

DNA damage-induced replication arrest in *Xenopus* egg extracts

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Chromosomal replication is sensitive to the presence of DNA-damaging alkylating agents, such as methyl methanesulfonate (MMS). MMS is known to inhibit replication through activation of the DNA damage checkpoint and through checkpoint-independent slowing of replication fork progression. Using *Xenopus* egg extracts, we now report an additional pathway that is stimulated by MMS-induced damage. We show that, upon incubation in egg extracts, MMS-treated DNA activates a diffusible inhibitor that blocks, in trans, chromosomal replication. The downstream effect of the inhibitor is a failure to recruit proliferating cell nuclear antigen, but not DNA polymerase α , to the

nascent replication fork. Thus, alkylation damage activates an inhibitor that intercepts the replication pathway at a point between the polymerase α and proliferating cell nuclear antigen execution steps. We also show that activation of the inhibitor does not require the DNA damage checkpoint; rather, stimulation of the pathway described here results in checkpoint activation. These data describe a novel replication arrest pathway, and they also provide an example of how subpathways within the DNA damage response network are integrated to promote efficient cell cycle arrest in response to damaged DNA.

Introduction

Chromosomal replication must occur in an error-free manner if cells are to faithfully propagate their genetic material. DNA damage, by either endogenous or exogenous agents, represents a major impediment to faithful replication. There are many ways that damaged templates are harmful to the replication process: damage can cause incorporation of improper nucleotides, which can lead to mutations; and it can stop the process outright as some lesions impose physical blockades to polymerase (pol) progression (Friedberg et al., 1995). Because the replication process is sensitive to the presence of damage, cells have evolved sophisticated damage response networks that shut down DNA replication when damage is present. A detailed understanding of how these networks function will be critical to a complete understanding of how cells cope with genotoxic stress.

Thus far, two mechanisms have been described that regulate DNA replication in response to damage. The first involves activation of DNA damage checkpoint pathways (for review see Nyberg et al., 2002). In metazoan cells, damage checkpoints are mediated by two key regulators, the ataxia-telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) protein

kinases (for review see Abraham, 2001). Activation of ATM/ATR by damaged DNA sets in motion signaling cascades that ultimately block DNA replication. The checkpoint blocks replication by preventing usage of origins of replication (Santocanale and Diffley, 1998; Shirahige et al., 1998; Costanzo et al., 2000; Falck et al., 2001; Costanzo et al., 2003), and the replication targets at the origin are now being defined. In human cells, ATM-mediated attenuation of the S-phase Cdk (S-Cdk) that initiate DNA replication is a major pathway that prevents origin firing in response to double-strand breaks (Falck et al., 2001). In *Xenopus*, ATM-mediated attenuation of S-Cdk has been documented previously (Costanzo et al., 2000), as has ATR-mediated negative regulation of another protein kinase required to initiate replication, Cdc7 (Costanzo et al., 2003).

Work in budding yeast has described recently a second mechanism through which damaged DNA controls replication (Tercero and Diffley, 2001). By monitoring replication fork advancement, Tercero and Diffley (2001) found that methyl methanesulfonate (MMS) causes a significant reduction in the rate of replication fork progression. Thus, damaged

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Abbreviations used in this paper: ATM, ataxia-telangiectasia mutated; ATR, A-T and Rad3 related; bio-dUTP, biotinylated-dUTP; MCM, minichromosome maintenance complex; MMS, methyl methanesulfonate; NPE, nucleoplasmic extract; ORC, origin recognition complex; PCNA, proliferating cell nuclear antigen; pol, polymerase; pre-IC, preinitiation complex; pre-RC, prereplication complex; RFC, replication factor C.

DNA can signal a block to the firing of replication origins, and it can slow down fork progression on preestablished replication forks. The former mechanism is dependent on an intact damage checkpoint, whereas the latter mechanism operates independently of checkpoint control. It is not currently understood if the reduction in the rate of fork progression is the result of an active signaling pathway, or if the MMS-induced lesions passively impose a physical blockade to replication fork advancement.

Damaged DNA negatively affects replication at both early (origin firing) and late (fork stalling) steps in the pathway, but there are many other possible points of intervention. Chromosomal replication occurs through an ordered series of initiation and elongation reactions (for review see Bell and Dutta, 2002; Blow and Hodgson, 2002; Diffley and Labib, 2002). Initiation begins in late mitosis when origin recognition complex (ORC) nucleates the assembly of a prereplication complex (pre-RC) on origins of replication. Besides ORC, the known pre-RC components include Cdc6, Cdt1, and the minichromosome maintenance complex (MCM). The transition from a pre-RC to a preinitiation complex (pre-IC) occurs as cells cross the G1/S boundary and involves the action of two protein kinases, S-Cdk and Cdc7-dbf4. Pre-IC assembly is considered complete when the Cdc45 protein binds to the complex. Upon Cdc45 binding, DNA unwinding commences and the single-stranded DNA binding protein replication protein A coats the unwound template strand. DNA replication initiates when pol α is recruited to the template strand and synthesizes an RNA-DNA hybrid primer. After primer synthesis, the replication clamp loader, replication factor C (RFC), binds to the primed site and loads the clamp protein proliferating cell nuclear antigen (PCNA). PCNA, a homotrimeric protein involved in many aspects of DNA metabolism, functions during replication as a processivity factor for DNA pol δ , allowing elongation synthesis to occur in an uninterrupted manner for long distances.

Our laboratory has been using *Xenopus* egg extracts to study how DNA damage response pathways and DNA replication pathways are integrated so that replication and cell cycle progression can be coordinated with the repair of damaged DNA (Michael et al., 2000; Stokes et al., 2002; Van Hatten et al., 2002). In a previous paper (Stokes et al., 2002), we found that MMS-induced damage checkpoint activation requires the assembly of replication forks. This finding suggested that the checkpoint does not directly sense MMS-induced lesions; rather, the stalled replication forks that form in response to the damage are the signals sensed by the checkpoint (Stokes et al., 2002). During the course of this analysis, we observed that when MMS-treated sperm chromatin was coincubated with undamaged sperm chromatin in the same extract, neither the damaged nor undamaged chromatin was replicated efficiently. This indicated that the MMS-treated sperm chromatin was influencing the replication of the undamaged chromatin, in a manner possibly analogous to one or both of the mechanisms described above. Here, we pursue this initial observation by analyzing in detail how damaged DNA influences replication of undamaged DNA. Our results show that, in frog egg extracts, MMS-damaged DNA controls replication of undamaged

DNA through a novel mechanism that is distinct from both checkpoint-dependent block to origin firing, and the slowing of replication fork progression.

