Replication factory activation can be decoupled from the replication timing program by modulating Cdk levels

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In the metazoan replication timing program, clusters of replication origins located in different sub-chromosomal domains fire at different times during S phase. We have used Xenopus laevis egg extracts to drive an accelerated replication timing program in mammalian nuclei. Although replicative stress caused checkpoint-induced slowing of the timing program, inhibition of checkpoint kinases in an unperturbed S phase did not accelerate it. Lowering cyclin-dependent kinase (Cdk) activity slowed both replication rate and progression through the timing program, whereas raising Cdk activity increased them. Surprisingly, modest alteration of Cdk activity changed the amount of DNA synthesized during different stages of the timing program. This was associated with a change in the number of active replication factories, whereas the distribution of origins within active factories remained relatively normal. The ability of Cdk to differentially affect replication initiation, factory activation, and progression through the timing program provides new insights into the way that chromosomal DNA replication is organized during S phase.

Introduction

The hundreds or thousands of replication origins distributed throughout each eukaryotic genome initiate replication forks at distinct and reproducible times during the course of S phase (Goren and Cedar, 2003; Donaldson, 2005; Zink, 2006). Replication timing profiles of the entire genome of budding yeast (Raghuraman et al., 2001), fission yeast (Heichinger et al., 2006; Eshaghi et al., 2007; Hayashi et al., 2007; Mickle et al., 2007), Drosophila melanogaster (Schübeler et al., 2002; MacAlpine et al., 2004), and mammals (White et al., 2004; Woodline et al., 2004, 2005; Jeon et al., 2005; Cohen et al., 2006; Farkash-Amar et al., 2008; Hiratani et al., 2008) have been described previously. In general, transcriptionally active euchromatin replicates early in S phase, whereas transcriptionally inactive heterochromatin replicates late. In metazoans, chromosome domains that replicate at similar times are clustered together within the nucleus so that replication occurring at different times in S phase displays distinct intranuclear patterns (Goren and Cedar, 2003; Zink, 2006).

Experiments in yeast suggest that replication origins become programmed for late replication at some stage during G1 (Raghuraman et al., 1997). A similar “timing decision point” was defined in metazoans as a stage in early G1 when tissue culture nuclei acquire the ability to support a normal replication timing program when subsequently driven into S phase by Xenopus laevis egg extracts (Dimitrova and Gilbert, 1999). This represents the establishment of the replication timing program and coincides with movement of early- or late-firing chromosomal regions to appropriate positions within the nucleus, which may involve chromatin modification of the region surrounding replication origins.

Relatively little is known about how the timing program is executed during S phase. Each replication origin is loaded with Mcm2-7 during late mitosis and G1 to form a prereplicative complex that licenses the origin for a single initiation event in the coming S phase (Nishitani and Lygerou, 2004; Blow and Dutta, 2005). During S phase, each prereplicative complex is acted on by Cdk complexes and the Dbf4-dependent kinases to induce initiation. It is likely that Mcm2-7 is the essential substrate for Cdc7 in the initiation of replication. In yeast, the
Sld2 and Sld3 proteins have been shown to be essential substrates for Cdks in the initiation of replication, although their vertebrate homologues are currently unknown (Masumoto et al., 2002; Tanaka et al., 2007; Zegerman and Diffley, 2007). Phosphorylation of Sld2 and Sld3 promotes the recruitment of other replisome proteins such as Cdc45 to Mcm2-7 at replication origins.

Replication forks from clusters of adjacent replication origins are organized into replication factories in the nucleus (Jackson and Pombo, 1998; Berezney et al., 2000; Frouin et al., 2003; Kitamura et al., 2006). To account for the number of DNA replication forks generated during S phase, most replication factories must contain multiple replication forks, probably in the range of 5–50 forks per factory (Berezney et al., 2000). Little is known about what causes the clustering of replication origins into factories, although some aspect of chromosomal structure may play a role.

In this study, we examine in detail how Cdk activity drives progression through the replication timing program. We use the experimental system developed by Gilbert et al. (1995), in which replication of mammalian G1 nuclei is driven by incubation in X. laevis egg extracts (Gilbert et al., 1995; Dimitrova and Gilbert, 1999). We provide evidence for a direct function of Cdks in activating replication factories and driving progression through the replication timing program that is distinct from the established function of Cdks in initiating the initiation of replication.

