DNA Damage-Dependent Inactivation of Complementary Strand Synthesis in *Xenopus laevis* Egg or HeLa Cell Lysates†

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ABSTRACT: Genotoxic lesions frequently arrest DNA synthesis and, as a consequence, result in the accumulation of incompletely replicated chromosomal segments containing long single-stranded regions of parental DNA. Here, we exploited complementary strand synthesis in *Xenopus laevis* egg or HeLa cell lysates to test how the eukaryotic replication machinery responds to such damaged single-stranded intermediates. Although both cell lysates promoted efficient conversion of M13 or ϕX174 single-stranded templates to double-stranded products, their replication activity was inhibited by DNA damage arising from ultraviolet (UV) radiation or exposure to the alkylating agent *N*-methyl-*N*-nitrosourea (MNU). When M13 single-stranded DNA containing UV- or MNU-induced lesions was coincubated with single-stranded substrates containing no lesions, we observed suppression of DNA synthesis on both damaged and undamaged templates. In contrast, complementary strand synthesis remained unaffected in coincubation reactions containing damaged DNA in the double-stranded form. Effective inhibition of complementary strand synthesis on undamaged templates required the presence of at least stoichiometric amounts of UV- or MNU-treated single-stranded DNA, indicating that a DNA polymerase or accessory protein is excluded from DNA synthesis by immobilization at or near the lesion sites. In support of this competitive mode of inhibition, we found that inactivation of DNA synthesis by coincubation with damaged single-stranded DNA was relieved by the addition of an exogenous DNA polymerase that catalyzes processive strand elongation. In summary, this study reveals sequestration of critical components of the eukaryotic replication machinery in a DNA damage-dependent and single-strand-specific manner, thereby providing a potential mechanism to sense arrested replication intermediates during an early recognition step of S phase checkpoint responses.

In eukaryotes, chromosomal DNA is duplicated during S phase of the cell cycle by the action of many replication forks that operate by continuous DNA synthesis on the leading strand and, in parallel, discontinuous DNA synthesis with formation of numerous Okazaki fragments on the lagging strand (*1*−*3*). This coordinated process generates two double-stranded branches of semiconservatively replicated DNA, each containing one parental strand and one daughter strand (*4*, *5*). Studies with synchronized cells have shown, however, that progression through S phase is also associated with the transient accumulation of long chromosomal regions in single-stranded rather than double-stranded form (*6*−*8*). These single-stranded intermediates consist mainly of parental DNA with lengths of up to approximately 100 kb[1] (*9*), and are believed to result from strand displacement when DNA synthesis on the leading and lagging strands is uncoupled and DNA replication proceeds in an asymmetric manner (*9*−*10*). Because template lesions often impede DNA replication, the fraction of such single-stranded intermediates is dramatically increased upon exposure to genotoxic agents (*11*−*14*). To complete DNA synthesis and, hence, allow progression into the next stage of cell cycle, these aborted replication intermediates must be efficiently processed, presumably by a replicative mechanism that is preferentially active on single-stranded substrates. Thus, the semiconservative conversion of long single-stranded templates to double-stranded DNA is central to the overall cellular response to genotoxic insults.

In the present study, we used complementary DNA synthesis in *Xenopus laevis* egg and HeLa cell lysates as model systems to test how the eukaryotic replication machinery interacts with single-stranded intermediates such as those arising upon DNA synthesis arrest at sites of damage. High-speed lysates derived from *Xenopus* eggs or HeLa cells have been shown to support efficient replication of single-stranded templates in vitro (*15*, *16*), and many basic enzymatic reactions catalyzed by these cell-free lysates mimic key aspects of eukaryotic replication. For example, DNA synthesis on circular single-stranded templates is initiated by the formation of oligoribonucleotides about 10 residues in length, and DNA polymerase α is required for the initial elongation of these RNA primers (*15*, *16*). Multiple cellular DNA polymerases have been implicated as essential enzymatic factors during the subsequent steps of complementary strand synthesis (*17*−*19*). Finally, these replication reactions in *Xenopus* egg or HeLa cell lysates produce covalently

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[1] Abbreviations: kb, kilobase(s); MNU, *N*-methyl-*N*-nitrosourea; SV40, simian virus 40; UV, ultraviolet.

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closed, double-stranded DNA molecules that are rapidly packed into nucleosomes (*16*).

Previous reports have demonstrated that DNA polymerases tend to stop DNA synthesis either 5′ to or opposite sites of damage in the template strand (*20*−*23*). Also, recent genetic studies showed that DNA polymerases and other replication factors are required for the execution of a cell cycle checkpoint that delays S phase progression in response to replication blocks (*24*−*27*). Although it is an attractive model that a subset of replicative factors, in particular DNA polymerases, may have an additional function in sensing replication blocking lesions (*24*), no biochemical link between DNA synthesis arrest and checkpoint activation could be established. Using *Xenopus* egg or HeLa cell lysates, we found that DNA lesions generated by ultraviolet (UV) radiation or *N*-methyl-*N*-nitrosourea (MNU) not only inhibit complementary DNA synthesis on damaged templates but, unexpectedly, also suppress DNA synthesis on undamaged substrates added to the same incubation mixtures. Dose dependence experiments with UV- or MNU-treated single-stranded DNA indicate that this intermolecular mode of inhibition occurs by replication factor trapping at or near sites of template lesions. Damage-dependent depletion of a replicative function is supported by experiments in which inhibition of DNA synthesis by coincubation with UV-irradiated single-stranded DNA was reversed upon addition of an exogenous DNA polymerase. Thus, in contrast to previous reports suggesting that DNA polymerases dissociate rapidly from sites of template damage (*28*−*30*), our study reveals damage-specific interactions between intrinsic components of the eukaryotic replication machinery and a subset of intractable replication intermediates. Because several DNA polymerases and accessory proteins are required for S phase checkpoint induction, these interactions with damaged single-stranded templates may be directly implicated in DNA damage recognition and subsequent events that link replication arrest to cell cycle regulation.