Results

Inhibition of sperm chromatin replication by MMS-treated plasmid DNA molecules

Previous work has shown that exposure of sperm chromatin to MMS caused a delay in the replication of that chromatin upon subsequent incubation in *Xenopus* egg extracts (Stokes et al., 2002). Furthermore, we found that coincubation of MMS-treated sperm chromatin with undamaged sperm chromatin caused a replication delay on both the damaged and undamaged templates. To more precisely determine how damaged DNA affects the replication of undamaged DNA, we used the *Xenopus* nucleus-free nucleoplasmic extract (NPE) system (Walter et al., 1998). To use NPE for replication studies, sperm chromatin templates are first incubated in a soluble cytosolic extract, termed egg cytosol, so that chromatin remodeling and pre-RC assembly can occur (Fig. 1 A). After incubation in egg cytosol, replication is triggered through the addition of a nuclear extract, termed NPE. Upon addition of NPE, origins are fired in a synchronous manner, and a single, complete round of replication occurs (Walter et al., 1998). An attractive feature of the NPE system is that it is completely soluble. Thus, the absence of nuclear envelopes that normally partition DNA templates from one another allows a unique opportunity to analyze how the presence of damaged DNA templates affect the replication of undamaged DNA. Critical to the analysis, however, is a means to specifically distinguish the damaged from undamaged DNA, and to also physically separate the two after incubation in NPE. To accomplish this, we coincubated MMS-treated plasmid DNA molecules with undamaged sperm chromatin templates and monitored replication of the sperm chromatin templates using a visual assay (Fig. 1 B). The large size difference between sperm chromatin templates and plasmid molecules allowed us to specifically monitor replication of sperm chromatin, as the plasmid molecules are too small to be visualized under these conditions. To visualize replication of the sperm chromatin templates, biotinylated-dUTP (bio-dUTP) was added to NPE during the replication reaction, and, after incubation, the samples were fixed and stained with fluorescent streptavidin to monitor uptake of the bio-dUTP. As a control, we also coincubated sperm chromatin with undamaged plasmid molecules. As shown in Fig. 1 C, addition of MMS-treated plasmid DNA resulted in a dose-dependent reduction in replication of sperm chromatin. This is inferred from the reduction in fluorescent intensity of the samples containing the damaged plasmid, relative to the sample that received the undamaged plasmid. After quantification of the data, we found that inclusion of 3 ng/ μ l alkylated plasmid reduced replication of sperm chromatin (present at 8 ng/ μ l in all samples) to, on average, 27% of the reaction that did not receive any plasmid (Fig. 1 D). Importantly, addition of 3 ng/ μ l undamaged plasmid had no effect on sperm chromatin replication. We conclude that the MMS-treated plasmid, but not the undamaged plasmid, blocked replication of

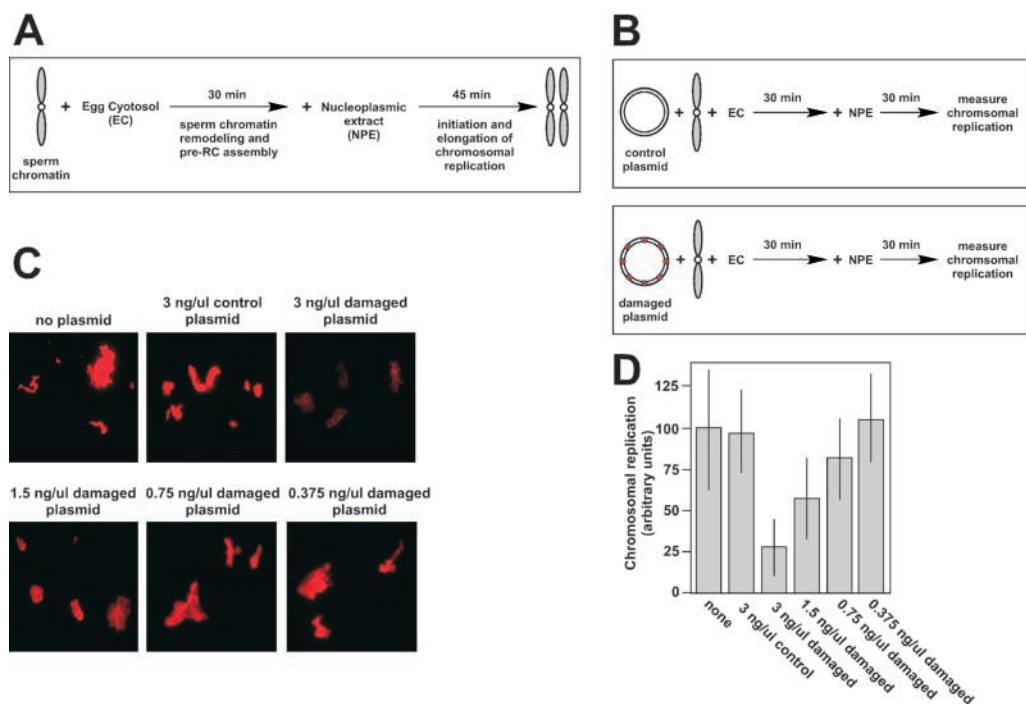


Figure 1. Dose-dependent inhibition of sperm chromatin replication by MMS-treated plasmid DNA. (A) Schematic depiction of the NPE DNA replication system. See Results for details. (B) Experimental design. Either control, undamaged plasmid DNA, or MMS-treated, damaged plasmid DNA, was coincubated in the NPE system with sperm chromatin. Replication of the sperm chromatin templates was then assessed. (C) The indicated amount of plasmid DNA was mixed with sperm chromatin, which was present at a final concentration of 2,000/ μ l in all samples. After incubation in egg cytosol for 30 min, NPE containing bio-dUTP was added and incubation was continued for an additional 30 min. The samples were fixed and stained with Texas red–labeled streptavidin, according to established procedures (Stokes et al., 2002), and viewed under a fluorescence microscope. The relative fluorescent intensity of each sample is an indicator of the efficiency of DNA replication. (D) Quantification of the data shown in C was achieved through measurement of the fluorescent intensity of 50 individual sperm chromatin templates for each experiment, using the Scion Image software package from images obtained from a fluorescent microscope attached to a Spot camera. The bars represent mean averages for each experiment, and the error bars refer to one SD from that mean.

sperm chromatin DNA. This result is consistent with our previous finding that MMS-treated sperm chromatin inhibited replication of undamaged sperm chromatin after incubation in the same NPE (Stokes et al., 2002).

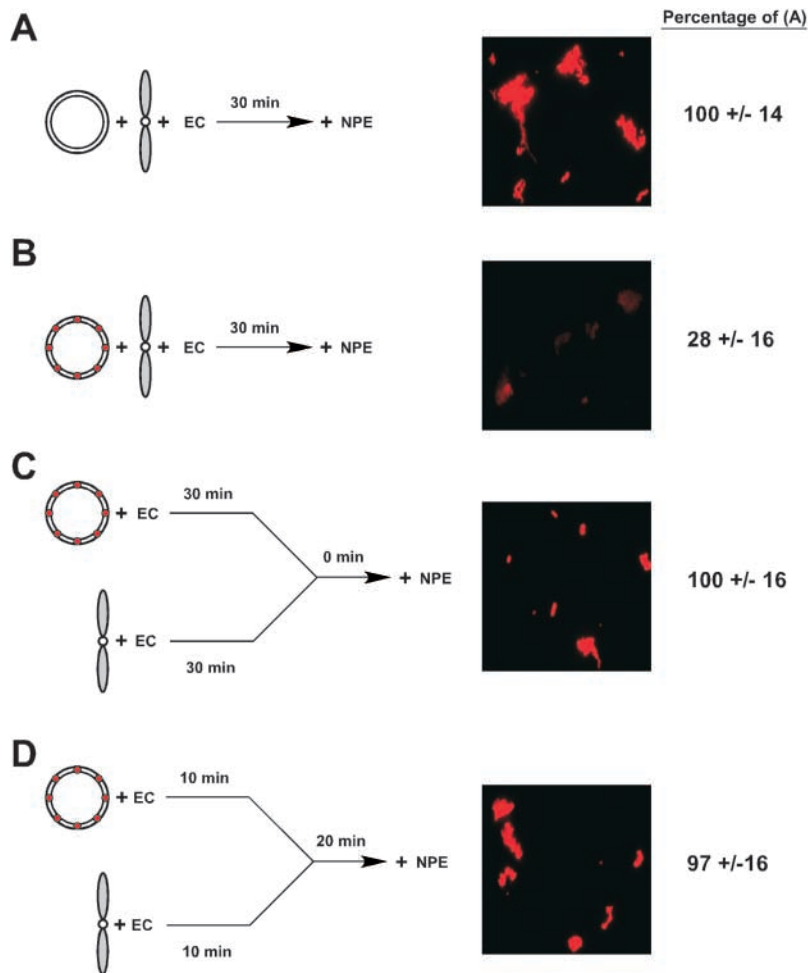
To better understand how the damaged plasmid DNA negatively affected replication of sperm chromatin, we asked which phase in the replication reaction was sensitive to the presence of the damaged plasmid. For this, we added sperm chromatin to egg cytosol and varied the time of addition of the damaged plasmid. The amount of sperm chromatin replication was measured, and the values were normalized to the replication observed in a control reaction containing undamaged plasmid (the control reaction is shown in Fig. 2 A). As was the case in Fig. 1, if both damaged plasmid and sperm chromatin are added simultaneously, then replication of the sperm chromatin was suppressed ($28 \pm 16\%$ of control; Fig. 2 B). Interestingly, if the damaged plasmid and sperm chromatin were incubated separately in egg cytosol, and the two templates were combined just before addition of NPE, then the damaged plasmid did not suppress sperm chromatin replication ($100 \pm 16\%$ of control; Fig. 2 C). Sperm chromatin replication also occurred normally if the damaged plasmid and sperm chromatin were separated for just the first 10 min of the egg cytosol incubation, and then combined for 20 min before addition of NPE ($97 \pm 16\%$ of control; Fig. 2 D). This indicates that replication of the

