Characterization of a novel ATR-dependent, Chk1-independent, intra-S-phase checkpoint that suppresses initiation of replication in Xenopus

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Summary
In most eukaryotes, replication origins fire asynchronously throughout S-phase according to a precise timing programme. When replication fork progression is inhibited, an intra-S-phase checkpoint is activated that blocks further origin firing and stabilizes existing replication forks to prevent them undergoing irreversible collapse. We show that chromatin incubated in Xenopus egg extracts displays a replication-timing programme in which firing of new replication origins during S phase depends on the continued activity of S-phase-inducing cyclin-dependent kinases. We also show that low concentrations of the DNA-polymerase inhibitor aphidicolin, which only slightly slows replication-fork progression, strongly suppress further initiation events. This intra-S-phase checkpoint can be overcome by caffeine, an inhibitor of the ATM/ATR checkpoint kinases, or by neutralizing antibodies to ATR. However, depletion or inhibition of Chk1 did not abolish the checkpoint. We could detect no significant effect on fork stability when this intra-S-phase checkpoint was inhibited. Interestingly, although caffeine could prevent the checkpoint from being activated, it could not rescue replication if added after the timing programme would normally have been executed. This suggests that special mechanisms might be necessary to reverse the effects of the intra-S-phase checkpoint once it has acted on particular origins.

Key words: Checkpoint, Xenopus, ATR, Replication timing

Introduction
During S-phase of the cell cycle, replication forks are initiated from many replication origins that are activated in a precise temporal order (Diffley, 1998; Gilbert, 2002). In higher eukaryotes, clusters of adjacent origins typically fire nearly synchronously but with different clusters of origins firing at different times throughout S phase (Hand, 1978). In sperm chromatin replicating in cell-free extracts of Xenopus eggs, origins within these clusters are roughly spaced at 5-15 kb intervals, the spacing of origins being independent of the DNA sequence (Blow, 2001; Blow et al., 2001; Hyrien et al., 2003). It is unclear how the time of activation of any individual origin is determined.

At the end of mitosis, each origin becomes licensed for subsequent replication by the sequential loading of the origin recognition complex (ORC), Cdc6, Cdt1 and multiple copies of the Mcm2-7 complex, thus forming a prereplicative complex (pre-RC) (Blow and Hodgson, 2002; Nishitani and Lygerou, 2002). During S phase, the initiation of replication forks at licensed origins is dependent on at least two different protein kinases: S-phase-inducing cyclin-dependent kinases (S-CDKs) and the Cdc7-Dbf4 kinase. These kinases promote the loading of the Cdc45 protein onto origins, creating a pre-initiation complex (Mimura and Takisawa, 1998; Zou and Stillman, 1998; Jares and Blow, 2000).

In the yeast Saccharomyces cerevisiae, Cdc7 activity is required throughout S phase to promote initiation at origins firing at different times (Bousset and Diffley, 1998; Donaldson et al., 1998a). Cyclin-dependent kinases (CDKs) have also been shown to play roles in the replication timing programme in S. cerevisiae (Donaldson et al., 1998b). CDKs are likely to be required throughout S phase to promote initiation (like Cdc7), because the CDK-dependent loading of Cdc45 onto late-firing origins only occurs late in S phase (Aparicio et al., 1999).

Genome stability is maintained by checkpoints that prevent cell-cycle progression if DNA replication is blocked or DNA is damaged (Hartwell and Weinert, 1989; Elledge, 1996). Checkpoint pathways include damage sensors, signal transducers and effectors (Hartwell and Weinert, 1989; Latif et al., 2001; Hutchins and Clarke, 2004). At the top of the signal-transduction pathway are the ATM and ATR kinases, members of the phosphoinositide-3-kinase-like kinase (PIKK) family. Although ATM seems mainly to be activated by DNA double-strand breaks (Khanna and Jackson, 2001; Valerie and Povirk, 2003), ATR can be activated by a range of DNA-damaging agents or by inhibition of replication forks (Cliby et al., 1998; Guo et al., 2000; Liu et al., 2000; Wright et al., 1998). Two further protein kinases, Chk1 and Chk2, are phosphorylated by and act downstream of ATM and ATR kinases.

In S. cerevisiae, checkpoint activation has been shown to have two distinct effects on DNA replication. First, late-firing origins are prevented from initiating replication (Santocanale and Diffley, 1998; Shirahige et al., 1998). Second, pre-existing...
replication forks are stabilized by the checkpoint to prevent them from undergoing irreversible collapse (Lopes et al., 2001; Tercero and Diffl ey, 2001). Similar responses appear to operate in mammalian cells: if DNA synthesis is inhibited, a caffeine-sensitive intra-S-phase checkpoint stabilizes components of existing forks and prevents initiation at late-firing origins (Dimitrova and Gilbert, 2000).

In this report, we show that the replication-timing programme of *Xenopus* requires the ongoing activity of S-CDKs and is responsive to checkpoint inhibition. Inhibition of DNA synthesis using aphidicolin leads to activation of checkpoint pathways that prevents further origin firing. However, we see no evidence for this checkpoint pathway affecting fork stability. We also show that the ATR kinase plays a major role in preventing late origin firing in this system.

Materials and Methods

Preparation and use of extracts

*Xenopus* egg extracts were prepared as described (Chong et al., 1997) and were supplemented with 100 µg ml⁻¹ cycloheximide, 25 mM phosphocreatine and 15 µg ml⁻¹ creatine phosphokinase before use. Metaphase-arrested extracts were released into interphase with 0.3 mM CaCl₂. DNA synthesis was assessed by measuring incorporation of [α³²P]dATP into acid-insoluble material, assuming an endogenous dATP pool of 50 µM (Blow and Laskey, 1986; Chong et al., 1997). Final DNA concentrations in the assays were kept at 10-20 ng DNA (µl extract)⁻¹. All incubations were performed at 23°C.