MATERIALS AND METHODS

Cell Lysates. Unfertilized eggs from *Xenopus laevis* were obtained after injection of chorionic gonadotropin (*31*). Egg lysate was prepared at 4 °C as described previously (*16*). Briefly, dejelled eggs were washed in lysis buffer [20 mM Hepes, pH 7.5, 70 mM potassium acetate, 1 mM dithiothreitol, 5% (w/v) sucrose] and broken by centrifugation at 12 000*g* for 30 min. The resulting supernatant was recentrifuged for 1 h at 40 000 rpm in a Sorvall TST60 rotor, and clear lysates with typical protein concentrations of 20 mg/mL were stored in small aliquots at −80 °C. HeLa cytosolic lysate (about 5 mg of protein/mL) was obtained from proliferating cells following a previously published method (*19*) that includes cell lysis in hypotonic buffer (20 mM Hepes, pH 7.8, 5 mM potassium acetate, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol) followed by disruption in a Dounce homogenizer. After removal of nuclei by centrifugation at 5000*g* for 5 min, the cell lysate was recentrifuged in the Sorvall TST60 rotor at 35 000 rpm for 1 h. All cell lysis and centrifugation steps were performed at 4 °C, and the resulting cytosolic supernatant was stored in small aliquots at −80 °C.

DNA Substrates. M13mp19 single-stranded DNA (7249 bases) was purified from bacteriophage-infected *Escherichia coli* DH5αF′ cell cultures (Gibco BRL), as described (*32*). Plasmids were purified by CsCl and sucrose gradient centrifugation (*33*) to eliminate all nicked molecules. Phage ϕX174 single-stranded DNA (5386 bases) was purchased from New England Biolabs. Plasmid pSB408 (10.7 kb) was a gift of C. Sengstag (Institute of Toxicology, ETH and University of Zürich) and was propagated in *Escherichia coli* DH5α (Gibco BRL). For UV irradiation, single-stranded or double-stranded DNA was dissolved in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA at a concentration of 100 ng/μL. Drops of 10 μL each were placed on ice in an open petri dish and irradiated at a dose rate of 1.6 $J \cdot m^{-2} \cdot s^{-1}$ using a germicidal lamp with peak output at 254 nm. The UV fluence was monitored with a Steritest dosimeter. UV-irradiated DNA was stored at −80 °C. A 100 mM stock solution of MNU (Sigma) was prepared in dimethyl sulfoxide. M13mp19 single-stranded DNA (100 ng/μL in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was exposed to different concentrations of MNU for 30 min, followed by addition of sodium acetate to 0.3 M and 2 volumes of ethanol. Samples were then immediately centrifuged for 30 min at 12 000*g* and 4 °C; the resulting pellets were washed 3 times with 70% (v/v) ethanol, dried, redissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and stored at −80 °C. The integrity of alkylated DNA was confirmed by 1% agarose gel electrophoresis in the presence of ethidium bromide (*32*), demonstrating that the fraction of linear single-stranded M13 molecules was not increased upon pretreatment with up to 1 mM MNU.

Complementary DNA Synthesis in Eukaryotic Cell Lysates. Standard incubations contained 25 μL of egg lysate supplemented with 3 mM ATP, 5 mM $MgCl_2$, 40 mM creatine phosphate, 150 μg/mL phosphocreatine kinase (Boehringer Mannheim), and 10^6 dpm of [α-^{32}P]dATP (Dupont NEN), essentially as described by Almouzni and Méchali (*16*). Varying amounts of damaged or undamaged DNA templates were added as outlined in the figure legends, and incubations were carried out at 23 °C for the indicated time periods. After addition of 240 μL of stop solution consisting of 20 mM Tris-HCl, pH 8.0, 0.5% (w/v) SDS, and 20 mM EDTA, samples were deproteinized by treatment with proteinase K (500 μg/mL; Boehringer Mannheim) for 1 h at 37 °C, followed by phenol extraction. DNA was ethanol-precipitated in the presence of 0.3 M sodium acetate and centrifuged at 14 000*g* for 30 min at 4 °C. The DNA pellets were washed with 70% (v/v) ethanol, dried, and redissolved in 10 μL of Tris-HCl, pH 8.0, 10 mM EDTA. Radiolabeled reaction products were resolved by electrophoresis on 1% agarose gels in the presence of ethidium bromide (*32*), and visualized by autoradiography. DNA synthesis was quantified by scanning densitometry of individual bands on the X-ray films using a Molecular Dynamics Computing Densitometer with ImageQuant software. In all cases, the relative densitometric intensities were confirmed by Cerenkov counting of the respective gel slices. Complementary DNA synthesis in HeLa cytosolic lysate was performed essentially as devised by Krude and Knippers (*19*). Briefly, reactions of 80 μL contained 50 μL of cytosolic lysate, 40 mM Hepes, pH 7.8, 4 mM $MgCl_2$, 3 mM ATP, 0.1 mM each of GTP, CTP, and UTP, 25 μM dATP, 0.1 mM each of dGTP, dCTP, and dTTP, 0.5 mM dithiothreitol, 40 mM phosphocreatine kinase, 100 μg/mL phosphocreatine kinase, 10^6 dpm of

[α-^{32}P]dATP, and the indicated amounts of damaged or undamaged DNA templates. After incubations for 2 h at 37 °C, replication products were analyzed and quantified as outlined before.