sperm chromatin is only sensitive to the presence of the damaged plasmid for, maximally, the first 10 min of incubation in egg cytosol. The experiment shown in Fig. 2 makes two important points. (1) The presence of the alkylated plasmid does not nonspecifically poison the sperm chromatin replication reaction. If this were the case, then replication should be inhibited no matter when the damaged plasmid was added, as in all cases the damaged plasmid was added before the initiation of replication (which only occurs upon addition of NPE). (2) The 10-min window of opportunity for the damaged plasmid to inhibit replication indicates that the step in chromosomal replication that is blocked by the damaged plasmid occurs very early in the process.

The data in Figs. 1 and 2 demonstrate that sperm chromatin replication is sensitive to the presence of damaged, but not undamaged, plasmid DNA. In addition, the data show that sperm chromatin must be exposed to the damaged plasmid very early in the replication process in order for the inhibition to occur. To further characterize this negative regulation, we next asked if transient exposure of egg cytosol to the damaged plasmid was sufficient to inactivate the extract toward sperm chromatin replication, or if continuous exposure of egg cytosol to the damaged plasmid was required for the inhibition to occur. For this, we incubated egg cytosol with either damaged or undamaged plasmid DNA that had been immobilized on magnetic

Figure 2. The block to replication of sperm chromatin is dependent on when the sperm chromatin is exposed to the damaged plasmid.

(A) Undamaged plasmid DNA or (B–D) MMS-treated plasmid DNA was mixed with egg cytosol (EC) at 3 ng/ μ l. In A and B, the plasmids were incubated together with the sperm chromatin (2,000/ μ l in all cases) for the entire 30-min incubation in EC. In C, the plasmid and sperm chromatin were incubated separately in EC and combined just before addition of NPE. In D, the plasmid and sperm chromatin samples were incubated separately in EC for 10 min, and combined for 20 min before addition of NPE. After a 30-min incubation in NPE, replication of sperm chromatin was assessed as in Fig. 1. The micrographs show representative results from each experiment, and the numbers (\pm 1 SD) refer to quantification of the fluorescence intensity data (see Fig. 1 D, for a description of the quantification). The average fluorescent signal intensity value for A was arbitrarily set to 100, and the other values adjusted accordingly.



beads. After a 30-min incubation, the plasmid beads were collected on a magnetic stand, and the supernatant was recovered. We define egg cytosol that had been transiently exposed to damaged plasmid as EC^D, and egg cytosol that had been transiently exposed to control plasmid as EC^C. The EC^C and EC^D extracts were then tested for the ability to support replication of subsequently added sperm chromatin (see Fig. 3 A for experimental design). Pilot experiments showed that EC^C promoted sperm chromatin replication just as well as naive egg cytosol, demonstrating that the manipulations involved did not nonspecifically inactivate the extracts (unpublished data). When replication of sperm chromatin was assessed in EC^D and compared with that measured in EC^C, we found that replication in EC^D was significantly lower than that observed in EC^C, even after prolonged incubation (Fig. 3 B). This result shows that even transient exposure of the extract to damaged DNA is sufficient to inactivate the extract toward replication of sperm chromatin templates.

A simple explanation for the data presented thus far is that the damaged plasmid DNA titrates an essential replication factor or factors away from the sperm chromatin template, thus preventing chromosomal replication. Alternatively, the damaged plasmid could activate a diffusible, transacting inhibitor that is responsible for the block to chromosomal replication. To distinguish between these possibilities, we asked if the EC^D

extract described in Fig. 3 (A and B) contained a transacting replication inhibitor. To assay for such an inhibitor, we mixed naive egg cytosol with either three parts buffer or three parts EC^D, and compared the ability of these mixtures to support replication of sperm chromatin. Egg cytosol that had been diluted with three parts buffer supported sperm chromatin replication to the same extent as undiluted egg cytosol (Fig. 3 C), which is consistent with previous observations that egg cytosol is refractory to fourfold dilution in the NPE system (Walter, J., personal communication). By contrast to buffer, addition of three parts EC^D to naive egg cytosol resulted in a nearly complete block to chromosomal replication (Fig. 3 C). This suggests that an activity contained within the EC^D inactivated the replication-promoting activity of the naive egg cytosol and, therefore, that EC^D contains an inhibitor of chromosomal replication. Titration experiments showed that three parts EC^D was the minimal amount of EC^D that we tested that resulted in a block to replication, lowering the ratio to 2:1 EC^D to egg cytosol was without significant effect (unpublished data). The inability of two parts EC^D to suppress replication indicates that the amount of inhibitor contained in the EC^D is, per unit volume, sufficient to inactivate a maximum of 1.33 vol of egg cytosol. Based on the experiments shown in Fig. 3, we conclude that the damaged plasmid-induced block to chromosomal replication is not due to simple sequestration of a replication factor by the damaged plasmid. If this were so, then the EC^D