Antibodies and reagents

Roscovitine, wortmannin and debromohymenialdisine (DBH) were dissolved in dimethyl sulfoxide (DMSO). Roscovitine was used at 0.5 mM, wortmannin at 400-800 nM and DBH at 2-100 µM; in all cases, the final DMSO concentration was <1%. Caffeine dissolved in H₂O was used in 5 mM. Neutralizing X-ATR antibody was a gift from K. Cimprich and was used as described (Costanzo et al., 2003). Antibodies for immunoblotting were as follows: anti-Cdc45 antibody was a gift from H. Takisawa (Mimura and Takisawa, 1998); anti-Rad17 antibody was a gift from H. Lindsay (Costanzo et al., 2003); anti-PCNA (PC10) was from Sigma; anti-human-phospho-Chk1 Ser345 was a gift from H. Lindsay (Costanzo et al., 2003). Antibodies for immunoblotting were as follows: anti-Cdc45 antibody was a gift from H. Takisawa (Mimura and Takisawa, 1998); anti-PCNA (PC10) was from Sigma; anti-human-phospho-Chk1 Ser345 was from Cell Signaling Technology; anti-human-Chk1 (sc-7898) was from Santa Cruz Biotechnology.

The bovine His6-AN-Cyclin A cDNA plasmid was a gift from J. Endicott (Brown et al., 1995). Cyclin A was expressed in BL21DE3 induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 4 hours at 37°C. Bacteria were lysed using Bugbuster (Merck). Cyclin A was solubilized in 8 M urea and purified on Ni-NTA beads (Qiagen) at 37°C. Bacteria were lysed using Bugbuster (Merck). Cyclin A was expressed in BL21DE3 induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 4 hours at 37°C. Bacteria were lysed using Bugbuster (Merck). Cyclin A was solubilized in 8 M urea and purified on Ni-NTA beads (Qiagen) following the manufacturer’s instructions. Urea was removed by dialysis against THED buffer (20 mM Hepes-KOH, 0.03% Triton X-100, 20% ethylene glycol, 150 mM KCl, pH 8).

Immunodepletion

Chk1 was immunodepleted from extract using a protein-A Dynabeads (Dynal Biotechnology) according to the manufacturer’s instructions. Briefly, Dynabeads were washed with 100 mM Hepes, pH 8, and saturated with anti-Chk1 antibody or non-immune-rabbit IgG (control depletion). After removing excess antibody, two rounds of depletion with 50% (v/v) beads were performed at 4°C for 5 minutes each. Dynabeads were recovered and the depleted extracts were stored in liquid N₂ for further use.

Chromatin and nuclear templates

Demembranated *Xenopus* sperm nuclei were prepared as described (Chong et al., 1997). For chromatin analysis, DNA was incubated in extract at 20 ng DNA µl⁻¹. Extract was then diluted 20-fold in nuclear isolation buffer (NIB; 50 mM KCl, 50 mM Hepes-KOH, pH 7.6, 5 mM MgCl₂, 2 mM dithiothreitol (DTT), 0.5 mM spermidine-3HCl, 0.15 mM spermine-4HCl, 1 µg ml⁻¹ each aprotinin, leupeptin and pepstatin) plus 0.1% Triton X-100, and then underlayered with the same buffer containing 15% (w/v) sucrose. Chromatin was pelleted at 4200 g in a swing-out rotor for 5 minutes at 4°C. The supernatant was removed and the sucrose interface washed three times with 150 µl NIB plus 0.1% Triton X-100. The overlying cushion was removed and the chromatin recentrifuged at 10,000 g in a fixed-angle rotor. The chromatin was resuspended in sodium dodecyl sulfate (SDS) sample buffer. For nuclear reisolation assays, nuclear isolation was performed as above except that Triton X-100 was omitted and only the first centrifugation was performed, after which the chromatin was resuspended to 100 ng DNA µl⁻¹ and kept on ice for further use.

For isolation of intact nuclei (Kumagai et al., 1998), extract containing nuclei was diluted tenfold in buffer containing 40% (v/v) sucrose, 50 mM Hepes-KOH, pH 7.5, 100 mM KCl and 2.5 mM MgCl₂, and pelleted at 5000 g in swing-out rotor for 5 minutes at 4°C. The pellet was resuspended in 1 ml of the same buffer and recentrifuged. The supernatant was removed and nuclear samples were resuspended in SDS sample buffer.

Biotin dUTP labelling was performed essentially as described (Blow and Watson, 1987). Biotin-11-dUTP (Roche) was added to extract at 20 µM. At the required time, the extract was diluted tenfold with Buffer A (60 mM KCl, 15 mM Tris-HCl, pH 7.4, 15 mM NaCl, 2 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 1 µg ml⁻¹ each aprotinin, leupeptin and pepstatin) plus 10% sucrose, underlayered with 200 µl Buffer A plus 30% sucrose and spun at 5000 g in a swing-out rotor. Most of the supernatant was removed and the nuclei gently resuspended. Nuclei were supplemented with 0.5 µl Texas Red Streptavidin (Pharmacia) and 2 µg ml⁻¹ Hoechst 33258, incubated at room temperature for 10 minutes, and visualized by fluorescence microscopy.