Complementary DNA Synthesis by Purified DNA Polymerases. A pUC19/M13 sequencing primer (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) was phosphorylated with ATP and T4 polynucleotide kinase (Gibco BRL) and annealed to single-stranded M13mp19 DNA by incubation at 70 °C for 5 min. Standard annealing reactions (10 μL) contained 20 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 50 mM NaCl, 4 pmol of phosphorylated primer, and 100 ng of M13 template. These mixtures were cooled to room temperature during 1 h and placed on ice. Incubations with 1 unit of T7 DNA polymerase (Amersham) were then performed in 20-μL reactions containing 10 mM Tris-HCl, pH 7.4, 25 mM NaCl, 1 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 10^6 dpm of [α-^{32}P]dATP, 5 units of T4 DNA ligase (Gibco BRL), and 100 ng of M13 template DNA annealed with 4 pmol of sequencing primer. Alternatively, reactions were carried out in the absence of sequencing primers, or with ϕX174 templates instead of M13 single-stranded DNA. After incubation at 37 °C for 60 min, DNA polymerase reactions were stopped by heating to 70 °C for 10 min and analyzed directly by agarose gel electrophoresis. Radiolabeled DNA products were visualized and quantified as indicated in the previous section. Incubation mixtures with 1 unit of T4 DNA polymerase (Gibco BRL) contained 33 mM Tris−acetate, pH 7.9, 66 mM sodium acetate, 10 mM magnesium acetate, 100 μg/mL bovine serum albumin, 2 mM dithiothreitol, 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 10^6 dpm of [α-^{32}P]dATP, and 5 units of T4 DNA ligase (Gibco BRL). DNA substrates were added, and the reactions were carried out and analyzed as outlined before for T7 DNA polymerase.

RESULTS

Inhibition of Complementary Strand Synthesis by UV Radiation Damage. Circular single-stranded M13mp19 genomes were converted to double-stranded DNA by incubation in *Xenopus laevis* egg lysate. The reaction mixtures were supplemented with ATP and an ATP-regenerating system consisting of phosphocreatine kinase and creatine phosphate. Additionally, radiolabeled [^{32}P]dATP was included in these incubations to monitor DNA synthesis in a quantitative manner. Under these conditions, complementary strand synthesis in *Xenopus laevis* egg lysate occurs with over 95% efficiency during incubations of 2 h (*15*,*16*). As illustrated in the autoradiography of Figure 1 (lane 1), the resulting double-stranded replication products were resolved by agarose gel electrophoresis into three radiolabeled fractions that represent covalently closed M13 circles (form I DNA), nicked M13 circles (form II DNA), and linear M13 DNA (form III). In its polylinker region, M13mp19 contains a single recognition site for *Bam*HI. Therefore, digestion of circular replication products by subsequent incubation with *Bam*HI generates a prominent radiolabeled band with exactly the same electrophoretic mobility as a linear M13mp19 DNA marker (data not shown).

To test the effect of template lesions on complementary strand synthesis, M13 single-stranded DNA was UV-irradiated prior to incubation with *Xenopus* egg lysate. As expected, the efficiency of complementary strand synthesis was progressively reduced with increasing UV doses (Figure 1). In particular, the formation of covalently closed M13 circles (form I DNA) was completely abolished after exposure to a UV dose of 100 J·m^{-2} (Figure 1, lane 5). This UV dose is expected to generate about 4−5 UV photoproducts per M13 substrate molecule of 7.3 kb (*34*,*35*), translating to roughly 1 UV lesion per 1500 template bases. Thus, complementary strand synthesis in *Xenopus* egg lysate is highly sensitive to template damage generated by UV radiation.