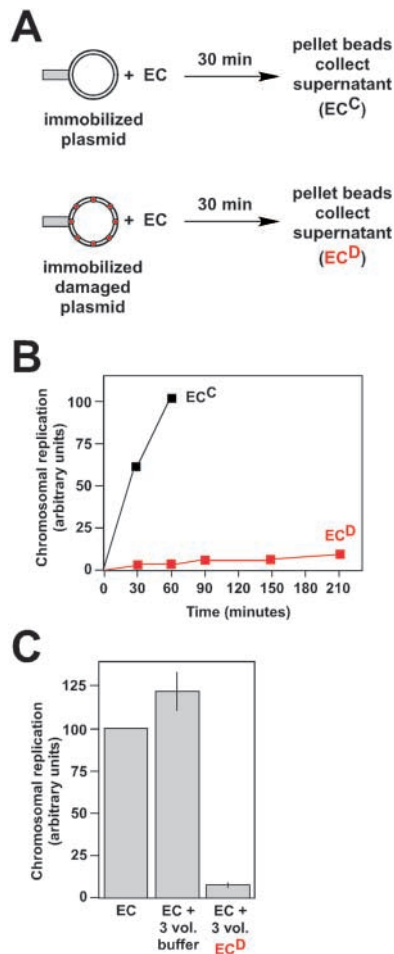


Figure 3. MMS-treated DNA generates a diffusible inhibitor of chromosomal replication. (A) Experimental strategy. Egg cytosol (EC) was mixed with either damaged or undamaged plasmid DNAs that had been immobilized on magnetic beads. After a 30-min incubation, the beads were separated from the extract by collection on a magnetic stand, and the extract was recovered. Extracts that had been exposed to undamaged, control plasmid are defined as EC^C, whereas extracts that had been exposed to MMS-treated plasmid are defined as EC^D. (B) Either EC^C or EC^D was mixed with 2,000/ μ l sperm chromatin for 30 min, followed by addition of NPE containing α -³²P-labeled dATP. Replication of the sperm chromatin was assessed after every 30 min of additional incubation by agarose gel electrophoresis as described previously (Walter and Newport, 1999). The dried gels were exposed to a PhosphorImager screen, and the amount of radioactivity incorporated into the DNA was determined by PhosphorImager analysis. The amount of DNA synthesis observed in the last time point for the EC^C extract was arbitrarily set to 100, and all other values were adjusted accordingly. (C) EC was mixed with three parts buffer (sperm dilution buffer; Murray, 1991), or three parts EC^D, and incubated with sperm chromatin for 30 min. NPE was added, and replication was assessed 30 min later. Replication in the diluted extracts was compared with that observed with undiluted EC, where the value was arbitrarily set to 100. All reactions contained a total of 12,000 sperm nuclei. Replication was analyzed as in B. The experiment was performed three times, and the bars represent the mean averages for each experiment. The error bars refer to one SD from that mean.

should have been inert, and should have behaved in a manner analogous to buffer in the mixing experiment reported in Fig. 3 C. Thus, the damaged plasmid generates a diffusible inhibitor of chromosomal replication.

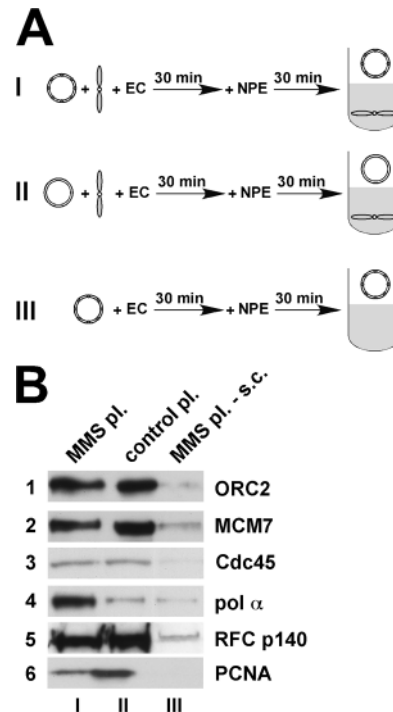


Figure 4. Damaged plasmid blocks sperm chromatin replication by preventing loading of the pol clamp protein PCNA onto the assembling replication complex. (A) Experimental strategy. Either damaged (I), or undamaged (II), plasmid was mixed together with sperm chromatin in EC and incubated for 30 min. NPE was added and, after an additional 30-min incubation, the reactions were centrifuged through a sucrose cushion to isolate the sperm chromatin. A third reaction (III), which contained damaged plasmid alone, was also included. Plasmids were included at a concentration of 3 ng/ μ l. (B) The pellet fractions from the sucrose density centrifugations depicted in A were probed, by immunoblotting, for the presence of the ORC2 subunit of ORC (row 1); the MCM7 subunit of the MCM complex (row 2); Cdc45 (row 3); the large, catalytic subunit of pol α (row 4); the large subunit of RFC (row 5); or PCNA (row 6). The lanes are demarcated I, II, and III, and refer to the reactions labeled I, II, and III, respectively, in A.

Identification of the damaged DNA-induced arrest point in sperm chromatin replication

To better understand how the MMS-induced inhibitor was blocking replication, it was of interest to map the step in the sperm chromatin replication pathway that was sensitive to this inhibitor. Our strategy for this was to first coinubate the damaged plasmid with sperm chromatin in NPE, and then, after incubation, to physically separate the two different DNA templates using sucrose density centrifugation. Plasmid DNAs lack the density required to cosediment, through concentrated sucrose cushions, with the heavier sperm chromatin templates. After separation from both the plasmid DNA and the rest of the extract, the sperm chromatin-associated replication proteins were detected by immunoblotting (see Fig. 4 A for experimental design). For controls, we included a reaction that contained undamaged plasmid and, to assess the specificity of the isolation procedure, a sample that contained damaged plasmid but no sperm chromatin. The results of this experiment are shown in Fig. 4 B. When undamaged plasmid was used in the experiment (lane II), all of the replication factors that we

probed for were found in the sperm chromatin fraction, as expected given that this reaction is replication competent. When we probed the sample derived from the reaction containing damaged plasmid (lane I), we found that ORC2 and MCM7, both components of the pre-RC, were bound to chromatin. In addition, the pre-IC factor Cdc45, as well as both pol α and the 140-kD subunit of RFC, were all associated with chromatin in this sample (lane I). Importantly, however, we detected a substantial reduction in the amount of PCNA that associated with sperm chromatin in the sample that received the damaged plasmid (lane I), relative to what was associated with sperm chromatin in the sample that received undamaged plasmid (lane II). Finally, we found no enrichment for any of the replication proteins analyzed in the pellet fraction from the reaction that received only damaged plasmid (lane III), demonstrating that the presence of the replication proteins in lanes I and II was due to association with sperm chromatin. This experiment shows that coincubation of the damaged plasmid, but not undamaged plasmid, results in a severe reduction in the amount of PCNA that binds to the sperm chromatin. Because PCNA is an essential replication factor, we conclude that the molecular basis for inhibition of chromosomal replication by the damaged plasmid is the inefficient recruitment of PCNA to the assembling replication complex.

The results presented thus far indicate that MMS-treated plasmid DNA generates a diffusible inhibitor that blocks chromosomal replication by preventing PCNA, but not pol α , from binding to chromatin. One possible explanation for this is that one of the two PCNA-dependent pols, pol δ or DNA pol ϵ , may be prevented from binding to chromatin in response to the inhibitor. A failure to recruit one or the other of these pols could, in principle, destabilize interaction between PCNA and the primed site. To examine this, sperm chromatin was incubated with either the EC^C or EC^D extracts (Fig. 3) for 30 min. NPE was added and, after an additional 30-min incubation, the chromatin was isolated and probed for the presence of pol δ and pol ϵ by immunoblotting. Both pol δ and pol ϵ were efficiently recruited to chromatin in the samples containing either the replication-incompetent EC^D extract or the control, replication-competent EC^C extract (Fig. 5 A). This is by contrast to PCNA, which associated with chromatin much more efficiently in the sample containing EC^C, relative to EC^D. We conclude that the damage-induced failure of PCNA to bind to sperm chromatin cannot be explained by a defect in the recruitment of either pol δ or pol ϵ to chromatin.