Alkaline agarose gels

For alkaline agarose-gel analysis, sperm nuclei were incubated at 10 ng µl⁻¹ in egg extract. Reactions were stopped with Stop N (20 mM Tris-HCl, pH 8, 200 mM NaCl, 5 mM EDTA, 0.5% SDS) containing 2 µg ml⁻¹ RNase A. DNA was extracted with phenol:chloroform:isoamyl-alcohol (25:24:1) using Phase Lock Gel tubes (Eppendorf), ethanol-precipitated and resuspended in alkaline loading buffer (25 mM NaOH, 3 mM EDTA, 1.25% Ficoll, 0.0125% bromocresol green). Agarose gels were poured in 50 mM NaCl, 1 mM EDTA and then equilibrated in 50 mM NaCl, 1 mM EDTA for 3 hours. Gels were run at 2 V cm⁻¹ for 14-16 hours at 4°C and then fixed in 7% trichloroacetic acid (w/v), 1.4% (w/v) sodium pyrophosphate for 20 minutes. Gels were dried between sheets of 3MM paper (Whatman) and exposed to X-ray film. As DNA size standards, 25-50 ng of λ-phage HindIII markers (New England Biolabs) end-labelled with [γ-³²P]dATP, were run alongside.

Results

*Xenopus* replication timing programme is driven by S-CDKs

When demembranated sperm nuclei are added to interphase *Xenopus* egg extract, the sperm chromatin decondenses and is assembled into interphase nuclei. S phase begins shortly after nuclear assembly is complete and continues for 20-30 minutes (Blow, 2001). Fig. 1A shows a typical reaction in which replication starts around 30 minutes and is completed by 60-70 minutes. Replication origins can fire at different times during S phase in this system (Herrick et al., 2000; Lucas et
Intra-S-phase checkpoint in *Xenopus* (al., 2000; Blow et al., 2001). To examine the kinetics of this replication-timing programme, we pulse-labelled nascent DNA with $[\alpha-32P]$dATP at different times; nascent DNA was separated on alkaline agarose gels and autoradiographed (Fig. 1B). The first short $[\alpha-32P]$dATP-labelled strands became visible around 35 minutes and increased in size, consistent with the reported fork rate of 10 nucleotides per second (Mahbubani et al., 1992). Small nascent DNA fragments were seen for 20-30 minutes from the start of S phase, consistent with new replication forks initiating throughout most of S phase.

S-CDKs such as Cdk2/cyclin-E and Cdk2/cyclin-A induce the initiation of DNA replication, but are not required for the elongation of forks once they have initiated (Strausfeld et al., 1994; Blow, 2001). We next investigated whether S-CDK activity was required only at the beginning of S-phase or whether it was required throughout S phase as new replication origins fired. The CDK inhibitor roscovitine (Meijer et al., 1997) was added to egg extract at different times throughout S phase and subsequent DNA synthesis measured (Fig. 2A). When roscovitine was added before the start of S phase (20-25 minutes; Fig. 2A, filled triangles and diamonds), there was virtually no DNA synthesis. However, addition of roscovitine during early to mid-S phase (30-40 minutes; Fig. 2A, filled circles, open triangles and diamonds) allowed limited DNA synthesis to occur. Addition of roscovitine in late S phase (50 minutes; Fig. 2A, open circles) had no effect on subsequent DNA synthesis, all replication forks presumably having been initiated by this time.

In the *Xenopus* system, individual nuclei can...
assemble and initiate replication asynchronously (Blow and Watson, 1987). In order to rule out the possibility that the asynchrony in initiation was simply due to asynchrony in the times that different nuclei started to replicate, we repeated the roscovitine addition experiment using biotindUTP instead of $[\alpha-3^2P]dATP$; nuclei were then isolated, the biotinylated DNA stained with fluorescent streptavidin and the proportion of nuclei that had started to replicate was determined by fluorescence microscopy. Fig. 2B shows that >80% of nuclei started replicating 25-30 minutes from the start of the incubation. This means that the asynchrony in initiation shown in Fig. 1B and Fig. 2A largely resulted from asynchrony between different replication origins within individual nuclei, thus representing a replication-timing programme as is seen in other eukaryotes. The partial inhibition of replication seen when roscovitine was added during early S phase (Fig. 2A) suggests that CDK activity is required throughout S phase to support continuing initiation events.

In order to confirm this, roscovitine was added to extracts during early or mid-S phase (35 or 45 minutes) and nascent DNA was pulse-labelled with $[\alpha-3^2P]dATP$ at different times and separated on alkaline gels (Fig. 2C,D). After addition of roscovitine, the smallest labelled DNA molecules moved up the gel at ~10 nucleotides per second [the normal fork rate (Mahbubani et al., 1992)]. This suggests that roscovitine inhibits new initiation events within ~2.5 minutes of being added to extract. Therefore, S-CDK activity is required throughout S phase to drive continuing initiation but is not required for elongation of replication forks (Strausfeld et al., 1994).

Late origin firing is inhibited in response to replication-fork inhibition

In response to replication-fork arrest in *S. cerevisiae* and mammals, ATM/ATR-dependent checkpoint pathways inhibit initiation from late-firing replication origins (Santocanale and Diffley, 1998; Shirahige et al., 1998; Weinert et al., 1994). We next investigated whether replication fork inhibition invokes a similar intra-S-phase checkpoint in *Xenopus*. Fig. 3A,B shows the effect on DNA synthesis of supplementing *Xenopus* extracts with different concentrations of aphidicolin, a competitive inhibitor of replicative DNA polymerases. Replication was significantly inhibited by concentrations of aphidicolin as low as 5 µM and, at 40 µM, inhibition was almost complete. Nascent strands synthesized in the presence of aphidicolin were analysed on alkaline gels (Fig. 3C). Despite the significant inhibition of DNA synthesis seen with 5 µM aphidicolin, nascent strands were of high