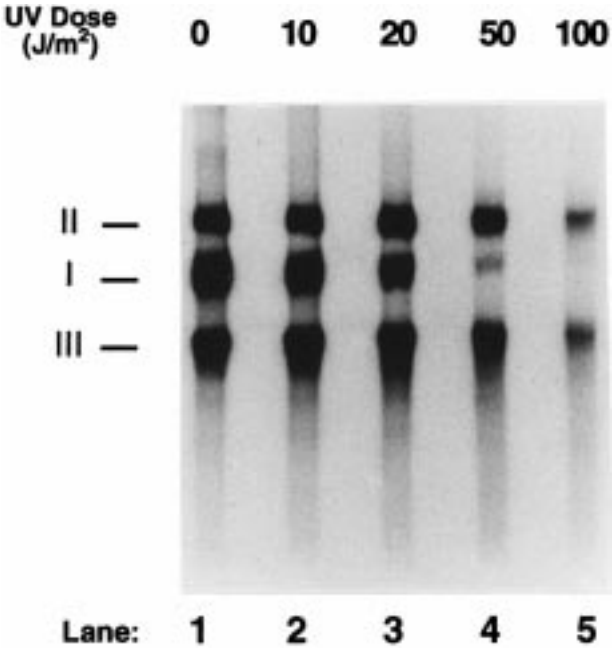

FIGURE 1: Complementary strand synthesis in *Xenopus laevis* egg lysate. M13 single-stranded DNA (300 ng) was incubated in *Xenopus* egg lysate supplemented with ATP, an ATP regenerating system, and radiolabeled [α-^{32}P]dATP. After 2 h at 23 °C, reaction products were purified and resolved by 1% agarose gel electrophoresis, and radiolabeled bands representing double-stranded replication products were visualized by autoradiography. For the reactions of lanes 2−5, M13 single-stranded DNA was UV-irradiated at the indicated doses prior to incubation in *Xenopus* egg lysate. A UV dose of 100 J·m^{-2} completely abolished the synthesis of covalently closed M13 circles (lane 5). The electrophoretic positions of form I (closed circles), form II (nicked circles), and form III (linear) M13 products are indicated.

UV-Induced Inactivation of Complementary Strand Synthesis. We performed competition assays to investigate the mechanistic basis underlying the strong inhibition of complementary strand synthesis in response to UV radiation damage. In the experiment of Figure 2, 300 ng each of undamaged and UV-irradiated (100 J·m^{-2}) M13 templates was combined in *Xenopus* egg lysate and incubated for 2 h. Surprisingly, these coincubation mixtures containing both undamaged and damaged single-stranded DNA yielded only marginal levels of overall DNA synthesis (Figure 2, lanes 3 and 4), indicating that the presence of UV lesions not only inhibited complementary strand synthesis on UV-irradiated templates but also suppressed complementary strand synthesis on undamaged templates. We used the term "inactivation" to denote this intermolecular mode of inhibition. Among the different double-stranded replication products, inactivation of complementary strand synthesis by coincubation with UV-irradiated

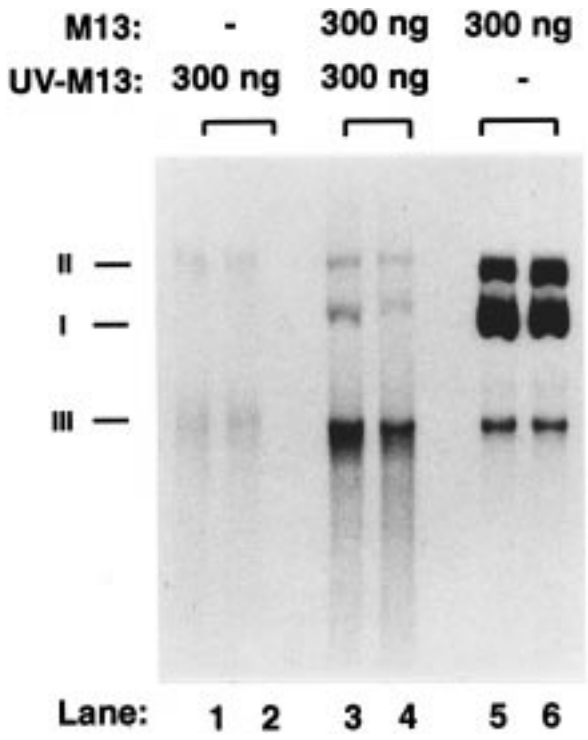

FIGURE 2: Coincubation reactions with UV-irradiated single-stranded DNA. Equal amounts (300 ng) of UV-irradiated (100 J·m^{-2}) and undamaged M13 single-stranded DNA were coincubated for 2 h in *Xenopus* egg lysates exactly as indicated in the legend of Figure 1, and the radiolabeled replication products were analyzed by agarose gel electrophoresis and autoradiography (lanes 3 and 4). Parallel control reactions contained only 300 ng of UV-irradiated DNA (100 J·m^{-2}; lanes 1 and 2) or only undamaged DNA (lanes 5 and 6).

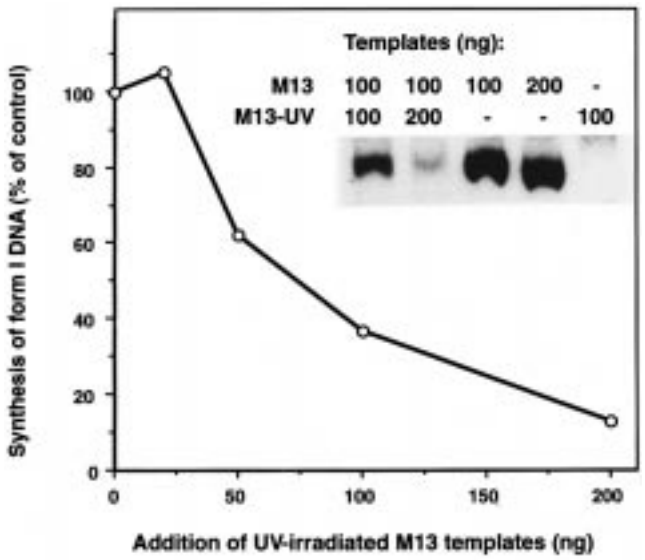

FIGURE 3: Inactivation of complementary strand synthesis by coincubation with UV-irradiated single-stranded DNA. Undamaged M13 single-stranded DNA (100 ng) was mixed with increasing amounts of UV-damaged M13 single-stranded DNA (100 J·m^{-2}) and incubated for 2 h in *Xenopus* egg lysate. Radiolabeled M13 replication products were resolved by agarose gel electrophoresis and visualized by autoradiography. The relative level of form I M13 DNA (covalently closed circles) was determined by laser scanning densitometry of the resulting X-ray films and expressed as the percentage of form I M13 DNA obtained in control reactions containing 100 ng of undamaged M13 single-stranded DNA only (mean values of three independent experiments). The inset shows radiolabeled form I replication products resulting from typical reactions containing either undamaged M13 templates ("M13"), UV-irradiated M13 templates ("M13-UV"), or both undamaged and UV-irradiated M13 templates, as indicated.