If egg extract is exposed either continuously (Fig. 4) or transiently (Fig. 5 A) to damaged plasmid DNA, then PCNA fails to load on to sperm chromatin. One possibility is that PCNA itself is regulated by the inhibitory system described here. If PCNA is rendered inactive by the inhibitory system, then it should fail to bind to even simple DNA substrates upon incubation in extract containing the inhibitor. To test this possibility, the ability of PCNA to associate with simple DNA structures after incubation in extract was assessed. Two structures were designed, one mimicked a replication fork (Fig. 5 B, fork), and the other contained a region of dsDNA followed by a long 3' overhang tail (Fig. 5 B, 3' overhang). PCNA would be expected to bind to the fork structure, as a primed site with a free 3' end is present

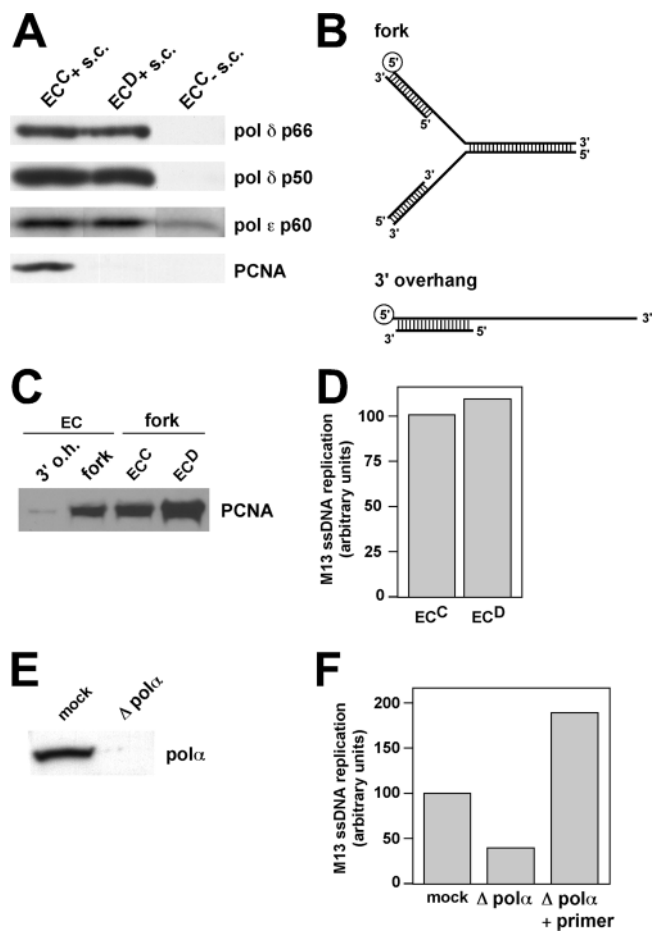


Figure 5. Pol α and PCNA are not directly down-regulated by the replication arrest pathway. (A) EC^C and EC^D were prepared as in Fig. 3. 2,000/ μ l sperm chromatin was added (to EC^C in EC^C + s.c. lane, and to EC^D in EC^D + s.c. lane) and the reactions were incubated for 30 min. NPE was added and incubation was continued for 30 min. The chromatin was isolated and probed for the presence of pol δ (50- and 66-kD subunit), pol ϵ (60-kD subunit), and PCNA. To control for the specificity of the chromatin isolation procedure, a sample containing EC^C but lacking sperm chromatin was also processed and analyzed (EC^C - s.c. lane). (B) Schematic renditions of the DNA structures used to assess PCNA DNA-binding activity. The circled "5'" refers to the position of the biotin group on the 5' end of the top strand in each structure. See text and methods for details on construction of these structures. (C) The indicated DNA structure was incubated in the indicated extract for 30 min (EC, EC^C, and EC^D refer to the extracts described in Fig. 3). The structures were recovered on a magnetic stand, the beads were washed, and bound proteins were eluted with SDS-PAGE sample buffer. The eluted proteins were probed for PCNA by immunoblotting. (D) M13 ssDNA was added, along with radio-labeled dATP and the indicated supplements, to either EC^C or EC^D. After a 30-min incubation, replication of the M13 ssDNA was assessed by agarose gel electrophoresis as described for sperm chromatin in Fig. 3 B. (E) EC^D extract was treated with immobilized antibodies against pol α (Δ pol α) or with immobilized nonspecific antibodies (mock), and the depleted extracts were probed by immunoblotting for pol α (p60 subunit). (F) M13 ssDNA replication was measured in the extracts described in E. The sample labeled " Δ pol α + primer" received M13 ssDNA to which the M13 universal primer had been preannealed.

within this structure, but not to the 3' overhang structure, which lacks a primed site with a free 3' end. Fig. 5 C shows that this was indeed the case. When naive egg cytosol was used as the source of extract, PCNA was found to associate

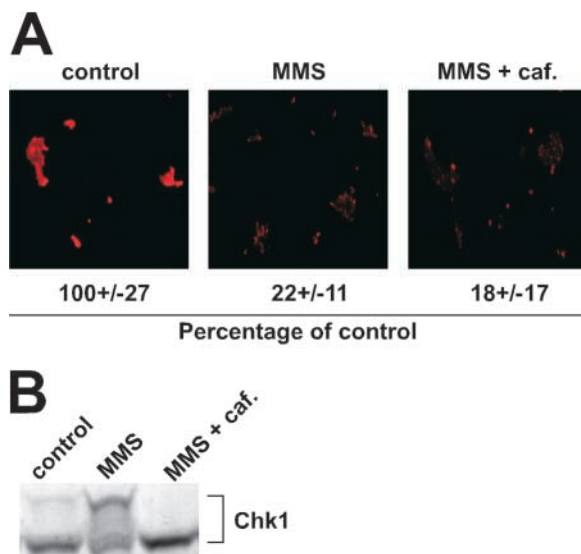


Figure 6. Checkpoint-independent activation of the replication arrest pathway. (A) Either undamaged, control plasmid (3 ng/ μ l, control lane), or damaged plasmid (3 ng/ μ l, MMS and MMS + caf. lanes) was added along with 2,000/ μ l sperm chromatin to egg cytosol. In addition, 5 mM caffeine was added to one of the samples receiving the MMS-treated plasmid (MMS + caf. lane). After a 30-min incubation, NPE was added. After an additional 30-min incubation, replication was assessed as in Fig. 1. (B) Reactions identical to those presented in A were set up, and 35 S-labeled Chk1 protein was added. After incubation in NPE, the samples were recovered and fractionated on SDS-PAGE gels to visualize the phosphorylation-induced Chk1 mobility shift. The gels were fixed and dried, and exposed to film to detect Chk1. The radio-labeled Chk1 protein was produced by coupled transcription/translation of the full-length *Xenopus* Chk1 cDNA in rabbit reticulocyte lysates in the presence of 35 S-labeled methionine.

efficiently with the fork structure, and not with the 3' overhang structure. Next, we asked if the replication incompetent EC^D extract could promote association of PCNA with the fork structure. As shown in Fig. 5 C, we found that it could, just as efficiently as the EC^C extract. Thus, although PCNA cannot bind to sperm chromatin in EC^D , it can bind to simple DNA substrates in EC^D . This suggests that PCNA itself is not directly controlled by the inhibitory system.

Another possibility is that pol α is down-regulated by the inhibitory system, which in turn would prevent PCNA from accessing sperm chromatin. To ask if pol α catalytic activity is negatively regulated, we measured M13 ssDNA replication in both EC^C and EC^D . It is known that M13 ssDNA replication in frog egg extracts is pol α dependent (Mechali and Harland, 1982). Thus, if pol α is negatively regulated, we would expect decreased M13 ssDNA replication in EC^D relative to EC^C . As shown in Fig. 5 D, this was not the case; we detected robust M13 ssDNA replication in both EC^C and EC^D . To ensure that M13 ssDNA replication was in fact pol α dependent in the EC^D extract, we depleted the pol α complex from EC^D (Fig. 5 E) and measured M13 ssDNA replication. Depletion of pol α from EC^D suppressed M13 ssDNA replication by 60% (Fig. 5 F), which is consistent with the level of suppression of M13 ssDNA replication observed when pol α is removed from naive egg cytosol (67%, not depicted). In addition, the inhibition of M13 ssDNA replication observed in pol

α -depleted EC^D was reversed when the requirement for pol α was bypassed through preannealing of an oligonucleotide primer to the M13 ssDNA (Fig. 5 F). Thus, pol α contributes to M13 ssDNA replication in EC^D . Together, the data in Fig. 5 (D and F) demonstrate that pol α is active toward simple ssDNA templates in EC^D as it is in the control EC^C extract, which rules out the possibility that inhibitor present in EC^D globally inactivates pol α . Consistent with this, we note that pol α stably associates with chromatin in the presence of the inhibitory system (Fig. 4 B), and DNA binding by pol α is known to be stabilized by primer synthesis (Yuzhakov et al., 1999).