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Fig. 3. Aphidicolin induces a caffeine-sensitive replication checkpoint. (A-D) Sperm nuclei were incubated at 15 ng DNA µl$^{-1}$ in interphase egg extract supplemented with $[\alpha-3^2P]dATP$ plus various concentrations of aphidicolin plus or minus 5 mM caffeine. (A) Total DNA synthesis was measured at 120 minutes. (B) DNA synthesis was measured between 15 minutes and 120 minutes as indicated. (C,D) At 120 minutes, DNA was separated on an alkaline agarose gel and autoradiographed. The migration of end-labelled λ-phage HindIII-digested DNA is also shown. (E,F) Sperm nuclei were incubated at 15 ng DNA µl$^{-1}$ for 35 minutes in interphase egg extract supplemented with 7.5 µM aphidicolin. The reaction was split in two and supplemented with 0.5 mM roscovitine minus (E) or plus (F) 5 mM caffeine. At 5 minute intervals, aliquots were pulse labelled for 2 minutes with $[\alpha-3^2P]dATP$ and then DNA was separated on an alkaline agarose gel and autoradiographed. The migration of end-labelled λ-phage HindIII-digested DNA is also shown.
molecular weight, well above the average replicon size of ~10 kb (Blow et al., 2001). This suggests that the primary cause for the inhibition of DNA synthesis at 5 µM aphidicolin is a reduction in the number of active replication forks. Only at higher aphidicolin concentrations was there a clear reduction in nascent strand length. This is consistent with aphidicolin activating an intra-S-phase checkpoint that blocks initiation.

To test this idea, we supplemented extracts with caffeine, an inhibitor of the ATM and ATR kinases (Blasina et al., 1999; Sarkaria et al., 1999). In the absence of aphidicolin, caffeine did not significantly change the overall S-phase kinetics (Fig. 3B, and data not shown). However, caffeine almost completely reversed the inhibition of replication observed with 5 µM aphidicolin (Fig. 3A,B,D). Only at 15 µM and above, at which concentrations aphidicolin had a marked effect on nascent strand length, was caffeine unable to prevent a marked reduction in DNA synthesis. The caffeine-dependent accumulation of short nascent strands in the presence of high aphidicolin concentrations has been previously reported in *Xenopus* (Yanow et al., 2003).

The lengths of nascent strands seen in extracts with and without caffeine were similar, although caffeine caused an increase in the proportion of strands >9 kb long at moderate aphidicolin concentrations (10-15 µM). This increase in strand size could be caused by increased nascent-strand fusion, or by an increased fork rate. We therefore measured the effect of caffeine on the fork rate in the presence of aphidicolin. Sperm nuclei were incubated in extract containing 7.5 µM aphidicolin for 35 minutes (the start of S phase) and then aliquots were supplemented with 0.5 mM roscovitine (to block further initiation) plus or minus 5 mM caffeine. At various times afterwards, aliquots were pulse-labelled with [α-32P]dATP and the nascent strands separated on alkaline agarose gels. Fig. 3E shows that, in the presence of 7.5 µM aphidicolin and no caffeine, nascent strands elongated to a size of ~8 kb after 90 minutes (an average fork rate of ~2.4 nucleotides per second). In the presence of caffeine (Fig. 3F), forks elongated at a similar rate, reaching ~9 kb after 90 minutes (an average fork rate of ~2.7 nucleotides per second). Although caffeine might therefore have a slight effect on the fork rate in the presence of aphidicolin, its major effect is to increase the number of active forks, which in turn will lead to increased strand fusion at later times. These results suggest that the inhibition of DNA synthesis by aphidicolin comprises two distinct components: a checkpoint-dependent inhibition of initiation that predominates at low aphidicolin concentrations and direct inhibition of fork elongation that becomes significant at higher aphidicolin concentrations.

**Aphidicolin reduces the number of active forks in a checkpoint-dependent manner**

We next devised a method to measure the number of active replication forks. Sperm nuclei were incubated in extract with increasing concentrations of aphidicolin; at the beginning of S-phase (35 minutes), the nuclei were transferred to fresh extract containing 0.5 mM roscovitine and [α-32P]dATP but lacking aphidicolin. The roscovitine present in the second extract inhibits further initiation so that the subsequent replication is dependent only on active replication forks present in the nuclei at the time of transfer. This technique showed that 7.5 µM or 15 µM aphidicolin caused a progressive decrease in the number of active replication forks [Fig. 4A; a similar tendency was observed with 40 µM and 120 µM (data not shown)], suggesting that aphidicolin reduced initiation in a concentration-dependent manner. Addition of caffeine to the first extract did not significantly change the number of active forks in the absence of aphidicolin. However, in the presence of caffeine, addition of aphidicolin no longer reduced the number of active forks but instead led to an increase (Fig. 4B). This result is consistent with aphidicolin inducing an intra-S-phase checkpoint that inhibits further origin firing. The increase in the number of active forks seen with a combination of caffeine and aphidicolin might be a consequence of the synchronization of replication forks that this combination of inhibitors is likely to induce (when forks are synchronized near the origin, more replication can occur in the second extract). It has also been shown in yeast that inhibition of checkpoint kinases permits the activation of late-firing and dormant replication origins (Santocanale and Diffley, 1998; Shirahige et al., 1998), and this might also occur in *Xenopus*.

We next investigated whether the timing programme still functions in the presence of aphidicolin. Sperm nuclei were incubated for 35 minutes, 45 minutes or 60 minutes in extract supplemented with 40 µM aphidicolin; nuclei were then isolated and transferred to fresh extract containing roscovitine [α-32P]dATP. Because 40 µM aphidicolin is sufficient to inhibit most fork elongation under these conditions (Fig. 3C,D), any forks initiated will accumulate close to the origins. The results showed a small but significant accumulation of active forks between 35 minutes and 60 minutes in aphidicolin (Fig. 4C), suggesting that the checkpoint slows, but does not abolish, the firing of new replication origins. When this experiment was repeated with both aphidicolin and caffeine in the first extract, a greater increase in the number of active forks was seen between 35 minutes and 60 minutes (Fig. 4D). This suggests that an approximately normal initiation timing programme occurs in the presence of aphidicolin when the checkpoint is abolished by caffeine.