Results

The kinetics of replication in vitro

Nuclei were prepared from CHOC-400 cells released for 4 h into G1 from mitotic synchrony. These had passed the timing decision point and were programmed to replicate according to their normal replication timing program (Dimitrova and Gilbert, 1999). The nuclei were incubated in X. laevis egg extract supplemented with geminin to ensure that only origins licensed in vivo were used (Okuno et al., 2001; Dimitrova et al., 2002). At different times, extracts were pulsed with Cy3-dUTP to label sites of ongoing DNA replication. As reported previously (Dimitrova and Gilbert, 1999), labeling patterns were observed that resembled those seen in vivo. We categorized these as patterns I–V, in accordance with previous nomenclature (Fig. 1 a; O’Keefe et al., 1992). However, it should be noted that the in vitro patterns we observed were not identical to the in vivo ones, particularly at later stages of S phase. In particular, we saw many combined patterns that we designated I/II, II/III, III/IV, and IV/V. The appearance of these combined patterns may be a result of the rapid S phase occurring in vitro (~2 h compared with ~12 h in vivo) and is consistent with the idea that the in vitro timing program can progress to later stages before finishing the replication of the earlier stages (see following paragraphs).

Fig. 1 b shows the proportion of different patterns seen every 10 min during an incubation in vitro. The replication patterns appeared in the same order as in vivo. Although most nuclei reached the late type IV and IV/V patterns after an incubation of 140 min, α-[3H]dATP incorporation suggested that overall replication was inefficient, averaging ~38% of template DNA replicated (Fig. 1 c). Inefficient replication of somatic nuclei in X. laevis egg extract has been reported previously (Dimitrova and Gilbert, 1998). It is not the result of poor-quality extracts, as CHOC-400 chromosomes and X. laevis sperm nuclei replicated efficiently (unpublished data).

Fig. 1 d shows the total time the egg extract spent replicating the different patterns in vitro compared with the times reported for CHO cells in vivo (Dimitrova and Gilbert, 1999). In vitro, relatively more time is spent replicating the later patterns, likely as a result of the lower rates of replication at later stages (Fig. 1 c). When the pattern proportions are normalized to the rate of replication (Fig. 1 d, bottom), they look roughly similar to the in vivo proportions. However, consistent with Dimitrova and Gilbert (1999), we saw very few pure type V labeling patterns in vivo. Preincubation of X. laevis extracts in the absence of nuclei only slightly altered the appearance of replication patterns once template nuclei were subsequently added (Fig. S1). This suggests that correct progression through the replication timing program requires an interaction between extract and template nuclei.

Initiation times associated with the different patterns

Cdks are required throughout S phase for individual origins to initiate replication. Shortly after addition of Cdk inhibitors such as roscovitine to X. laevis egg extracts, most new initiation events are blocked without affecting forks that have already initiated (Strausfeld et al., 1994, 1996; Luciani et al., 2004). To see how this affected the timing program, we added 1 mM roscovitine to extract at different times after addition of CHO nuclei. Initiation events (as indicated by roscovitine-sensitive DNA replication) took place over a period of >80 min (Fig. 2 a and not depicted). This is significantly longer than the ~25-min initiation period when X. laevis sperm nuclei replicate in X. laevis egg extract (Luciani et al., 2004). Fig. 2 b shows that roscovitine addition blocked the appearance of new replication patterns as expected of an initiation inhibitor. For example, addition of roscovitine at 20 min prevented the appearance of most type II/III, III, and III/IV patterns, suggesting that few initiation events associated with type III DNA had occurred by 20 min. Fig. 2 c shows a hypothetical time course of initiation events associated with each different replication pattern that would be consistent with our results. There is considerable overlap between the times, which may partly explain the labeling of mixed pattern types (I/II and II/III, etc).

Regulation of the timing program by checkpoint kinases

We next supplemented extracts with the DNA polymerase inhibitor aphidicolin at 3 µM, which slows replication forks by two- to three-fold and activates checkpoint kinases in egg extracts (Luciani et al., 2004). Fig. 3 a shows that 3 µM aphidicolin led to a dramatic slowing of the replication timing program, so even after 140 min, most CHO nuclei still showed
possibly by reducing the stability of stalled replication forks (Fig. S2 d).

The effect of Cdk activity on the timing program

We next investigated whether, in addition to being required to drive replication initiation, Cdk5s also play a role in driving the replication timing program. If there was a strict coupling between the rate of initiation and the rate of progression through the timing program, the two would be expected to be reduced together in response to reduction of Cdk activity. However, the replication timing program might be completely independent of both Cdk activity and initiation, and in this case, it would proceed unchanged despite a reduction in the frequency of initiation. Fig. 4 a shows the effect of increasing roscovitine concentrations on total histone H1 kinase activity. The decline in Cdk activity was mirrored by a similar decrease in replication rates (Fig. 4 b), suggesting that Cdk activity is rate limiting for DNA replication (Strausfeld et al., 1994; Moore et al., 2002).
A premature mitosis (Strausfeld et al., 1996; unpublished data). Because protein synthesis in our extracts is blocked with cycloheximide, the majority of Cdk activity is supplied by cyclin E–Cdk2 (cyclins A and B having been degraded during mitosis and D-type cyclins not being present at this stage of development; Howe et al., 1995; Rempel et al., 1995; Hartley et al., 1996; Vernon and Philpott, 2003).

