependent on Cdc6, which is required for DNA replication initiation but not for progression of a cell cycle (CDK activation, etc.). Therefore, replication factory formation is likely to be a consequence of DNA replication in budding yeast. This is probably also the case in vertebrate cells because the loading of replication fork proteins onto DNA during S phase in *Xenopus* is dependent on the DNA having previously been licensed, which in turn is dependent on Cdc6 activity (Jares and Blow, 2000; Mimura et al., 2000). Because replisomes are assembled at replication origins dependent upon S-CDK activation, Cdc6, and origin unwinding (Tanaka and Nasmyth, 1998), we suggest that replication factories are mainly, if not exclusively, composed of replisomes at advancing replication forks. Our data are not readily consistent with formation of factories by replisome components prior to DNA replication.

In contrast to our results, a previous study using immunostaining of fixed nuclei detected globular patterns of Pol2 and Pol3 (a catalytic subunit of polymerase δ) in the nucleus throughout the cell cycle (Ohya et al., 2002). Such signals might correspond to the weak globular Pol2 (and Pol1, PCNA) signals that we also observed throughout the cell cycle. In fact, we found that after fixing cells for immunostaining, the S phase-specific bright globular signals of Pol1, Pol2, and PCNA were less evident (data not shown). On the other hand, it is unlikely that the bright globular *POL1-4GFP*, *POL2-4GFP*, and *YFP-PCNA* signals in S phase of live cells were artifacts of GFP/YFP tagging because (1) such signals were strictly dependent on DNA replication, (2) new DNA synthesis occurred specifically at these signals, and (3) *POL2-4GFP* bright globular signals were reduced in DNA primase mutants (data not shown).

Our observations of replication factories in budding yeast raise questions about whether multiple replicons are processed in a single replication factory at a given time during S phase, as in vertebrate cells, and, if so, how many replicons are processed simultaneously. A genome-wide replication profile in *S. cerevisiae* suggested that up to 80 replicons are undergoing replication at any given time in S phase of haploid cells (Raghuraman et al., 2001). Because we observed up to eight replication factories in haploid cells, this suggests that an average of ten replicons are processed in each factory at any given time. However, this number could be highly variable as factories have a wide range of brightness when visualized with GFP.

Obviously, budding yeast cells process a lower number of replicons in a single replication factory than do vertebrate cells (where 10–100 replicons are processed per factory). This difference accounts for the different size of replication factories between budding yeast (180–280 nm) and vertebrate cells (up to 500 nm–1 μm; Leonhardt et al., 2000; Somanathan et al., 2001; Sporbert et al., 2002) when visualized with GFP. Replication factories show more dynamic behavior in budding yeast, presumably because factories containing a smaller number of replicons can change their shapes and sizes more readily as replicons join and leave factories as they initiate and terminate replication. It will be intriguing to address using our experimental system which replicons are processed in the same factory, the timing by which these replicons join the factory, and how these processes could differ from cell to cell.

Why should cells keep sister replisomes closely associated, and why do they assemble groups of replisomes in factories? One possible benefit might be to avoid only half of a replicon being replicated. Provided that replication origins lose their ability to initiate replication once the origin is unwound and replication forks are generated (Blow and Dutta, 2005), a half replicon might fail to replicate if one replisome could initiate replication without

waiting for the other replisome to be loaded onto the origin. In addition, associated sister forks may coordinate the DNA polymerase operation for two leading and two lagging strands to avoid chromosome entanglement and to facilitate smooth reeling in and out of unreplicated and replicated DNA strands (Falaschi, 2000). Moreover, the assembly of multiple replisome pairs into a replication factory may be a way to increase the local concentration of DNA components (e.g., deoxyribonucleotides) and/or replication accessory proteins (e.g., PCNA and RPA), which could lead to more efficient DNA replication. Furthermore, cohesion between sister chromatids is also established during S phase (Uhlmann and Nasmyth, 1998). Proteins involved in the establishment of cohesion might be also concentrated in replication factories, which may account for coupled DNA replication and cohesion establishment.