To gain direct evidence for the changes in the number of active forks, we examined proteins assembled onto chromatin in the presence of aphidicolin. Sperm nuclei were incubated in extracts containing increasing concentrations of aphidicolin, plus or minus caffeine; at 50 minutes (mid-S-phase) chromatin was isolated and immunoblotted for Rad17 (a component of the 9-1-1 or RSR damage recognition clamp loader that localizes with DNA-damage foci) (Ellison and Stillman, 2003; Post et al., 2003; Stokes and Michael, 2003) and the pre-initiation/replication-fork protein Cdc45. Fig. 5A shows that the amount of Rad17 on chromatin increased with increasing concentrations of aphidicolin, consistent with increased activation of the checkpoint pathway. As expected, Rad17 recruitment was not inhibited by caffeine. In contrast to Rad17, the amount of Cdc45 loaded onto chromatin was significantly reduced in the presence of 5 µM aphidicolin. This is consistent with our observations that 5 µM aphidicolin can almost fully activate the intra-S-phase checkpoint (Fig. 3). In the presence of both aphidicolin and caffeine, there was a dramatic increase in the amount of Cdc45 loaded onto chromatin, consistent with a previous report (Yanow et al., 2003). This increase might represent Cdc45 loading onto excess Mcm2-7 present at all origins (Mahbubani et al., 1997).
Fig. 5B shows time courses of Cdc45 and PCNA loading onto chromatin in the presence or absence of 15 µM aphidicolin and caffeine. PCNA is a processivity factor for DNA polymerase δ present at replication forks. Aphidicolin induced a dramatic reduction in Cdc45 loading and a less dramatic reduction in PCNA loading. The relatively high levels of PCNA seen in the presence of aphidicolin might reflect the fact that PCNA is involved in DNA repair as well as DNA replication. The reduced levels of Cdc45 and PCNA seen in the presence of aphidicolin remained approximately constant even at later times, suggesting a lack of fork termination. However, in the presence of 15 µM aphidicolin and caffeine, the higher levels of Cdc45 loaded onto chromatin declined at later times, which suggests that, consistent with the high levels of DNA synthesis observed (Fig. 3), fork termination can occur under these conditions. By contrast, PCNA levels remained surprisingly high on the chromatin in the presence of aphidicolin (Fig. 5B). This might reflect ongoing DNA repair that is required after DNA synthesis has occurred in the presence of aphidicolin. Some of the chromatin-bound PCNA in the presence of aphidicolin and caffeine showed a retarded electrophoretic mobility, consistent with the ubiquitination of PCNA that occurs during post-replicative repair (Hoege et al., 2002).

Neither aphidicolin nor caffeine destabilize active replication forks

Our results have so far shown that aphidicolin induces a
caffeine-sensitive reduction in the number of active replication forks in *Xenopus* egg extracts, and are consistent with an intra-S-phase checkpoint that inhibits initiation. However, an alternative explanation might be that aphidicolin reduces the number of active forks by inactivating and disassembling them. In order to distinguish between these possibilities, sperm nuclei were incubated in extract for 40 minutes (mid-S-phase) to allow some origins to fire, and then aphidicolin to a final concentration of 40 µM was added (high enough to inhibit significant fork progression) and roscovitine (to prevent further initiation). At various times afterwards, nuclei were isolated and the number of active replication forks assessed by analysing the rate of replication in fresh extracts supplemented with [α-32P]dATP and roscovitine (Fig. 6A). Fig. 6B shows that the replication forks remained fairly stable in the presence of aphidicolin, with more than >75% activity remaining after a 90 minute incubation. Results consistent with this were also obtained when fork elongation in the presence of aphidicolin was analysed on alkaline agarose gels (data not shown). These results suggest that fork instability is not a major factor in our experiments and cannot explain the decreased number of active replication forks that are seen in the presence of aphidicolin.

Work in yeast and mammalian cells has indicated that, in addition to inhibiting further initiation, checkpoint pathways play an important role in stabilizing replication forks under conditions where replication is blocked (Dimitrova and Gilbert, 2000; Tercero and Diffley, 2001; Lopes et al., 2001; Tercero et al., 2003). *S. cerevisiae* cells lacking *MEC1* (an ATR homologue) or *RAD53* (a Chk2 homologue) are unable to recover from a replication block because replication forks collapse irreversibly (Tercero and Diffley, 2001; Lopes et al., 2001). We therefore investigated whether we could see a similar checkpoint-dependent stabilization of replication forks in *Xenopus*. The experiments shown in Fig. 6A,B were repeated but, this time, the first extract contained caffeine in addition to aphidicolin. Fig. 6C shows that following transfer to fresh extract supplemented with [α-32P]dATP and roscovitine, nuclei previously arrested for up to 45 minutes with aphidicolin and caffeine showed no significant loss of functional replication forks.

This experiment involved inhibiting new initiation events with the CDK inhibitor roscovitine, so this formally left open the possibility that CDK activity is required to destabilize the arrested forks when the replication checkpoint is inhibited. In order to address this, we incubated sperm nuclei in untreated egg extract and then, just at the start of S phase (35 minutes), added 7.5 µM aphidicolin and caffeine, plus or minus roscovitine. At different times thereafter, nascent DNA was pulse labelled with [α-32P]dATP and the nascent DNA analysed on an alkaline agarose gel (Fig. 6D). In the absence of roscovitine, the nascent strands elongated at a reduced rate of ~3 nucleotides per second (compared with 10 nucleotides per second in the absence of aphidicolin). This rate was maintained over the full 15 minute time course, consistent with the replication forks remaining active over this time. In addition, new nascent strands were initiated over this time, because the checkpoint had been blocked by caffeine. When roscovitine was added to the extract at the same time as aphidicolin, the existing forks elongated at a similar rate and extent to that seen in the absence of caffeine, suggesting that CDK activity had no major effect on fork stability. However, roscovitine clearly prevented new origins from firing, as evidenced by the lack of short nascent strands at later times (Fig. 6D, right). Taken together, these results suggest that, when inhibited by aphidicolin, replication forks remain fairly stable in *Xenopus* extracts irrespective of the activity of the ATM/ATR or S-CDK kinases.