MMS-induced replication arrest and the S-phase checkpoint

The data presented thus far describe a pathway that is activated by MMS-induced damage and which results in a failure to recruit the essential replication factor PCNA to sperm chromatin. In budding yeast, MMS treatment activates a checkpoint-dependent block to the firing of late origins (Shirahige et al., 1998). Although the inhibitory system described here blocks replication after origin firing, it was nonetheless of interest to determine if activation of the inhibitory system was dependent on damage checkpoint signaling. To do this, we determined if the damaged plasmid-induced block to chromosomal replication occurs under conditions where the checkpoint kinases ATM and ATR are inactivated. To inactivate ATM and ATR, we used caffeine, a potent small molecule inhibitor of ATM/ATR kinase activity (Sarkaria et al., 1999). Sperm chromatin and MMS-treated plasmid were added to egg cytosol that either contained or lacked caffeine. After a 30-min incubation, NPE was added and replication of the sperm chromatin was monitored by uptake of bio-dUTP. The results are shown in Fig. 6 A. The MMS-treated plasmid prevented replication of the sperm chromatin to the same extent in the extract containing caffeine as it did in the extract lacking caffeine. To ensure that the caffeine was functional under these conditions, we simultaneously examined phosphorylation of the ATR substrate Chk1. ATR-mediated phosphorylation of Chk1 causes a mobility shift on SDS-PAGE gels (Guo et al., 2000). Fig. 6 B shows that Chk1 phosphorylation was stimulated in the sample containing the damaged plasmid, relative to the sample containing the control plasmid. Importantly, caffeine completely suppressed the damaged-induced phosphorylation of Chk1, as inferred by loss of the Chk1 mobility shift on SDS-PAGE (Fig. 6 B). Together, the data in Fig. 6 (A and B) demonstrate that, under conditions where ATR kinase activity toward its substrate Chk1 is suppressed by caffeine, the ability of the MMS-treated plasmid to block replication of the sperm chromatin is unaffected. Consistent with this, we also found that PCNA still failed to load onto sperm chromatin in extracts containing both MMS-treated plasmid and caffeine (unpublished data). From this, we conclude that the MMS-induced replication arrest described in this paper is not controlled by the canonical DNA damage checkpoint.

We have shown here that MMS-induced damage blocks replication of undamaged DNA, and that the arrest point for the replication block occurs after pol α binding to chromatin

and before PCNA loading. Interestingly, the DNA replication inhibitor aphidicolin, which activates the S-phase checkpoint in frog egg extracts (Dasso and Newport, 1990), also allows binding of pol α and prevents binding of PCNA (Michael et al., 2000). Aphidicolin also induces a dramatic increase in the amount of pol α associated with chromatin (Michael et al., 2000), and consistent with this, we note that the amount of pol α bound to chromatin when the damaged plasmid is coincubated with sperm chromatin noticeably exceeds that which is bound when the undamaged plasmid is included (Fig. 4 B). Thus, these similarities prompted us to speculate that the replication arrest observed on undamaged DNA in response to the MMS-induced inhibitor might signal an S-phase checkpoint response. To explore this, either undamaged or damaged plasmid was coincubated with sperm chromatin in egg cytosol for 30 min, and NPE was added. After continued incubation in the NPE, the sperm chromatin was isolated and binding of both PCNA and the checkpoint protein Rad17 was assessed by indirect immunofluorescence. We probed for Rad17 because previous work has shown that in *Xenopus*, Rad17 binds specifically to chromatin that is undergoing a checkpoint response, and does not bind tightly to chromatin that is actively replicating (Stokes et al., 2002; Lee et al., 2003). Thus, Rad17 binding is a reliable indicator of activation of the S-phase checkpoint. The results are shown in Fig. 7 A. PCNA was detected on the sperm chromatin derived from the sample that received undamaged plasmid, whereas the sample receiving the damaged plasmid displayed no detectable binding of PCNA (Fig. 6 C). Therefore, this result is consistent with the data shown in Figs. 4 and 5. When Rad17 was assessed, we could not detect the protein in association with sperm chromatin that was coincubated with undamaged plasmid. This was also expected, given that this chromatin is actively replicating. By contrast, Rad17 was easily detected on the sperm chromatin that had been prevented from replicating due to coincubation with the damaged plasmid, indicating that this chromatin was undergoing a checkpoint response. Rad17 association with sperm chromatin also occurred after incubation of sperm chromatin in the EC^D extract that is free of plasmid (unpublished data), thus eliminating the possibility that the signal observed in Fig. 7 A was due to entanglement of the damaged plasmid DNA with the sperm chromatin. We conclude that coincubation of sperm chromatin with damaged, but not undamaged, plasmid DNA induces binding of the checkpoint protein Rad17 to sperm chromatin.

The data in Fig. 7 A suggest that when replication of sperm chromatin is blocked by the MMS-induced replication arrest pathway, structures form on the sperm chromatin that trigger a checkpoint response. If so, then the sperm chromatin would be expected to contribute to the MMS plasmid-induced Chk1 phosphorylation observed in Fig. 6 B. To address this, we compared Chk1 phosphorylation in an NPE reaction that contained MMS-treated plasmid alone to a reaction that contained both MMS-treated plasmid and sperm chromatin. As controls, we also assessed Chk1 phosphorylation in NPE reactions containing sperm chromatin alone, or with sperm chromatin together undamaged plasmid DNA. Fig. 7 B shows that a Chk1 mobility shift could not be detected in the samples containing either sperm chro-

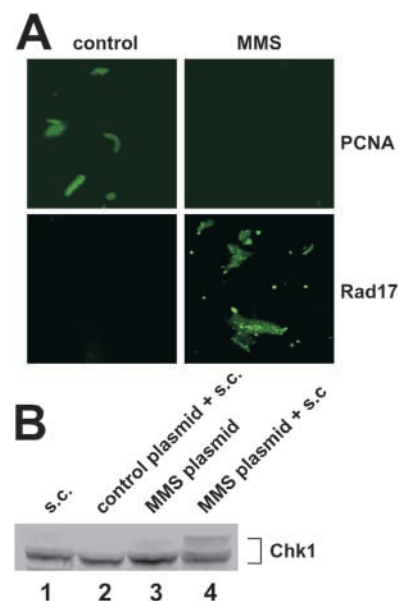


Figure 7. Amplification of the checkpoint signal through replication arrest on undamaged DNA. (A) 2,000/ μ l sperm chromatin was mixed together with either control plasmid, or MMS-treated plasmid, in egg cytosol for 30 min before the addition of NPE. 30 min after NPE addition, the sperm chromatin templates were fixed and stained with antibodies against either PCNA or frog Rad17. The samples were visualized by fluorescence microscopy. Plasmids were included at a concentration of 3 ng/ μ l. (B) Egg cytosol containing ³⁵S-labeled Chk1 was mixed with sperm chromatin (lane 1), or sperm chromatin plus control plasmid (lane 2), or MMS-treated plasmid (lane 3), or MMS-treated plasmid plus sperm chromatin (lane 4). After a 30-min incubation, NPE was added and incubation was continued for an additional 30 min. The phosphorylation status of Chk1 was determined by migration on SDS-PAGE as in Fig. 6 B. Plasmids were included at a concentration of 3 ng/ μ l.