The ability of cyclin A to stimulate DNA replication without significantly increasing total H1 kinase activity is likely explained by it being 10–100 times more effective at inducing replication initiation than cyclin E (Strausfeld et al., 1996; Moore et al., 2002).

Fig. 5c shows that cyclin A also slightly accelerated the replication timing program. This was most evident at 90 min, when cyclin A induced the appearance of type IV patterns. Fig. 5d shows that 1 pM cyclin A not only accelerated the timing program but also induced appearance of pure type V patterns, which were very rare in control samples. These experiments show that the replication program can be accelerated by increasing Cdk levels.

As shown in Fig. 1, even though during a normal S phase most CHO nuclei reached type IV and IV/V patterns, only ~40% of the template DNA was typically replicated. Therefore, we wondered whether raising Cdk levels could enhance the rate or extent of replication. We tested this using recombinant cyclin A, which when added to X. laevis egg extracts, binds to Cdk1 and provides an S phase–inducing Cdk activity (Strausfeld et al., 1994, 1996). Fig. 5a and b shows that although recombinant cyclin A barely increased total histone H1 kinase activity, 1 fM–1 nM cyclin A stimulated replication of CHO nuclei with an optimum at ~1 pM. At 1 µM, cyclin A inhibited replication by forcing entry into a premature mitosis (Strausfeld et al., 1996; unpublished data). Because protein synthesis in our extracts is blocked with cycloheximide, the majority of Cdk activity is supplied by cyclin E–Cdk2 (cyclins A and B having been degraded during mitosis and D-type cyclins not being present at this stage of development; Howe et al., 1995; Rempel et al., 1995; Hartley et al., 1996; Vernon and Philpott, 2003).

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The role of Cdk5 in the activation of replication factories

Comparison of Fig. 4 (b and c) suggests that 1–10 µM roscovitine inhibited replication rates more strongly than it inhibited progression through the replication timing program so that nuclei had replicated less DNA than normal when they embarked on later replication patterns. This effect is directly demonstrated in Fig. 6 a. First, a detailed time course was performed with 10 µM roscovitine, with total DNA synthesis and replication patterns measured every 10 min (Fig. S3). The proportion of nuclei showing each pattern was plotted on the vertical axis as previously but with the horizontal axis representing total DNA replication at each time point (Fig. 6 a). This shows that 10 µM roscovitine made the later replication patterns (III, III/IV, IV, and IV) appear at lower levels of DNA replication than in the control. Therefore, lowering Cdk levels decouples initiation from progression through the timing program. The converse effect was seen when extracts were supplemented with 1 pM cyclin A: more DNA replication was seen at each corresponding replication pattern (Fig. 6 b).

To confirm this, we performed experiments in which total DNA synthesis was measured in individual nuclei. CHO nuclei were continuously labeled with either α-[32P]dATP or Cy5.5-dCTP in extract optionally supplemented with 10 µM roscovitine or 1 pM cyclin A. DNA synthesis in α-[32P]dATP-labeled samples was measured by TCA precipitation and scintillation counting (Fig. S4, a and c). DNA synthesis in the Cy5.5-dCTP–labeled samples was measured by microscopically quantifying Cy5.5 fluorescence in randomly selected nuclei (Fig. S4, b and d). When α-[32P]dATP labeling was plotted against Cy5.5 fluorescence at different times, the result was approximately linear, demonstrating the concordance between the two measurements (Fig. S4, e and f). Cy5.5-dCTP–labeled samples were also pulse labeled with Cy3-dUTP, allowing the determination of replication patterns in nuclei whose total DNA synthesis is known. The results shown in Fig. 6 c reveal two important features. First, in the control sample (Fig. 6 c, black bars), the amount of DNA replication associated with each particular replication pattern fell within fairly narrow confines, as expected of a true replication timing program. Second, 10 µM roscovitine (Fig. 6 c, green bars) significantly lowered the total amount of DNA synthesis associated with each different pattern, whereas cyclin A (Fig. 6 c, red bars) increased it. This shows that altering Cdk activity alters the total rates of replication initiation to a greater degree than progression through the replication timing program and directly demonstrates decoupling of the two processes.
When progression into a new stage of the timing program occurs, new initiation events must occur in newly activated replication factories. Therefore, we investigated which of these aspects were most strongly affected when Cdk activity was varied. CHO nuclei replicating in vitro plus or minus roscovitine or cyclin A were pulsed with Cy3-dUTP in mid- or late S phase (50 or 90 min). Cy3-dUTP labeling revealed the distribution of replication foci, each of which is presumed to consist of one or a small number of replicon clusters (Fig. 7, a–c). We next quantified the number of replication foci present under different Cdk levels and the mean Cy3 intensity of individual foci (which provides an indication of the number of replication forks that they contain). Surprisingly, treatment of extracts with up to 10 µM roscovitine predominantly reduced the total number of foci, leaving their intensity largely unchanged (Fig. 7, d and e). Higher concentrations of roscovitine, which more strongly suppressed both Cdk activity and total DNA replication (Fig. 4, a and b), inhibited both foci number and intensity (Fig. 7, d and e). Conversely, stimulating DNA replication with cyclin A increased the number of replication foci. This effect is highly reproducible (Fig. S5 a) and is also seen when replication factories are visualized with an anti-PCNA antibody (Fig. S5, b–e). Consistent with these results, DNA fiber analysis showed that treatment of extracts with 10 µM roscovitine did not significantly change either replication fork speed or the density of replication forks within active replicon clusters (Fig. 8). These experiments
show that modest changes to Cdk activity preferentially alter the activation of replication factories without significantly changing the rate of initiation within active factories.