Fig. 6. Fork stability is not significantly affected by the intra-S-phase checkpoint. (A-C) Sperm nuclei were incubated at 10 ng DNA µl⁻¹ in *Xenopus* extract. After 35 minutes (early S phase) extract was supplemented with 40 µM aphidicolin and 0.5 mM roscovitine minus (B) or plus (C) 5 mM caffeine. 0 minutes, 10 minutes, 25 minutes or 55 minutes after this, nuclei were isolated and transferred to fresh extract supplemented with 0.5 mM roscovitine and [α-32P]dATP. Total DNA synthesis was measured at different times after transfer. A schematic outline of the experiment is shown in A. (D) Sperm nuclei were incubated at 10 ng DNA µl⁻¹ in extract. At 35 minutes, the extract was supplemented with 7.5 µM aphidicolin and 5 mM caffeine, minus or plus 0.5 mM roscovitine. At the indicated times, samples were pulse labelled with [α-32P]dATP for 2 minutes, the DNA was isolated and analysed by agarose electrophoresis and autoradiography. The migration of end-labelled λ-phage HindIII-digested DNA is also shown.
Replication checkpoint involves ATR and S-CDKs

We have shown that the intra-S-phase checkpoint in *Xenopus* blocks the loading of Cdc45 onto chromatin (Fig. 5B). Because S-CDK activity is required for Cdc45 recruitment to chromatin (Mimura and Takisawa, 1998; Zou and Stillman, 1998; Jares and Blow, 2000) and we have shown that the replication-timing programme in *Xenopus* is driven by S-CDKs (Fig. 2), we investigated the potential role of S-CDK inhibition in the checkpoint response. We first checked whether caffeine-induced recovery from the checkpoint required CDK activity. Sperm nuclei were added to *Xenopus* egg extracts supplemented with 15 μM aphidicolin and [α-32P]dATP; then, at various times, the extract was supplemented with caffeine (to block the checkpoint) plus or minus roscovitine, and the total amount of DNA synthesised at 2 hours was measured (Fig. 7A). As expected, when caffeine was added to the extract before the onset of S phase, replication was efficiently rescued. Efficient rescue also occurred when caffeine was added up to 40 minutes (mid-S phase), but rescue rapidly tailed off, so that, when caffeine was added at 50 minutes, there was virtually no rescue of replication. The timescale of the decline in caffeine’s ability to rescue the aphidicolin-dependent block of replication is similar to that of the replication-timing programme itself. This is consistent with caffeine being able to prevent activation of the checkpoint that blocks initiation but being unable to reverse this block once it has been implemented. When both caffeine and roscovitine were added together, there was virtually no rescue of replication for any time (Fig. 7A). This suggests that the checkpoint inhibits replication at a stage before S-CDK activity, consistent with the observed inhibition of Cdc45 loading.

We next investigated whether we could overcome the intra-S-phase checkpoint by increasing S-CDK activity. When cyclin A is added to *Xenopus* egg extract, it activates the large pool of free Cdk1 and provides S-CDK activity, although higher cyclin A additions induce nuclear envelope breakdown and the onset of mitosis (Strausfeld et al., 1996). We therefore performed a replication assay in the presence of 10 μM aphidicolin, with or without caffeine, and increasing concentrations of recombinant cyclin A (Fig. 7B). In the absence of caffeine, only a slight rescue of replication was detected with increasing concentrations of cyclin A (Fig. 7B, diagonal shading). At the highest cyclin-A concentration (1000 nM), nuclear envelope breakdown occurred, showing that functional CDK activity was generated in the extract. In the presence of both aphidicolin and caffeine, cyclin A showed a dose-dependent stimulation of DNA synthesis (Fig. 7B, black bars). These results suggest that, although S-CDK activity is required to exit from the checkpoint response, inhibition of S-CDK activity is unlikely to be the sole cause of the checkpoint-induced inhibition of initiation.

We next investigated which kinase pathways are involved in the intra-S-phase checkpoint. We performed replication assays in the presence of aphidicolin and caffeine, wortmannin, or a neutralizing antibody for *Xenopus* ATR (Costanzo et al., 2003). The anti-ATR antibody rescued DNA-replication inhibition to levels >60% of those seen with caffeine (Fig. 8A), suggesting that ATR plays a major role in the intra-S-phase checkpoint in *Xenopus*. By contrast, wortmannin was not able to rescue the inhibition of replication induced by even low concentrations of aphidicolin (Fig. 8B) and failed to rescue the aphidicolin-induced inhibition of Cdc45 loading onto chromatin (Fig. 8C). Because wortmannin inhibits ATM and DNA-dependent protein kinase (DNA-PK) more strongly than ATR (Sarkaria et al., 1998), the inability of wortmannin to rescue aphidicolin-dependent block of replication suggests that ATM does not play an essential role in the intra-S-phase checkpoint. A combination of the anti-ATR antibody and wortmannin did not enhance the rescue above that seen with the anti-ATR antibody alone (data not shown).