templates affected primarily the yield of form I and form II DNA, and, in particular, the synthesis of covalently closed M13 circles (form I DNA) was reduced by more than 90% relative to the control incubations carried out in the absence of damaged templates (Figure 2, lanes 3 and 4). The relative increase of material migrating near the position of form III DNA is likely to result from partial degradation of poorly replicated templates.

We then performed dose dependence experiments in which 100 ng of undamaged M13 DNA templates was coincubated with increasing amounts of M13 single-stranded DNA that had been exposed to a UV radiation dose of 100 J·m^{-2}. After reactions of 2 h, the synthesis of circular covalently closed replication products (form I DNA) was determined by gel electrophoresis and laser scanning densitometry of the respective autoradiographs, and the results were compared with the yield of form I DNA in control reactions containing only undamaged templates (Figure 3). These quantitative evaluations revealed partial inhibition of complementary strand synthesis when 100 ng of undamaged templates was coincubated with 50 or 100 ng of UV-irradiated single-stranded DNA, whereas nearly complete inhibition of complementary strand synthesis was obtained by increasing the amount of UV-irradiated single-stranded DNA to 200 ng. The autoradiogram in the inset of Figure 3 illustrates the low level of form I M13 DNA obtained in typical coincubation reactions supplemented with either 100 or 200 ng of UV-irradiated templates. The inset of Figure 3 also demonstrates that inactivation of complementary strand synthesis was strictly dependent on the presence of damaged bases. In fact, the recovery of form I replication products was not diminished by increasing the amount of undamaged substrate to 200 ng. Similarly, inactivation of complementary strand synthesis was strictly dependent on the presence of damaged DNA in the single-stranded form. In fact, even high amounts (200 ng) of UV-irradiated double-stranded DNA (plasmid pSB408 of 10.7 kb pairs exposed to UV doses ranging from 100 J·m^{-2} to 10 kJ·m^{-2}) were completely unable to reduce complementary strand synthesis operating on undamaged M13 single-stranded substrates (Figure 4). Thus, effective inactivation of complementary DNA synthesis is restricted to those coincubation mixtures that contain at least stoichiometric amounts of damaged DNA in the single-stranded form.

When complementary strand synthesis in *Xenopus* egg lysate was prematurely stopped after only 30 min of incubation, we detected mainly unligated products that migrated near the position of nicked circles (Figure 5, lanes 7 and 8). To test whether these early replication stages were prone to inactivation of complementary strand synthesis, we coincubated equal amounts (100 ng) of undamaged and UV-irradiated (100 J·m^{-2}) M13 templates for 30 min. On the average, 30-min incubations containing both undamaged and UV-irradiated single-stranded DNA yielded 40.7% less replication intermediates than the control incubations containing only undamaged single-stranded templates (Figure 5, lanes 2 and 3). The yield of double-stranded DNA was again not reduced by simply increasing the amount of undamaged templates without addition of UV-irradiated

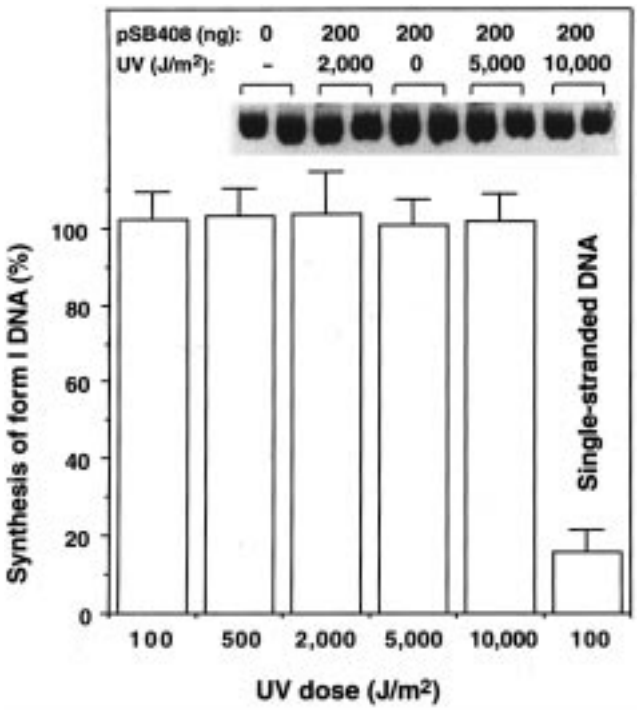

FIGURE 4: Coincubation of undamaged M13 templates with UV-irradiated double-stranded DNA. M13 single-stranded DNA (100 ng) was mixed with 200 ng of plasmid pSB408 irradiated at UV doses ranging from 100 to 10 000 J·m^{-2}. After incubations of 2 h in *Xenopus* egg lysate, radiolabeled M13 replication products were resolved by agarose gel electrophoresis and visualized by autoradiography. The relative level of form I M13 DNA (covalently closed circles) was determined by laser scanning densitometry of the resulting X-ray films and expressed as the percentage of form I M13 DNA obtained in control reactions containing 100 ng of undamaged M13 templates only (mean values of three independent experiments). For comparison, the inhibition obtained upon coincubation of the same substrate with 200 ng of UV-irradiated (100 J·m^{-2}) single-stranded DNA is shown. The inset is a representative autoradiograph showing form I DNA generated in coincubation reactions containing unirradiated or UV-irradiated plasmids.