Discussion

The timing program in X. laevis egg extracts

When X. laevis sperm nuclei are incubated in X. laevis egg extract (mimicking events occurring at fertilization), the sperm DNA is replicated completely in ~30 min. A rudimentary replication timing program is observed with certain chromosome domains replicating at different stages of this rapid S phase (Labit et al., 2008). In this study, we have examined how the replication timing program is executed in X. laevis egg extracts replicating mammalian CHO-C400 nuclei. As described by Dimitrova and Gilbert (1999), CHO nuclei incubated in X. laevis egg extracts are induced to replicate according to a timing program similar to that occurring normally in CHO cells. In this study, we show that initiation events in the CHO nuclei occurred over 1–2 h in vitro, which is considerably faster than the ~12 h seen in vivo. Initiation events in vitro associated with the five major labeling patterns occurred in order, but unlike the situation in vivo, they showed considerable overlap.

Given the distribution of forks within origin clusters, it would take >40 min to replicate a typical origin cluster in X. laevis egg extracts, which is similar to the time required in vivo. Because the five stages of the timing program are compressed into ~2 h in vitro, this makes it inevitable that different replication patterns are seen concurrently. Addition of very high concentrations of roscovitine at different stages through S phase provide further evidence that initiation events associated with different timing stages can occur at the same time. This means that progression from one stage of the timing program to another is not dependent on all of the replication associated with the earlier timing stage having been completed. Indeed, CHO nuclei replicating in vitro progress to late replication patterns with less than half of the template DNA replicated (Dimitrova and Gilbert, 1998; this study).

Progression through the timing program was slowed when fork progression was inhibited by aphidicolin. This slowing of the timing program involved checkpoint kinases and could be partially reversed by coaddition of the checkpoint inhibitor caffeine. When caffeine was added to extract replicating CHO nuclei in the absence of replicative stress, no acceleration of the timing program was seen. This is consistent with work in yeast and mammalian cells suggesting that although checkpoints delay origin firing in response to replicative stress, they do not significantly slow the timing program in unperturbed S phases (Santocanale and...
point kinases did not greatly accelerate the timing program (Luciani et al., 2004; Shechter et al., 2004; Woodward et al., 2006). This suggests that the high concentrations of sperm nuclei used by Shechter et al. (2004) created replicative stress leading to checkpoint-mediated slowing of the replication timing program.

Cdk activity and the structure of S phase

Cdns are required throughout S phase to induce replication initiation by phosphorylating Sld2 and Sld3 (or their metazoan...
Conversely, stimulation of Cdk activity increased the number of active replication factories. Treatment of intact U20S cells with roscovitine can also cause a similar reduction in the number of active replication factories (unpublished data). This suggests that the role of Cdks in promoting the activation of new replication factories is conserved throughout higher eukaryotes.

Modest changes to Cdk levels changed the number of active replication factories and thus overall replication rate without significantly changing progression through the different replication timing patterns. This changed the amount of total DNA counterparts) and allowing them to recruit Cdc45 and other replication fork proteins to licensed origins (Masumoto et al., 2002; Tanaka et al., 2007; Zegerman and Diffley, 2007). How then is this initiation function of Cdks coordinated with the timing program so that it drives initiation at origins appropriate for particular stages of S phase? Our results show that Cdk activity also promotes the activation of replication factories (Fig. 9). Reduction of Cdk activity to ~50% was associated with a reduced number of active replication factories, whereas the number of forks within each factory remained largely unchanged.