The ATM/ATR kinases mediate many of their checkpoint functions by activating the downstream kinases Chk1 and Chk2. ATM/ATR activate *Xenopus* Chk1 by phosphorylating it on Ser344 (equivalent to Ser345 in human Chk1) (Guo et al., 2000; Liu et al., 2000; Capasso et al., 2002). We used a phosphorylation-specific antibody capable of recognizing phospho-Ser344 on *Xenopus* Chk1 to monitor its activation. Concentrations of aphidicolin as low as 5 μM were enough to
cause phosphorylation of Chk1 on Ser344 in egg extract (Fig. 8D and data not shown). Aphidicolin-induced phosphorylation of endogenous Chk1 was prevented by either caffeine or wortmannin. Because wortmannin blocks the phosphorylation of Chk1 but does not functionally inhibit the checkpoint, this suggests that Chk1 is not an essential component of the checkpoint response. DBH is a relatively weak inhibitor of both Chk1 and Chk2 kinases (IC$_{50}$=3 µM) (Curman et al., 2001), and was unable significantly to restore DNA synthesis to extracts treated with 12 µM aphidicolin (Fig. 8E). Finally, we immunodepleted extracts of Chk1 and examined the sensitivity of the depleted extract to aphidicolin (Fig. 8F,G). DNA replication in Chk1-depleted extracts was still sensitive to 10 µM aphidicolin and could be rescued by the addition of caffeine. Taken together, these results suggest that the intra-S-phase checkpoint is largely mediated by ATR, not ATM, and that, although there might be a minor involvement of Chk2 and/or Chk1, they are not major mediators of the response.

Discussion

Xenopus egg extracts represent a versatile system to analyse cell-cycle control of DNA replication (Blow, 2001). In this report, we have taken advantage of this system to investigate the regulation of the replication-timing programme. Our data show that aphidicolin induces an intra-S-phase checkpoint that results in a block to late origin firing but does not significantly affect fork stability. We provide evidence that the ATR kinase is the most likely mediator of this checkpoint.

Fig. 8. ATR is necessary for the aphidicolin-induced intra-S-phase checkpoint. (A,B) Sperm nuclei were incubated at 15 ng DNA µl$^{-1}$ in extract supplemented with [α-32P]dATP, and various concentrations of aphidicolin, plus or minus 5 mM caffeine and (A) an antibody neutralizing the ATR kinase (α-XATR) or (B) 800 nM wortmannin. At 120 minutes total DNA synthesis was measured. As control, aphidicolin was substituted with DMSO. (C,D) Sperm nuclei were incubated at 15 ng DNA µl$^{-1}$ in extract supplemented with 0 µM, 15 µM or 120 µM aphidicolin, plus or minus 800 nM wortmannin or 5 mM caffeine. (C) At 50 minutes (mid-S-phase), chromatin was isolated and immunoblotted for Cdc45 and PCNA. (D) At 60 minutes, intact nuclei were isolated and immunoblotted for phospho-Ser344-Chk1. (E) Sperm nuclei were incubated at 15 ng DNA µl$^{-1}$ in extract supplemented with 12 µM aphidicolin plus or minus 5 mM caffeine or 5 µM DBH. At 120 minutes, total DNA synthesis was measured. (F,G) Extract was immunodepleted with anti-Chk1 antibodies. (F) Immunoblotting of whole nuclei assembled in either untreated extract, mock depleted or Chk1-depleted extracts showed the removal of Chk1. '?' indicates an unknown cross-reacting protein. (G) Sperm nuclei were incubated at 15 ng DNA µl$^{-1}$ in Chk1-depleted extract, non-immune-depleted extract or untreated extract, all supplemented with [α-32P]dATP and combinations of 10 µM aphidicolin and/or 5 mM caffeine. At 120 minutes, total DNA synthesis was measured.

Replication-timing programme in the Xenopus early embryo

In Xenopus, replication origins are spaced roughly 5-15 kb apart and are organized into small clusters (typically containing between two and ten origins) that fire almost synchronously (Blow et al., 2001). However, different clusters are activated at different times in S phase. Consistent with this, we show here that short nascent DNA strands appear throughout most of S phase. Using roscovitine, a specific CDK inhibitor, we have found that, when CDK activity is inhibited during S phase, subsets of origins fail to initiate and DNA replication cannot be completed. Moreover, we show that new initiation events are blocked within minutes of the addition of roscovitine, suggesting that the CDK execution point is very close to the time at which origins are activated. By contrast, Xenopus Cdc7 acts on replication origins before the S-CDKs and can act on a significant subset of replication origins before the onset of S phase (Jares and Blow, 2000; Walter, 2000). Taken together, these results indicate that S-CDKs play a key role in driving the replication timing programme in Xenopus. Moreover, the observation that addition of caffeine to the extract does not
significantly change S phase kinetics suggests that, in the absence of replication inhibitors, the normal replication programme does not require the activity of checkpoint kinases.

Aphidicolin induces a caffeine-sensitive checkpoint that prevents Cdc45 recruitment and inhibits origin firing

We show that low concentrations of aphidicolin (5-15 μM), which lower the rate of fork progression only slightly, dramatically inhibit DNA replication. This occurs as a consequence of a reduction in the total number of active forks and a reduction in the quantity of fork-associated proteins present on the DNA. All these effects can be reversed by caffeine and appear to be due to an ATR-dependent intra-S-phase checkpoint pathway. The checkpoint we describe here is clearly distinct from a previous effect noted in Xenopus, in which very high concentrations of aphidicolin (~300 μM) blocked the firing of replication origins in a caffeine-insensitive manner (Marheineke and Hyrien, 1991).

In S. cerevisiae, Rad53 (a Chk2 homologue) is required to maintain stable replication forks properly, because rad53 mutants are unable to recover from a replication block (Lopes et al., 2001). Moreover, replication forks in checkpoint mutants collapse irreversibly in the presence of DNA damage (Tercero and Diffley, 2001). However, we find no evidence for significant destabilization of forks in the Xenopus system. We have investigated fork stability by analysing nascent strands on alkaline gels, by using a nuclear re-isolation assay and by immunoblotting chromatin for replication-fork proteins. In none of these experiments did we see evidence for significant destabilisation of forks, whether or not the checkpoint response had been inhibited with caffeine. It is, however, possible that replication forks transiently stall and then resume, leaving little permanent evidence of the event. We therefore conclude that the major function of the intra-S-phase checkpoint in Xenopus egg extracts is to inhibit the initiation of further replication forks. It should, however, be noted that, owing to the limitations of cell-free extracts, we have only been able to investigate fork stability over a couple of hours, which is a shorter period than has been investigated for living yeast cells. It therefore remains possible that, over longer time periods, checkpoint kinases might play a role in stabilizing forks in Xenopus.