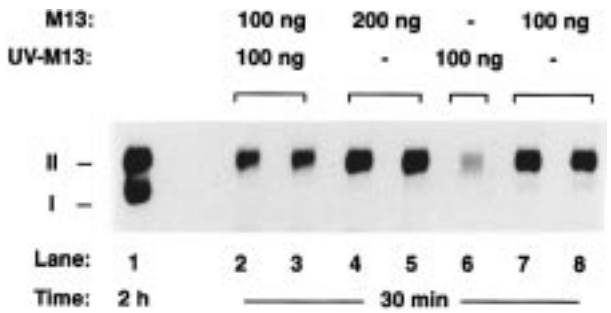

FIGURE 5: Inactivation of complementary strand synthesis during incubations of 30 min. Reaction mixtures in *Xenopus* egg lysate contained undamaged ("M13") and UV-irradiated ("UV-M13"; 100 J·m^{-2}) single-stranded DNA templates, as indicated. After 30 min (lanes 2−8) or 2 h (lane 1), the formation of double-stranded M13 replication products was determined by agarose gel electrophoresis and autoradiography. The reactions that were prematurely stopped after 30 min of incubation yielded essentially only form II DNA products (nicked M13 circles).

single-stranded DNA (Figure 5, lanes 4 and 5). On the other hand, inactivation of complementary DNA synthesis by UV-irradiated single-stranded DNA was also detected when the same series of reactions were stopped after 15 min of incubation, although the inhibition was less pronounced than after 30 min or 2 h (gels not shown). These time course

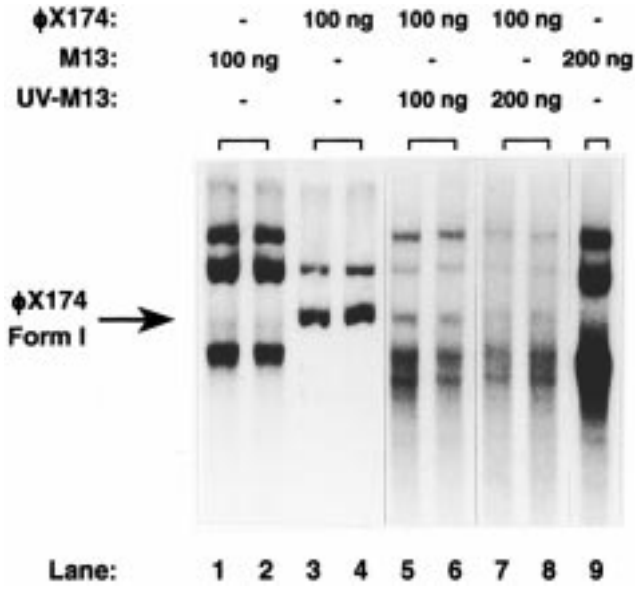

FIGURE 6: Inactivation of complementary strand synthesis on ϕX174 templates by coincubation with UV-irradiated M13 single-stranded DNA. Different amounts of ϕX174, M13, or UV-irradiated (100 J·m^{-2}) M13 single-stranded DNA were incubated for 2 h in *Xenopus* egg lysates. The resulting reaction products were resolved by agarose gel electrophoresis and visualized by autoradiography. The position of ϕX174 form I DNA (covalently closed circles) is shown by the arrow. The other bands represent ϕX174 form II and form III DNA, as well as the different forms of M13 DNA. This gel demonstrates that complementary strand synthesis on undamaged ϕX174 templates is inactivated by coincubation with 100 ng (lanes 5 and 6) or 200 ng (lanes 7 and 8) of UV-irradiated M13 single-stranded DNA.

experiments indicate that inactivation by UV-irradiated single-stranded DNA affects an enzymatic step of complementary strand synthesis that precedes the final ligation of nicked or linear intermediates to form covalently closed M13 circles.