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Modest changes to Cdk levels changed the number of active replication factories and thus overall replication rate without significantly changing progression through the different replication timing patterns. This changed the amount of total DNA
replication associated with each labeling stage of the timing program. However, the timing program was not entirely independent of Cdk activity. Addition of cyclin A caused a modest acceleration of the timing program, whereas high concentrations of roscovitine strongly inhibited progression through the timing program and the appearance of later replication patterns. At present, it is unclear whether the effect of Cdns on the timing program is an indirect consequence of their effect on factory activation and origin initiation or whether Cdns have a separate role in driving the progression from one timing stage to another.

In our experiments, the activation of replication factories showed the highest sensitivity to a change in Cdk levels (Fig. 9). A major effect of Cdns on the activation of replicon clusters has also been reported when sperm nuclei replicate in *X. laevis* egg extract (Krasinska et al., 2008). This sensitivity to Cdk levels might depend on there being additional Cdk substrates distinct from Cdk substrates required for individual origins to initiate, whose phosphorylation is required to allow the activation of new factories. Alternatively, it might be that the first origin to initiate within a factory requires the highest Cdk activity, but that once this has occurred, initiation of additional origins might be less dependent on high Cdk activity. For example, when initiation occurs in a factory, it might induce a change to the structure of the factory, making it easier for other origins associated with the factory to initiate. Previous work has suggested that the intra–S phase checkpoint may preferentially inhibit the activation of new replication factories, thereby directing new initiation events to inefficient dormant origins within active factories (Woodward et al., 2006; Ge et al., 2007; Blow and Ge, 2009). Our current results suggest that this could be achieved by checkpoint-mediated lowering of Cdk activity (Karlsson-Rosenthal and Millar, 2006; Boutros et al., 2007) or by checkpoint-mediated inhibition of Cdk targets required for factory activation.

Although the mechanisms remain obscure at present, our results show for the first time the multiple ways that Cdk activity can drive progression through S phase, differentially affecting the timing program, factory activation, and replication initiation. Identifying potential Cdk substrates for these transitions is an exciting new goal.

### Materials and methods

**Cell culture and synchrony**

CHOC-400 cells were propagated in DME (Invitrogen) supplemented with nonessential amino acids, 10% fetal calf serum (Perbio), and 10 U/ml streptomycin/penicillin at 37°C in 5% CO₂. G1 phase CHOC-400 cells were obtained using mitotic selection as described previously (Gilbert et al., 1995). Essentially, cells were supplemented with fresh medium containing 50 ng/ml nocodazole (Sigma-Aldrich) for 4 h to block cells in metaphase. Mitotic cells were washed in warm medium and released into G1 for 4 h to obtain post–origin decision point cells. Cells were prepared fresh for each experiment.

**Preparation of nuclei**

Intact nuclei were prepared as described previously (Wu et al., 1997). CHOC-400 cells were washed with ice-cold transport buffer (20 mM Hepes, 110 mM K acetate, 5 mM Na acetate, 2 mM Mg acetate, and 1 mM EGTA, pH 7.6, with KOH), counted, resuspended at 10–15 × 10⁶ cells/ml in transport buffer, and stored on ice. Samples were supplemented with 50 µg/ml digitonin (EMD), incubated on ice for 5 min, and the reaction was stopped by addition of 3% BSA (w/vol) in transport buffer. Nuclei were recovered by centrifugation at 1,500 rpm for 5 min at 4°C. Cell and nuclear morphology were examined by phase-contrast microscopy. Permeabilization of the plasma and nuclear membranes was verified by staining with 0.1 µg/ml DAPI and 150 kD IgG/FITC exclusion (Dako). Typically, >90% intact nuclei were obtained.