matin alone (lane 1), or sperm chromatin coincubated with control plasmid (lane 2). This was expected, given that these reactions are replication competent. Interestingly, when the samples containing the MMS-treated plasmid were compared, we found that Chk1 phosphorylation was enhanced in the reaction containing both MMS-treated plasmid and sperm chromatin (lane 4), relative to the reaction that contained only the MMS-treated plasmid (lane 3). This shows that both the MMS-treated plasmid and the sperm chromatin are required to initiate checkpoint signaling under these conditions. We note that in a previous publication, we reported that MMS-treated plasmid alone stimulated Chk1 phosphorylation in the NPE system (Stokes et al., 2002), however the amount of plasmid required to see that affect (25 ng/ μ l) greatly exceeded the amount used in the experiment shown in Fig. 7 B (3 ng/ μ l). Thus, under conditions where the damaged plasmid DNA is not present at high enough concentration to trigger a checkpoint response on its own, the addition of sperm chromatin allows checkpoint activation to occur. Based on the data in Figs. 6 and 7, we conclude that although the canonical S-phase checkpoint is not required for MMS-induced replication arrest, MMS-induced replication arrest results in activation of the checkpoint, even on undamaged DNA. This result has interesting implications for the mechanism of checkpoint activation,

and for the possibility of signal amplification during the DNA damage response (see Discussion).

Discussion

A novel replication arrest pathway

To date, two general mechanisms have been described to explain how damage-induced replication arrest occurs, a checkpoint-dependent block to origin firing (Santocanale and Diffley, 1998; Shirahige et al., 1998; Costanzo et al., 2000, 2003; Falck et al., 2001), and a checkpoint-independent block to fork progression (Tercero and Diffley, 2001). Here, we describe an additional mechanism that prevents DNA replication in response to DNA damage. This pathway is activated by alkylation damage of DNA. Activation of the pathway produces a diffusible inhibitor of DNA replication, which ultimately blocks chromosomal replication by preventing the essential replication factor PCNA from loading onto the template strand.

That this pathway is distinct from the previously described mechanisms is supported by the following observations: First, the pathway described here is operational under conditions that inactivate the canonical DNA damage checkpoint (Fig. 6). This shows that signaling through the ATM/ATR kinase family is not an essential component of the pathway, and this distinguishes the pathway described here from the caffeine-sensitive, checkpoint-dependent pathways that have previously been shown to block origin firing in *Xenopus* (Costanzo et al., 2000, 2003). Second, the target of the pathway, loading of PCNA onto the template strand, occurs after origin firing and recruitment of DNA pol. This also distinguishes the pathway described here from the checkpoint-dependent mechanism, as the known checkpoint pathways all target events occurring before the Cdc45 recruitment and origin-unwinding step (Introduction). Lastly, the pathway described here is distinct from the recently described mechanism that blocks elongation (Tercero and Diffley, 2001), as elongation synthesis of M13 ssDNA templates occurs normally in the presence of the MMS-activated inhibitory system (Fig. 5).

The inhibitor and its target

The inhibitory system described here operates through activation of a diffusible inhibitor of chromosomal replication. Data supporting this conclusion are shown in Fig. 3, where the EC^D extract dominantly suppresses the replication-promoting activity of naive egg cytosol. Furthermore, experimentation has shown that the inhibitory activity contained in EC^D is of high molecular weight, as extensive dialysis of EC^D did not reduce the ability of EC^D to block replication when mixed with egg cytosol (unpublished data). Another characteristic of the inhibitor is that it appears to act stoichiometrically, given that the ability of EC^D to inhibit egg cytosol is very sensitive to the ratio of EC^D to egg cytosol (a 3:1 ratio inhibits replication, whereas a 2:1 ratio does not). This sensitivity is indicative of a factor that stably interacts with its target because once the level of target surpasses the amount of inhibitor, such as when the ratio of EC^D to egg cytosol is modestly reduced, then the block to replication is lost. How does this inhibitor act? Two general possibilities are that the inhibitor binds to sperm chromatin, and thereby

renders the chromatin unable to replicate. Alternatively, the inhibitor could act by binding to its target in solution and, by doing so, prevent the target from binding to sperm chromatin. We favor the latter, as preliminary experiments indicate that sperm chromatin that is assembled in the presence of the inhibitor, and then isolated and transferred to a naive extract, is replication competent (unpublished data). Thus, the inhibitor does not appear to bind tightly to chromatin.

An interesting feature of the damage-induced inhibitory pathway described here is that the inhibitor can only block chromosomal replication if it is present very early in the chromosomal replication pathway, during the first 10 min of incubation in egg cytosol (Fig. 2). This would suggest that the target of the inhibitor is some component of the pre-RC, as pre-RC assembly represents the major activity that occurs in the first 10 min of incubation. Paradoxically, though, loading of ORC and MCM, two pre-RC components, occurs normally in the presence of the inhibitor. Indeed, the replication pathway only fails at a step after recruitment of pol α and RFC to chromatin, and before PCNA loading. The timing experiments shown in Fig. 2, however, make it unlikely that either RFC or PCNA are the direct targets of the inhibitor. It is important to consider that if the sperm chromatin is separated from the MMS-treated DNA for just 10 min, then the block to replication is lost (Fig. 2 D). This 10-min window of sensitivity occurs long before RFC or PCNA act during initiation. Thus, in the experiment shown in Fig. 2 D, both RFC and PCNA are functional in the presence of the inhibitor because an early step in replication was allowed to occur away from the inhibitor. This is powerful evidence that the inhibitor is not acting by physically sequestering RFC or PCNA, and the data shown in Fig. 5 using simple DNA templates support this conclusion.

One possibility, that would reconcile all of the data, is to postulate the existence of a factor X that has two critical properties. One, factor X is required to load PCNA onto chromosomal DNA templates. Two, chromosomal loading of factor X itself occurs early, during pre-RC formation (Fig. 8 A). In this scenario, damaged DNA generates an inhibitor (I^X) that directly targets factor X and prevents it from loading onto the pre-RC (Fig. 8 B). This would prevent PCNA from binding later on, after the G1/S transition. The failure of PCNA to bind would then, in turn, activate the replication checkpoint. In this scenario, the direct target of the inhibitory system is factor X, and not PCNA. This would explain why PCNA loads onto simple DNA substrates, but not onto chromosomes, in the presence of the inhibitor. If factor X were no longer subject to negative regulation by the inhibitor after it had loaded on to the pre-RC, then this would explain the timing effects described in Fig. 2. This model is clearly highly speculative, and further work, including identification of the inhibitor and its immediate target, will be required to definitively test its validity.