Inhibition of DNA Synthesis on Undamaged ϕX174 Single-Stranded DNA by UV-Irradiated M13 Templates. To unequivocally show that DNA damage located on one DNA molecule may effectively inhibit DNA synthesis on a second DNA molecule containing no lesions, we coincubated UV-irradiated M13 single-stranded DNA with undamaged ϕX174 templates. Because of the diverging sizes of these single-stranded phage genomes, the different double-stranded products resulting from complementary strand synthesis can be easily resolved by agarose gel electrophoresis (Figure 6). Compared to M13 DNA, circular ϕX174 single-stranded molecules constitute less efficient templates for complementary strand synthesis, but, after incubations of 2 h in *Xenopus* egg lysate, semiconservative replication of these ϕX174 templates generated mainly covalently closed circles of 5386 bp (Figure 6, lanes 3 and 4; the position of form I ϕX174 is indicated by the arrow). The synthesis of these ϕX174 form I products was markedly reduced when the reactions in *Xenopus* egg lysate were supplemented with equivalent amounts (100 ng) of UV-irradiated M13 templates (Figure 6, lanes 5 and 6). An even stronger inhibition of complementary strand synthesis on ϕX174 DNA was observed when 100 ng of ϕX174 templates was coincubated with 200 ng of UV-irradiated M13 single-stranded DNA (Figure 6, lanes 7 and 8). In contrast, no inhibition was detected when the amount of undamaged template was increased to 200 ng

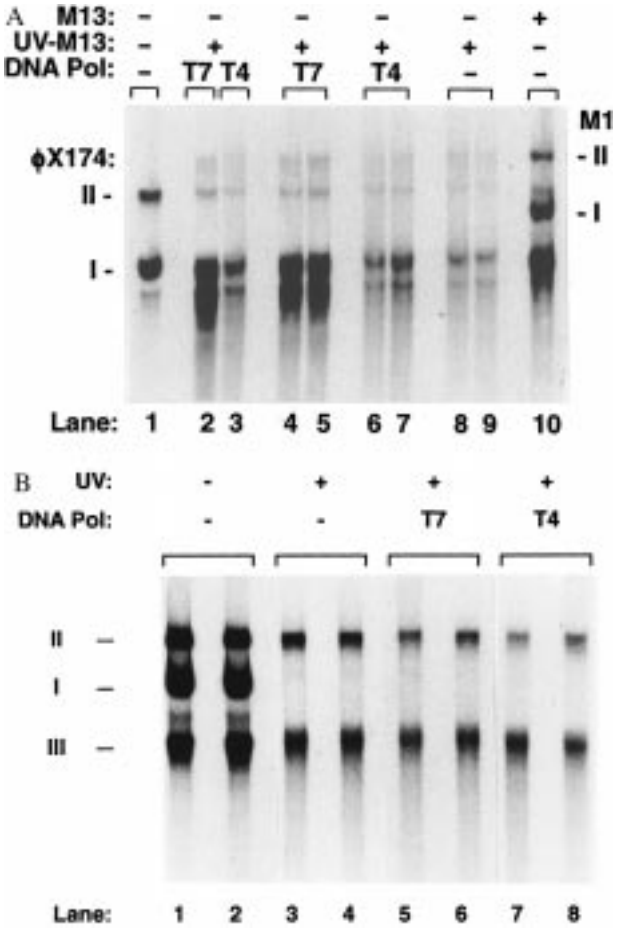

FIGURE 7: Rescue of complementary strand synthesis by an exogenous DNA polymerase. (A) Incubation mixtures in *Xenopus* egg lysate contained 100 ng of undamaged ϕX174 single-stranded DNA with additions of M13 DNA and DNA polymerases as indicated. Lane 1 shows a control reaction with undamaged ϕX174 single-stranded template only. Lanes 2−9: reactions supplemented with 100 ng of UV-irradiated (100 J·m^{-2}) M13 single-stranded DNA. The incubations of lanes 2, 4, and 5 contained 1 unit of T7 DNA polymerase, whereas the incubations of lanes 3, 6, and 7 contained 1 unit of T4 DNA polymerase. Lane 10: reaction containing 100 ng each of undamaged ϕX174 and undamaged M13 single-stranded DNA. (B) Control experiment in *Xenopus* egg lysate demonstrating that neither T7 nor T4 DNA polymerase was able to promote complementary strand synthesis on UV-irradiated (100 J·m^{-2}) M13 templates. Lanes 1 and 2, reactions containing 100 ng of undamaged M13 single-stranded DNA; lanes 3−8, reactions containing 100 ng of UV-irradiated M13 single-stranded DNA. The incubations of lanes 5−8 were performed in the presence of 1 unit of T7 or T4 DNA polymerase.

(Figure 6, lane 9). Similarly, we found that reactions containing 100 ng each of undamaged ϕX174 and M13 single-stranded DNA produced the expected levels of ϕX174 and M13 double-stranded products (shown in lane 10 of Figure 7A), indicating that *Xenopus* egg lysate contains all factors in sufficient amounts for the simultaneous replication of both templates. In summary, these results confirmed that damaged templates are able to suppress complementary strand synthesis on undamaged templates when coincubated in *Xenopus* egg lysate.

Rescue of DNA Synthesis by the Addition of Exogenous DNA Polymerases. Inactivation of complementary strand synthesis may occur by damage-dependent depletion of a critical factor (see Discussion). This hypothesis led us to test whether inactivation of complementary DNA synthesis may be relieved by supplementing the *Xenopus* egg lysate with excess amounts of an appropriate enzymatic activity. For that purpose, we inactivated complementary strand synthesis by coincubating 100 ng of undamaged ϕX174 templates with 100 ng of UV-irradiated M13 single-stranded DNA (Figure 7A, lanes 8 and 9). The synthesis of ϕX174 form I DNA was rescued when these coincubation reactions were enriched with T7 DNA polymerase (Figure 7A, lanes 2, 4, and 5). The addition of T7 DNA polymerase also generated substantial amounts of linear products, presumably because of abnormal DNA nicking or, alternatively, insufficient DNA ligation in this heterologous complementation system. Restoration of complementary strand synthesis was not observed when another prokaryotic factor, T4 DNA polymerase, was added to the coincubation mixtures (Figure 7A, lanes 3, 6, and 7). Apparently, T7 but not T4 DNA polymerase was able to substitute for an enzymatic activity that becomes limiting in the presence of UV-irradiated single-stranded DNA. Importantly, the added DNA polymerase only promoted DNA synthesis on undamaged templates. In fact, neither T7 DNA polymerase nor its T4 counterpart was able to rescue complementary strand synthesis in reaction mixtures containing only UV-irradiated single-stranded M13 molecules (Figure 7B, lanes 5−8).