**Replication assay**

*X. laevis* egg extract was prepared as described previously (Chong et al., 1997). In brief, unfertilized *X. laevis* eggs were dejellied and spin crushed.
at 12,000 g at 4°C for 20 min in a swinging bucket rotor. Cytoplasm was withdrawn, supplemented with 10 mg/ml cytochalasin B and 15% (vol/vol) extract dilution buffer (50 mM KCl, 50 mM Hepes KOH, pH 7.6, 2 mM DTT, 0.4 mM MgCl₂, 0.4 mM EGTA, 1 mg/ml each of pepstatin, leupeptin, and aprotinin, and 10% sucrose), and spun at 20,000 g at 4°C for 20 min in a swinging bucket rotor. Cytoplasm was withdrawn and frozen in 20% drops in liquid nitrogen. After thawing for use, extracts were supplemented with 250 µg/ml cycloheximide, 25 mM phosphocreatine, 15 µg/ml creatine phosphokinase, and 0.3 mM CaCl₂ and incubated for 15 min to promote metaphase exit. They were supplemented with 100 µg/ml geminin and incubated for a further 10 min to prevent further licensing of CHO nuclei. CHOC-400 nuclei were added to extract at 10,000 nuclei/ml. All incubations were performed at 23°C. To measure timing pattern labeling, nuclei were supplemented with 25 µM Cy3-dUTP for 5 min. Reactions were stopped, fixed, and prepared for microscopy exactly as described for replication pattern labeling. In parallel, total DNA synthesis at either 50 or 90 min was measured in extract supplemented with α-[32P]dATP by TCA precipitation. Datasets were acquired using a camera (Micromax) on a restoration microscope (DeltaVision DV3) built around a stand (Eclipse TE200; Olympus) with a 100× oil immersion objective and a cooled camera (CoolSNAP HQ; Photometrics) on a restoration microscope (DeltaVision SpectrI; Applied Precision) built around a stand (IX70; Olympus) with a 60× 1.4 NA Plan Apo lens (Applied Precision). For each nucleus, 22 optical sections were recorded every 0.5 µm, and 3D datasets were deconvolved using the constrained iterative algorithm software (SoftWoRx). For each nucleus, the section with the largest surface area (presumed middle of the nucleus) was selected for quantitation. A 4,000-pixel square (4 x 4 µm) was drawn in the physical center of the section. The number of discernible foci and the total incorporation of label within this area were measured. The mean label incorporated per focus was calculated. Data were generated from 20 nuclei for every condition and time point in each experiment. The OME Remote Objects (OMERO) insight program was used for quantitative analysis (Swedlow, 2003; Goldberg et al., 2005).

Analysis of Cy3-labeled foci
Labeling of replication foci was performed at either 50 or 90 min by supplementing X. laevis egg extract with 25 µM Cy3-dUTP for 5 min. Reactions were stopped, fixed, and prepared for microscopy exactly as described for replication pattern labeling. In parallel, total DNA synthesis at either 50 or 90 min was measured in extract supplemented with α-[32P]dATP by TCA precipitation. Datasets were acquired using a camera (Micromax) on a restoration microscope (DeltaVision DV3) built around a stand (Eclipse TE200; Nikon) with a 100× 1.40 NA Plan Apo lens (Nikon). For each nucleus, optical sections were recorded every 0.5 µm, and datasets were deconvolved using the constrained iterative algorithm software (SoftWoRx). For each nucleus, the section with the largest surface area (presumed middle of the nucleus) was selected for quantitation. A 4,000-pixel square (4 x 4 µm) was drawn in the physical center of the section. The number of discernible foci and the total incorporation of label within this area were measured. The mean label incorporated per focus was calculated. Data were generated from 20 nuclei for every condition and time point in each experiment. The OME Remote Objects (OMERO) insight program was used for quantitative analysis (Swedlow, 2003; Goldberg et al., 2005).

Analysis of PCNA foci
For immunodetection of PCNA foci, CHO nuclei incubated in vitro were isolated, fixed, and pelleted onto coverslips as described for replication pattern labeling. Coverslips were washed extensively with PBS plus 0.1% Triton X-100 (vol/vol; PBS-T). Samples were blocked for 1 h in the same buffer plus 3% BSA and incubated for a further 1 h with PC10 anti-PCNA antibody. After extensive washing in PBS-T, cells were labeled with FITC-labeled anti–mouse IgG followed by extensive washing in PBS-T. DNA was stained with 1 µg/ml DAPI for 5 min at 23°C. Coverslips were mounted with Vectashield mounting medium, sealed, and dried before visualization.

3D datasets were acquired using a cooled camera (CoolSNAP HQ; Photometrics) on a restoration microscope (DeltaVision SpectrI; Applied Precision) built around a stand (IX70; Olympus) with a 60× 1.4 NA Plan Apo lens (Applied Precision). For each nucleus, 22 optical sections were recorded every 0.5 µm, and 3D datasets were deconvolved using the constrained iterative algorithm software (SoftWoRx; Applied Precision). Spatiotemporal patterns could then be visualized and analyzed. The Open Microscopy Environment (OME; Swedlow, 2003; Goldberg et al., 2005) was used for quantitative analysis of images. Quantification of the Cy3.5 intensity was performed using the FindSpots algorithm, and results were exported into an OME XML file for analysis (Fiatano et al., 2000).