To address possible benefits of colocalizing replisomes in factories, we need to intervene in these structures without interfering with other processes, and we need to follow the outcome. Such studies will be facilitated by use of the technology that we describe here.

## EXPERIMENTAL PROCEDURES

### Yeast Genetics and Molecular Biology

Yeast strain background (W303), methods for yeast culture and for cell-cycle synchronization using α factor treatment or elutriation, and the TetR-GFP/*tet* and GFP-lacI/*lac* operator system were described previously (Piatti et al., 1996; Straight et al., 1996; Michaelis et al., 1997; Tanaka et al., 1997; Amberg et al., 2005). Cells were cultured at 25°C in YP medium containing glucose unless otherwise stated.

*POL1* and *POL2* were tagged with four tandem copies of GFP at their C termini, and *PCNA* was tagged with YFP at its N terminus. All tagging was done at the original gene loci by PCR methods as previously described (Prein et al., 2000; Maekawa et al., 2003), and all tagged genes were expressed with their authentic promoters. *POL1*, *POL2*, and *PCNA* are all essential for DNA replication and cell growth (Sugino, 1995; Waga and Stillman, 1998). Haploid cells containing *POL1-4GFP* and *POL2-4GFP* grew normally, suggesting that these tagged genes were functional. *YFP-PCNA* severely retarded haploid cell growth, indicating that the tagged construct was not fully functional. Therefore, we used haploid or homozygous diploid cells containing *POL1-4GFP* or *POL2-4GFP* and diploid cells containing heterozygous *YFP-PCNA* (i.e., *PCNA* on the other homologous chromosome is not tagged), all of which showed normal growth.

To integrate *tetO×224* and *lacO×256* arrays into chromosomal loci (*tetOs-1*, *tetOs-2*, and *lacOs*) as shown in Figures 2A and 4A,*K. lactis URA3* was first inserted at noncoding regions between *YDL089w* and *YDL088c*, *YDL020c* and *YDL019c*, and *YDL055c* and *YDL054c*, using a one-step PCR method (Amberg et al., 2005). Two 650–700 bp DNA fragments flanking the insertion sites were amplified by PCR and cloned into the pUC18 plasmid (GenBank/EMBL accession number L09136). *tetO×224* and *lacO×256* arrays were inserted between the two DNA fragments. Using these constructs, *K. lactis URA3* was replaced with *tetO×224* and *lacO×256* arrays by negative selection against *URA3* on culture plates containing 5-fluoroorotic acid (Amberg et al., 2005). Correct insertion of *URA3* and subsequent replacement were confirmed by PCR amplification of relevant chromosomal regions. TetR-3CFP and GFP-lacI constructs were as described previously (Straight et al., 1996; Bressan et al., 2004). *MATa/a* diploid cells were made by expressing the *HO* gene from the *GAL1-10* promoter in *MATa/α* diploid cells (Herskowitz and Jensen, 1991).

To facilitate BrdU incorporation (Vernis et al., 2003), five copies of the herpes simplex thymidine kinase gene were expressed from *GPD1* promoters (Dahmann et al., 1995), and the human equilibrative nucleoside transporter 1 (*ENT1*) gene (its codon usage was optimized for yeast) was expressed from *ADH1* promoter (K Shirahige *et al.*, personal communication).

### Microscopy and Image Analyses

The general procedures for time-lapse microscopy were described previously (Tanaka et al., 2005). Time-lapse images were collected at indicated time intervals at 23°C (ambient temperature) unless otherwise stated. Using the Deltavision microscope (Applied Precision), we acquired 5–9 (0.7 μm apart) z-sections, unless otherwise stated, which were subsequently deconvoluted and projected to two-dimensional images using SoftWoRx (Applied Precision) and Volocity (Improvision) software. To distinguish GFP and CFP signals, the JP4 filter set (Chroma) was used. GFP and CFP signals were quantified using Volocity. Replication factories were judged to colocalize with *tetOs* dots when Pol1-4GFP bright globular signals overlapped with *tetOs*-CFP dots on the focal plane for *tetOs*-CFP dots for 2 min or longer. To measure the distance between *tetOs*-CFP and *lacOs*-GFP dots, the distance between their centers was quantified in three-dimensional space.