DNA replication intra-S checkpoint involves ATR not ATM

The intra-S-phase checkpoint described in this report is sensitive to caffeine but not wortmannin. Both caffeine and wortmannin are inhibitors of the phosphoinositide-3-kinase family but wortmannin has a greater specificity for ATM than for ATR (Sarkaria et al., 1998). Even though wortmannin efficiently blocked Chk1 activation by aphidicolin, it neither significantly rescued the inhibition of DNA replication nor allowed Cdc45 to be recruited onto chromatin. By contrast, a neutralizing antibody against Xenopus ATR kinase (Lupardus et al., 2002; Costanzo et al., 2003) was also able to revert the aphidicolin-induced block to DNA replication, suggesting that the intra-S-phase checkpoint depends on ATR activity. This is consistent with results from other organisms suggesting that ATR mediates the checkpoint response to the inhibition of DNA replication (Zhao and Elledge, 2000; Cortez et al., 2001; Osborn et al., 2002).

Downstream from the ATR kinase in the checkpoint pathway are the checkpoint kinases Chk1 and Chk2. In Xenopus, aphidicolin-induced inhibition of S phase leads to activation of ATR and Chk1, and inhibits entry into mitosis (Michael et al., 2000). In mammalian cells, an aphidicolin-induced checkpoint that blocks late origin firing (Dimitrova and Gilbert, 2000) could be overcome with UCN-01, an inhibitor of Chk1 (Feijoo et al., 2001). This suggests that, in mammalian cells, the intra-S-phase checkpoint that blocks late origin firing is mediated by Chk1. However, other related kinases inhibited by UCN-01 (e.g. Hutchins et al., 2003; Komander et al., 2003) could also be involved.

Although we detected Chk1 phosphorylation in response to low levels of aphidicolin, three lines of evidence suggest that Chk1 does not play an essential role in the intra-S-phase checkpoint pathway we describe here. First, Chk1 depletion showed no sign of abolishing the checkpoint. Second, even though wortmannin efficiently blocked Chk1 activation in response to aphidicolin, Cdc45 still failed to be recruited onto chromatin and there was little rescue of replication. Third, we observed no significant rescue of the aphidicolin-induced replication block in the presence of DBH, an inhibitor of the Chk1 and Chk2 kinases (Curman et al., 2001). We therefore believe that Chk1 is not essential for the intra-S-phase checkpoint in Xenopus. The lack of rescue seen with DBH, and the fact that Chk2 is typically activated by ATM not ATR, also raises doubts about whether Chk2 plays an essential role in the checkpoint we describe here. Instead, the Xenopus intra-S-phase checkpoint might be mediated directly by ATR.

The targets of the intra-S-phase checkpoint are currently unclear. Chk1/Chk2-dependent checkpoints inhibit the dual-specificity phosphatase Cdc25, thereby maintaining the inhibitory phosphorylation of the CDK subunit at Thr14/Tyr15 and blocking CDK activation (Costanzo et al., 2000; Falck et al., 2001). However, we failed to rescue the checkpoint by addition of high levels of cyclin A, suggesting that the checkpoint does not depend solely on inhibition of S-CDKs. CDK activity was required to exit from the checkpoint-induced arrest, suggesting that initiation had been blocked at a point upstream of CDK function. These results are consistent with the intra-S-phase checkpoint inhibiting the Cdc7-Dbf4 kinase, because, in Xenopus, Cdc7 acts upstream of S-CDKs (Jares and Blow, 2000; Walter, 2000). In Schizosaccharomyces pombe, Hsk1/Cdc7 phosphorylation in response to replication inhibition by hydroxyurea is dependent on the Chk2 homologue Cds1 (Brown and Kelly, 1999; Snaith et al., 2000). Similarly, S. cerevisiae and S. pombe Rad53/Cds1 phosphorlyates Dbf4/Dfp1 and causes dissociation of the complex from the chromatin (Weinreich and Stillman, 1999; Snaith et al., 2000; Pasero et al., 1999). In Xenopus, the topoisomerase-II inhibitor etoposide induces an ATR-dependent checkpoint that inactivates the Cdc7-Dbf4 complex (Costanzo et al., 2003). Treatment of egg extracts with aphidicolin also results in accumulation of an alternative Cdc7 regulatory subunit Drf1 onto chromatin; this accumulation is blocked by addition of caffeine and by immunodepletion of either ATR or claspin (Yanow et al., 2003). Investigation of the role of these proteins in the Xenopus intra-S-phase checkpoint would therefore be of interest.
Ability of caffeine to rescue the checkpoint declines in parallel with the timing programme

We have shown that, when added at the start of a reaction, caffeine could efficiently prevent the inhibition of initiation induced by low concentrations of aphidicolin. However, we also found that the ability of caffeine to rescue DNA replication declined with time. When caffeine was added in late S phase, it was almost completely unable to restore synthesis to reactions in which replication had been blocked with low concentrations of aphidicolin. There was a close parallel between the period over which the ability of caffeine to rescue replication declined and the time over which the replication-timing programme is executed. One possible explanation for this behaviour is that the intra-S-phase checkpoint inhibits origins as they are about to be brought into play by the replication-timing programme and that, although caffeine can block the pathway that inhibits initiation, it cannot reverse this inhibition once it has occurred. This would be consistent with a model in which the inhibition of initiation proteins by ATR is either irreversible or its reversal requires a special mechanism in addition to simply turning off the kinase activity.

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