Timing pattern analysis
The following classification of different replication patterns based on previous work (O’Keefe et al., 1992) was used: type I, faintly punctate labeling throughout euchromatic regions; type II, intense and diffuse but incomplete labeling of euchromatic regions with distinct lack of nucleolar labeling; type II/III, diffuse labeling of euchromatic regions plus some labeling of the peripheral ring and perinucleolar regions; type III, intense labeling of the peripheral ring, possibly with some perinucleolar labeling; type III/IV, punctate labeling of the peripheral ring plus small-speckled heterochromatic foci within the nuclear interior; type IV, mainly labeling of small-speckled heterochromatic foci within the nuclear interior or at the periphery, with some of the speckled foci forming chain-like structures; type IV/V, labeling of large internal foci plus speckled labeling of small heterochromatic foci within the nuclear interior, which are more punctate than the previous (some peripheral replication may also persist); and type V, predominant labeling of large internal replication foci and at the periphery of the nucleus.

The timing of initiation events associated with different replication patterns was performed as follows: at different times after the start of the in vitro reaction (20, 40, 60, and 90 min), aliquots were taken to be supplemented with 1 mM roscovitine. At different times afterward, subaliquots of these were taken, pulsed with 25 µM Cy3-dUTP for 5 min, and the replication patterns were analyzed. If addition of roscovitine at a particular time significantly blocked the subsequent appearance of a particular pattern, the initiation events associated with that pattern were considered to have taken place exclusively prior to the roscovitine addition time. If addition of roscovitine at a particular time had no significant effect (relative to a control with no added roscovitine) on the appearance of a particular pattern, the initiation events associated with that pattern were considered to have taken place exclusively before the roscovitine addition time. Intermediate cases (in which roscovitine delayed but did not abolish the appearance of a pattern) were considered to indicate that the initiation events associated with that pattern were occurring at the roscovitine addition time.

DNA fiber labeling
CHO nuclei were incubated in extract supplemented with 100 µM BrdU under the desired conditions [5-min incubation at 45–50 min]. Reactions (100 µl) were stopped by resuspension in 400 µl TBS. The resuspended extract was underlayered with 1 ml TBS plus 0.1% Triton X-100 (vol/vol) and 20% sucrose (wt/vol) and was spun at ~300 g in a swinging bucket rotor for 5 min at 4°C. The supernatant was removed to leave only the sucrose cushion (100 µl; equal to the starting volume), and the cell pellet was resuspended and stored on ice. DNA was spread on glass slides (Superfrost; VWR) according to the following conditions: nuclei were diluted (1:5) in TBS buffer (~2,000 nuclei/µl). A 1-µl sample was spotted onto the glass slide followed by addition of µl of 1% triton buffer (75% SDS, 200 mM

Cdk5 and DNA replication • Thomson et al.
Tris-HCl, pH 7.4, and 50 mM EDTA] and incubated for ~4 min. DNA fibers were spread by tipping the slides at 36°. After migration down the DNA, slides were fixed in methanol/acidic acid [3:1] for >10 min. Slides were rehydrated with H2O and incubated in 2.5 M HCl for 1 h to denature the DNA. Slides were briefly rinsed in TBS and incubated for 1 h in blocking solution containing TBS, 1% [w/v] BSA, and 0.1% [v/v] Tween 20. Slides were incubated with monoclonal anti-BrdU (BD) at 20 µg/ml in this buffer for 1 h. Slides were washed several times in TBS + Tween, in TBS + Tween + BSA, briefly rinsed in TBS alone, and labeled with 1 µg/ml Alexa Fluor 555 anti-mouse antibody [Invitrogen] in TBS + Tween + BSA for 2 h. Samples were washed extensively in TBS, and DNA was stained with YOYO (Y-3601; diluted at 1:10,000 from a 1 mM stock; Invitrogen) for 10 min. Samples were rinsed five times in TBS and mounted in Vectashield.

Fiber analysis was performed as described previously [Ge et al., 2007]. The mean and standard deviation of track lengths were first determined by measuring the length of labeled tracks that were well separated from other tracks (thereby minimizing the risk that they represented fusions between adjacent replicons). Track clusters were selected for the determination of intracluster fork density and origin spacing by the following criteria: clusters (a) consisted of single DNA fibers and not fiber bundles based on YOYO staining, (b) were located in a relatively isolated area, (c) contained at least four consecutive tracks, and (d) each track in the cluster was no longer than the mean track length plus one standard deviation to minimize the risk of including clusters where termination and fusion of neighboring replicons had occurred. For each sample, at least 100 measurements were performed.

Online supplemental material

Fig. S1 shows the effect of preincubation of egg extract on the timing program. Fig. S2 shows that aphidicolin slows the timing program and caffeine causes fork instability. Fig. S3 shows the effect of roscovitine on DNA synthesis in individual nuclei ± 10 µM roscovitine or 1 pM caffeine causes fork instability. Fig. S4 shows that aphidicolin slows the timing program and caffeine causes fork instability. Fig. S5 shows the measurement of DNA synthesis in individual nuclei ± 10 µM roscovitine or 1 pM cyclin A. Fig. S5 shows the effect of roscovitine and cyclin A on replication foci. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200911037/DC1.