### Other Methods

FACS DNA-content analyses, BrdU incorporation, and subsequent chromatin immunoprecipitation and indirect immunostaining of epitope tags and incorporated BrdU were as described previously (Pichler et al., 1997; Dimitrova et al., 1999; Lengronne et al., 2001; Amberg et al., 2005). Find more methods in Note S5.

### Supplemental Data

Supplemental Data include 5 notes and 14 figures and can be found with this article online at http://www.cell.com/cgi/content/full/125/7/1297/DC1/.

## ACKNOWLEDGMENTS

We thank M.K. Raghuraman, A.D. Donaldson, S. Hiraga, E. Schwob, J.R. Swedlow, and all members of the Tanaka and Blow laboratories for discussions; L. Clayton for reading the manuscript; K. Shirahige, K. Nasmyth, R. Ciosk, S. Piatti, A.W. Murray, A.F. Straight, J.E. Haber, E. Schiebel, R. Tsien, Euroscarf, and the Yeast Resource Centre for reagents; Y. Kitamura and S. Swift for technical help; F. Wheatley and the media kitchen for media preparation. This work was supported by Cancer Research UK grants C303/A3289 and C303/A3135. TUT was also supported by The Wellcome Trust, Lister Research Prize, and Human Frontier Science Program.

Received: January 25, 2006
Revised: March 14, 2006
Accepted: April 13, 2006
Published: June 29, 2006

## REFERENCES

Amberg, D.C., Burke, D.J., and Strathern, J.N. (2005). Methods in Yeast Genetics (New York: CSHL press).

Baker, T.A., and Bell, S.P. (1998). Polymerases and the replisome: machines within machines. Cell *92*, 295–305.

Berezney, R., Dubey, D.D., and Huberman, J.A. (2000). Heterogeneity of eukaryotic replicons, replicon clusters, and replication foci. Chromosoma *108*, 471–484.

Blow, J.J., and Dutta, A. (2005). Preventing re-replication of chromosomal DNA. Nat. Rev. Mol. Cell Biol. *6*, 476–486.

Blow, J.J., and Tanaka, T.U. (2005). The chromosome cycle: coordinating replication and segregation. Second in the cycles review series. EMBO Rep. *6*, 1028–1034.

Bressan, D.A., Vazquez, J., and Haber, J.E. (2004). Mating type-dependent constraints on the mobility of the left arm of yeast chromosome III. J. Cell Biol. *164*, 361–371.

Bussiere, D.E., and Bastia, D. (1999). Termination of DNA replication of bacterial and plasmid chromosomes. Mol. Microbiol. *31*, 1611–1618.

Dahmann, C., Diffley, J.F., and Nasmyth, K.A. (1995). S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. Curr. Biol. *5*, 1257–1269.

Dimitrova, D.S., Todorov, I.T., Melendy, T., and Gilbert, D.M. (1999). Mcm2, but not RPA, is a component of the mammalian early G1-phase prereplication complex. J. Cell Biol. *146*, 709–722.

Dingman, C.W. (1974). Bidirectional chromosome replication: some topological considerations. J. Theor. Biol. *43*, 187–195.

Falaschi, A. (2000). Eukaryotic DNA replication: a model for a fixed double replisome. Trends Genet. *16*, 88–92.

Frouin, I., Montecucco, A., Spadari, S., and Maga, G. (2003). DNA replication: a complex matter. EMBO Rep. *4*, 666–670.

Herendeen, D., and Kelly, T.J. (1996). SV40 DNA replication. In In Eukaryotic DNA Replication, J.J. Blow, ed. (Oxford: IRL Press), pp. 29–65.

Herskowitz, I., and Jensen, R.E. (1991). Putting the HO Gene to work: practical uses for mating-type switching. In In Methods in Enzymology, C. Guthrie and G.R. Fink, eds. (San Diego: Academic Press), pp. 132–146.