The relationship between MMS-induced replication arrest and activation of the S-phase checkpoint

Another interesting feature of the pathway described here is that MMS-induced replication arrest generates an S-phase checkpoint response. This is most clearly seen in Fig. 7 A,

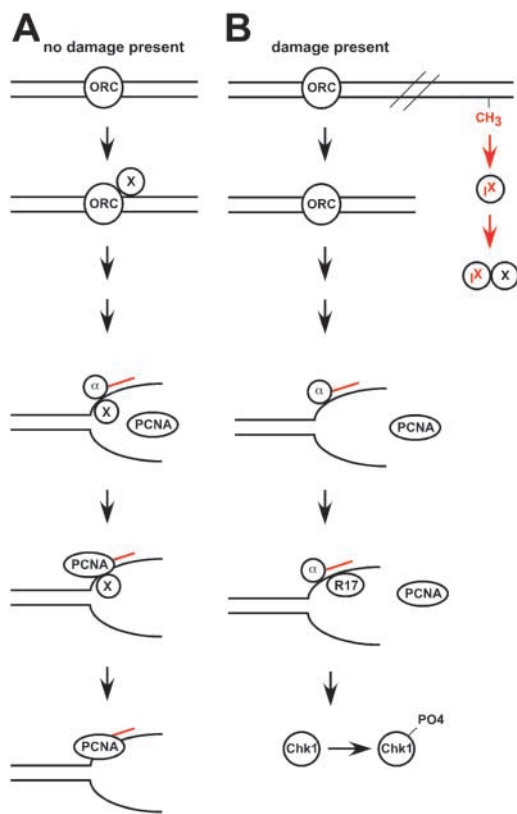


Figure 8. **A model for the replication arrest pathway, and how it is coupled to S-phase checkpoint activation.** Pol α is designated by " α ", and Rad17 is designated by "R17". See Results for details.

when coincubation of the damaged plasmid with undamaged sperm chromatin induced binding of the Rad17 checkpoint protein to the sperm chromatin, and in Fig. 7 B, when the sperm chromatin was required to initiate checkpoint signaling in NPE reactions containing low levels of MMS-treated plasmid. In this regard, it is intriguing that the step in replication that is sensitive to the MMS-induced replication arrest pathway, PCNA-binding, is just one step after the minimal step that must be completed in order to ensure that a replication checkpoint response is activated. Previous work in *Xenopus* egg extracts has shown that unreplicated DNA only triggers a checkpoint response if primer synthesis by pol α is allowed to occur (Michael et al., 2000). If replication is blocked before the pol α -dependent step, then the checkpoint fails to be activated; if it is blocked afterwards, then the checkpoint is activated. Thus, by targeting PCNA-binding, the replication arrest pathway ensures that MMS-induced damage also activates, indirectly, a canonical checkpoint response. Therefore, the coupling of replication arrest and checkpoint activation pathways suggests an integrated response to MMS-induced damage when it is sensed before entrance into S phase. Sensing of the damage activates the replication arrest pathway, which blocks replication by preventing PCNA from loading onto chromatin. The failure of PCNA to load then triggers a canonical S-phase checkpoint response, which stabilizes the partially assembled replication forks, and delays entrance into mitosis. This type of relay mechanism could also allow for a signal amplification step

during the DNA damage response, if, for example, a single MMS-induced lesion signals to delay replication at multiple neighboring origins. Our finding that low levels of MMS-treated plasmid actually require undamaged sperm chromatin to trigger a checkpoint response strongly suggests that such an amplification mechanism does indeed occur.

Materials and methods

Egg extract preparation

Egg cytosol and NPE were prepared as described previously (Walter et al., 1998). Immunodepletion of pol α was performed as described previously (Michael et al., 2000).

Plasmid DNA alkylation

Plasmid pSP72 (Promega), at 225 ng/ μ l, was mixed with an equal volume of buffer AB (1 \times M9 salts [Sambrook et al., 1989], 0.10 mM MgSO₄). MMS (Sigma-Aldrich) was added to 450 mM, and incubated for 30 min at 30°C. The DNA was diluted 1:10 in TE (10 mM Tris-HCl, and 1 mM EDTA, pH 7.5), and ethanol precipitated. The control plasmid used throughout this work was prepared in an identical fashion, except that MMS was omitted from the reaction.

DNA replication analysis

Fluorescence-based DNA replication assays were performed as described previously (Stokes et al., 2002). Radioactivity-based DNA replication assays were also performed as described previously (Walter and Newport, 1999).

Preparation of immobilized plasmid DNAs and simple DNA structures

Plasmid pSP72 was linearized with EcoRI (New England Biolabs). Linear pSP72 was labeled with biotin 14-dATP (GIBCO BRL) in a reaction containing 33 μ M biotin-14-dATP, and 10 U DNA pol I Klenow fragment (New England Biolabs, Inc.) per microgram of DNA, for 30 min at room temperature. Reaction progression was stopped by addition of EDTA to 10 mM, and reactions were loaded onto G-50 micro columns (Amersham Biosciences). Flow-through was ethanol precipitated and resuspended in autoclaved water. Biotinylated pSP72 was treated with MMS as described above (Plasmid DNA alkylation). Damaged or undamaged pSP72-biotin was coupled to streptavidin beads (Dynabeads M280; Dynal), at 1 mg of beads per 5 pmol DNA, according to the manufacturer's instructions. To make the replication fork structure used in Fig. 5 D, the following oligonucleotides were annealed together: Oligo 1 (5'biotin-GACGCTGCCG-AATTCTGGCGTTAGGAGATACCGATAAGCTTCGGCTTAAG3'), Oligo 2 (5'CGCCAGAATTCGGCAGCGTC3'), Oligo 3 (5'CTTAAGCCGAAGCTT-ATCGGTATCTTGCTTACGACGCTAGCAAGTGATCA3'), and Oligo 4 (5'TGATCACTTGCTAGCGTCG3'). To make the 3' overhang structure, Oligo 1 and Oligo 2 were annealed together. Annealed structures were then coupled to Dynabeads.

Isolation of sperm chromatin

Immunofluorescent staining of isolated sperm chromatin templates was performed as described previously (Walter and Newport, 1999). Isolation of sperm chromatin for immunoblotting was performed as described previously (Van Hatten et al., 2002).

Chk1 phosphorylation assay

Full-length *Xenopus* Chk1 cDNA was isolated by PCR amplification using oocyte cDNA as template. The PCR fragment was digested with BamHI and EcoRI and subcloned into pCDNA1/mycB (Michael et al., 1997). ³⁵S-labeled Chk1 was produced by coupled transcription and translation reactions in rabbit reticulocyte lysates (TnT; Promega). The radio-labeled protein was added to egg cytosol. After a 30-min incubation, NPE was added. 15 min after addition of NPE, the phosphatase inhibitor tautomycin was added, and incubation was continued for an additional 15 min before harvesting of the samples. The use of tautomycin in Chk1 phosphorylation assays in *Xenopus* egg extracts has been documented previously (Kumagai and Dunphy, 2000).

Antibodies

Antibodies against ORC2 and MCM7 were a gift of J. Newport (University of California, San Diego, La Jolla, CA); antibodies against Cdc45 were a

gift of J. Walter (Harvard Medical School, Boston, MA); antibodies against pol α , pol δ , and pol ϵ were a gift of S. Waga (Osaka University, Osaka, Japan); and antibodies against the large subunit of RFC were a gift of B. Stillman (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). Antibodies against frog Rad17 have been described previously (Stokes et al., 2002), and were a gift of H. Lindsay (Sussex University, Brighton, UK). Antibodies against PCNA were purchased from Santa Cruz Biotechnology, Inc. The antibodies used to immunodepleted pol α were raised in our laboratory against the 60-kD subunit of the pol α complex.

Image acquisition

All images were collected on a microscope (model BX51 TF; Olympus). The type, magnification, and NA of the objective lenses was UPlanAPO, 60 \times oil, NA = 1.40, respectively. The experiments were performed at room temperature using Hoechst 33258, Texas red-labeled streptavidin, and FITC-labeled secondary antibodies as fluorochromes. Images were captured on a camera (model 2.1.1; Diagnostic Instruments) and processed using SPOT Advanced version 3.2.4 software. Quantification of fluorescence was performed using the Scion Image β version 4.0.2 software by calculating mean density values.

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