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References


Krasinska, L., E. Besnard, E. Cot, C. Dohet, M. Méchali, J.M. Lemaitre, and D. Fisher. 2008. Cdk1 and Cdk2 activity levels determine the
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Figure S1. **Effect of preincubation of egg extract on the timing program.** (a and b) X. laevis egg extracts were preincubated at 23°C for different times after addition of CaCl$_2$ to promote exit from metaphase. They were supplemented with 10,000 nuclei/µl CHO-C400 nuclei and geminin. At either 55 or 115 min after nuclear addition, nascent DNA was pulse labeled for 5 min with Cy3-dUTP. At the end of the pulse, nuclei were isolated, and the percentage of nuclei showing different replication patterns was measured. (a) Cartoon of experimental protocol. (b) Percentage of replication patterns at either 60 (left) or 120 min (right) after addition of nuclei. Note that all other experiments in this study were performed with extracts preincubated for 15 min.
Figure S2. *Aphidicolin slows the timing program and caffeine causes fork instability.* (a–c) CHOC-400 nuclei were incubated at 10,000 nuclei/µl in *X. laevis* egg extracts supplemented with geminin ± 3 µM aphidicolin or 5 mM caffeine. (a and c) Extract was also supplemented with α-[³²P]dATP. At different times, DNA was TCA precipitated, and the amount of total DNA synthesis was assessed by scintillation counting. (b) At different times, nascent DNA was pulse labeled with Cy3-dUTP. Nuclei were isolated, and the percentage of nuclei showing different replication patterns was assessed. (d) CHOC-400 nuclei were incubated at 10,000 nuclei/µl in *X. laevis* egg extracts supplemented with geminin. After 35 min, extracts were supplemented with 100 µM aphidicolin, 1 mM roscovitine ± 5 mM caffeine, and the incubation was continued for an additional 60 min. Nuclei were isolated and incubated in fresh extract supplemented with α-[³²P]dATP and roscovitine. At different times, DNA was TCA precipitated, and the amount of total DNA synthesis was assessed by scintillation counting. The rate of synthesis provides an indication of the number of active forks that had remained after the incubation with aphidicolin plus or minus caffeine.
Figure S3. Effect of roscovitine and cyclin A on the timing program. (a–c) CHOC-400 nuclei were incubated at 10,000 nuclei/µl in X. laevis egg extracts supplemented with geminin ± 10 µM roscovitine (a and b) or 1 pM cyclin A (c). (a and c) Extract was also supplemented with α-[32P]dATP. At different times, DNA was TCA precipitated, and the amount of total DNA synthesis was assessed by scintillation counting. (b) At different times, nascent DNA was pulse labeled with Cy3-dUTP. Nuclei were isolated, and the percentage of nuclei showing different replication patterns was assessed.
Figure S4. Measurement of DNA synthesis in individual nuclei ± 10 μM roscovitine or 1 pM cyclin A. (a–f) CHOC-400 nuclei were incubated at 10,000 nuclei/μl in X. laevis egg extracts supplemented with geminin ± 10 μM roscovitine (a, b, and e) or 1 pM cyclin A (c, d, and f). (a and c) Extract was also supplemented with α-[32P]dATP. At different times, DNA was TCA precipitated, and the amount of total DNA synthesis was assessed by scintillation counting. (b and d) Extract was also supplemented with Cy5.5-dCTP. At different times, nuclei were isolated, the total Cy5.5 fluorescence of 20 randomly selected nuclei was determined by microscopy, and the mean value was determined. (e and f) For each time point in a–d, DNA synthesis as derived by α-[32P]dATP incorporation was plotted against mean nuclear replication as derived from Cy5.5 intensity.
Figure S5. **Effect of roscovitine and cyclin A on replication foci.** CHO nuclei were incubated in *X. laevis* egg extract plus or minus the indicated concentrations of roscovitine (rosc) or cyclin A (cyc A). Parallel incubations were supplemented with α-[\(\text{\textsuperscript{32}}\text{P}\)]dATP to measure total DNA synthesis. (a) At 50 min, extract was pulsed for 5 min with Cy3-dUTP. The number and intensity of Cy3-labeled foci was measured. Results from three independent experiments were normalized to values obtained in untreated extract and combined. Error bars indicate SEM. (b–e) At 50 min, nuclei were isolated, stained with anti-PCNA antibody, and examined for the presence of replication foci. (b) The number of foci in a 4 × 4-μm square in the center of 50 nuclei was determined. (c) The mean intensity of the 10 brightest foci in 50 nuclei was determined. Mean values of total DNA synthesis, number of PCNA foci, and PCNA content of foci shown in d. (e) Representative images are shown.