Hiraga, S., Hagihara-Hayashi, A., Ohya, T., and Sugino, A. (2005). DNA polymerases alpha, delta, and epsilon localize and function together at replication forks in Saccharomyces cerevisiae. Genes Cells *10*, 297–309.

Hozak, P., Hassan, A.B., Jackson, D.A., and Cook, P.R. (1993). Visualization of replication factories attached to nucleoskeleton. Cell *73*, 361–373.

Jackson, P.K., Chevalier, S., Philippe, M., and Kirschner, M.W. (1995). Early events in DNA replication require cyclin E and are blocked by p21CIP1. J. Cell Biol. *130*, 755–769.

Jares, P., and Blow, J.J. (2000). Xenopus cdc7 function is dependent on licensing but not on XORC, XCdc6, or CDK activity and is required for XCdc45 loading. Genes Dev. *14*, 1528–1540.

Jensen, R.B., Wang, S.C., and Shapiro, L. (2001). A moving DNA replication factory in Caulobacter crescentus. EMBO J. *20*, 4952–4963.

Johnson, A., and O'Donnell, M. (2005). Cellular DNA replicases: components and dynamics at the replication fork. Annu. Rev. Biochem. *74*, 283–315.

Lau, I.F., Filipe, S.R., Soballe, B., Okstad, O.A., Barre, F.X., and Sherratt, D.J. (2003). Spatial and temporal organization of replicating Escherichia coli chromosomes. Mol. Microbiol. *49*, 731–743.

Lemon, K.P., and Grossman, A.D. (1998). Localization of bacterial DNA polymerase: evidence for a factory model of replication. Science *282*, 1516–1519.

Lemon, K.P., and Grossman, A.D. (2000). Movement of replicating DNA through a stationary replisome. Mol. Cell *6*, 1321–1330.

Lengronne, A., Pasero, P., Bensimon, A., and Schwob, E. (2001). Monitoring S phase progression globally and locally using BrdU incorporation in TK(+) yeast strains. Nucleic Acids Res. *29*, 1433–1442.

Leonhardt, H., Rahn, H.P., Weinzierl, P., Sporbert, A., Cremer, T., Zink, D., and Cardoso, M.C. (2000). Dynamics of DNA replication factories in living cells. J. Cell Biol. *149*, 271–280.

Machida, Y.J., Hamlin, J.L., and Dutta, A. (2005). Right place, right time, and only once: replication initiation in metazoans. Cell *123*, 13–24.

Maekawa, H., Usui, T., Knop, M., and Schiebel, E. (2003). Yeast Cdk1 translocates to the plus end of cytoplasmic microtubules to regulate bud cortex interactions. EMBO J. *22*, 438–449.

Meister, P., Taddei, A., Vernis, L., Poidevin, M., Gasser, S.M., and Baldacci, G. (2005). Temporal separation of replication and recombination requires the intra-S checkpoint. J. Cell Biol. *168*, 537–544.

Michaelis, C., Ciosk, R., and Nasmyth, K. (1997). Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell *91*, 35–45.

Migocki, M.D., Lewis, P.J., Wake, R.G., and Harry, E.J. (2004). The midcell replication factory in Bacillus subtilis is highly mobile: implications for coordinating chromosome replication with other cell cycle events. Mol. Microbiol. *54*, 452–463.

Mimura, S., Masuda, T., Matsui, T., and Takisawa, H. (2000). Central role for cdc45 in establishing an initiation complex of DNA replication in Xenopus egg extracts. Genes Cells *5*, 439–452.

Nakamura, H., Morita, T., and Sato, C. (1986). Structural organizations of replicon domains during DNA synthetic phase in the mammalian nucleus. Exp. Cell Res. *165*, 291–297.

Newport, J., and Yan, H. (1996). Organization of DNA into foci during replication. Curr. Opin. Cell Biol. *8*, 365–368.

Ohya, T., Kawasaki, Y., Hiraga, S., Kanbara, S., Nakajo, K., Nakashima, N., Suzuki, A., and Sugino, A. (2002). The DNA polymerase domain of pol(epsilon) is required for rapid, efficient, and highly accurate chromosomal DNA replication, telomere length maintenance, and normal cell senescence in Saccharomyces cerevisiae. J. Biol. Chem. *277*, 28099–28108.

Pasero, P., Braguglia, D., and Gasser, S.M. (1997). ORC-dependent and origin-specific initiation of DNA replication at defined foci in isolated yeast nuclei. Genes Dev. *11*, 1504–1518.

Piatti, S., Lengauer, C., and Nasmyth, K. (1995). Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast Saccharomyces cerevisiae. EMBO J. *14*, 3788–3799.

Piatti, S., Bohm, T., Cocker, J.H., Diffley, J.F., and Nasmyth, K. (1996). Activation of S-phase-promoting CDKs in late G1 defines a "point of no return" after which Cdc6 synthesis cannot promote DNA replication in yeast. Genes Dev. *10*, 1516–1531.

Pichler, S., Piatti, S., and Nasmyth, K. (1997). Is the yeast anaphase promoting complex needed to prevent re-replication during G2 and M phases? EMBO J. *16*, 5988–5997.

Prein, B., Natter, K., and Kohlwein, S.D. (2000). A novel strategy for constructing N-terminal chromosomal fusions to green fluorescent protein in the yeast Saccharomyces cerevisiae. FEBS Lett. *485*, 29–34.

Raghuraman, M.K., Winzeler, E.A., Collingwood, D., Hunt, S., Wodicka, L., Conway, A., Lockhart, D.J., Davis, R.W., Brewer, B.J., and Fangman, W.L. (2001). Replication dynamics of the yeast genome. Science *294*, 115–121.

Santocanale, C., and Diffley, J.F. (1998). A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. Nature *395*, 615–618.

Schwob, E. (2004). Flexibility and governance in eukaryotic DNA replication. Curr. Opin. Microbiol. *7*, 680–690.

Schwob, E., and Nasmyth, K. (1993). CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in Saccharomyces cerevisiae. Genes Dev. *7*, 1160–1175.

Somanathan, S., Suchyna, T.M., Siegel, A.J., and Berezney, R. (2001). Targeting of PCNA to sites of DNA replication in the mammalian cell nucleus. J. Cell. Biochem. *81*, 56–67.

Sporbert, A., Gahl, A., Ankerhold, R., Leonhardt, H., and Cardoso, M.C. (2002). DNA polymerase clamp shows little turnover at established replication sites but sequential de novo assembly at adjacent origin clusters. Mol. Cell *10*, 1355–1365.

Straight, A.F., Belmont, A.S., Robinett, C.C., and Murray, A.W. (1996). GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion. Curr. Biol. *6*, 1599–1608.

Sugino, A. (1995). Yeast DNA polymerases and their role at the replication fork. Trends Biochem. Sci. *20*, 319–323.

Tanaka, T., and Nasmyth, K. (1998). Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases. EMBO J. *17*, 5182–5191.

Tanaka, T., Knapp, D., and Nasmyth, K. (1997). Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. Cell *90*, 649–660.

Tanaka, K., Mukae, N., Dewar, H., van Breugel, M., James, E.K., Prescott, A.R., Antony, C., and Tanaka, T.U. (2005). Molecular mechanisms of kinetochore capture by spindle microtubules. Nature *434*, 987–994.

Uhlmann, F., and Nasmyth, K. (1998). Cohesion between sister chromatids must be established during DNA replication. Curr. Biol. *8*, 1095–1101.

Vernis, L., Piskur, J., and Diffley, J.F. (2003). Reconstitution of an efficient thymidine salvage pathway in Saccharomyces cerevisiae. Nucleic Acids Res. *31*, e120.

Waga, S., and Stillman, B. (1998). The DNA replication fork in eukaryotic cells. Annu. Rev. Biochem. *67*, 721–751.

Wessel, R., Schweizer, J., and Stahl, H. (1992). Simian virus 40 T-antigen DNA helicase is a hexamer which forms a binary complex during bidirectional unwinding from the viral origin of DNA replication. J. Virol. *66*, 804–815.