Rescue of DNA synthesis by T7 DNA polymerase is strictly dependent on the capacity of *Xenopus* egg lysate to form initial RNA/DNA primers. In parallel experiments, M13 templates were annealed with complementary oligonucleotides phosphorylated at their 5′ ends, and mixed with either T7 or T4 DNA polymerase in incubation mixtures that also contained T4 DNA ligase but no *Xenopus* egg lysate. These primer elongation reactions yielded high levels of covalently closed circular M13 products. As expected, however, the formation of complementary strands on either M13 or ϕX174 single-stranded DNA was completely abolished when the DNA synthesis primers were omitted from the reaction mixtures (gels not shown). Because of this absolute requirement for preformed primers, the observation that T7 DNA polymerase is able to reverse inactivation of complementary DNA synthesis in *Xenopus* egg lysate (Figure 7A) indicates that this mode of inhibition affects an enzymatic reaction that occurs after the initial synthesis of RNA/DNA primers. In combination with the time course experiments of Figure 5, these results also exclude the final ligation reaction as a possible target of damaged-dependent inactivation.

MNU-Induced Inactivation of Complementary Strand Synthesis. To test whether inactivation of DNA synthesis is dependent on the presence of bulky DNA damage, we challenged complementary strand synthesis in *Xenopus* egg lysate by the addition of single-stranded DNA containing nonbulky lesions resulting from exposure to MNU. This monofunctional alkylating agent forms multiple methylation products in DNA, including N^7-methylguanine, O^6-methylguanine, N^3-methyladenine, and O^4-methylthymine (*36*). In MNU dose dependence experiments, we first assessed the drug concentration that is necessary to inhibit complementary strand synthesis to a significant extent. For that purpose, M13 single-stranded DNA was pretreated with various MNU concentrations, purified by ethanol precipitation, and incubated in *Xenopus* egg lysate for 2 h. Marked inhibition of complementary strand synthesis was observed only after

pretreatment of DNA with MNU at 1 mM (Figure 8A, lanes 7 and 8). This requirement for high drug concentrations is consistent with previous reports indicating that a fraction of rare methylation products such as O^6-methylguanine and N^3-methyladenine is mainly responsible for blocking DNA synthesis (*22*, *37*).

We then performed coincubation experiments in which 100 ng of undamaged M13 templates was coincubated with single-stranded DNA that was pretreated with 1 mM MNU (Figure 8B). As found in the previous coincubation reactions with UV-irradiated DNA (Figure 3), the presence of increasing amounts of MNU-damaged single-stranded DNA progressively reduced the synthesis of form I DNA on undamaged M13 templates. Again, effective inactivation of complementary DNA synthesis required at least stoichiometric amounts of damaged single-stranded DNA (Figure 8B). The inset of Figure 8B illustrates the yield of form I M13 DNA in typical coincubation reactions containing 100 ng of undamaged templates and between 20 and 200 ng of MNU-treated single-stranded DNA. In a parallel control incubation, increasing the amount of undamaged templates to a total of 200 ng per reaction had no effect on the level of form I products, confirming that inactivation of complementary strand synthesis is strictly dependent on the presence of DNA damage (Figure 8B, inset). We conclude from these results that inactivation of complementary strand synthesis is a general property of replication blocking lesions, including nonbulky methylation products.

Inactivation of Complementary Strand Synthesis in HeLa Cytosolic Lysate. An in vitro system based on HeLa cytosolic lysate has been shown to promote complementary strand synthesis on circular M13 templates (*19*). Therefore, we tested whether inactivation of complementary strand synthesis may be extended to this mammalian cell lysate. Compared to the *Xenopus* system, HeLa cytosolic lysate promoted complementary strand synthesis at lower rates and with a higher proportion of linear double-stranded products. Like in the *Xenopus* egg lysate, however, complementary strand synthesis was completely suppressed when the single-stranded template had been exposed to a UV dose of 100 $J \cdot m^{-2}$ (gels not shown). Also, as observed in *Xenopus* egg lysate, coincubation of undamaged templates with UV-irradiated M13 single-stranded DNA efficiently suppressed complementary strand synthesis (Figure 9). As previously found in *Xenopus* egg lysate, this inactivation of DNA synthesis by damaged single-stranded DNA affected primarily the yield of circular M13 products. In particular, the synthesis of form I M13 DNA was reduced by approximately 80% when the reactions (containing 100 ng of undamaged single-stranded DNA) were supplemented with 200 ng of UV-irradiated single-stranded DNA (Figure 9). Thus, inactivation of complementary strand synthesis by coincubation with damaged templates is observed in lysates from very different eukaryotic sources, indicating that this mode of inhibition is a characteristic eukaryotic response to the accumulation of damaged single-stranded replication intermediates.

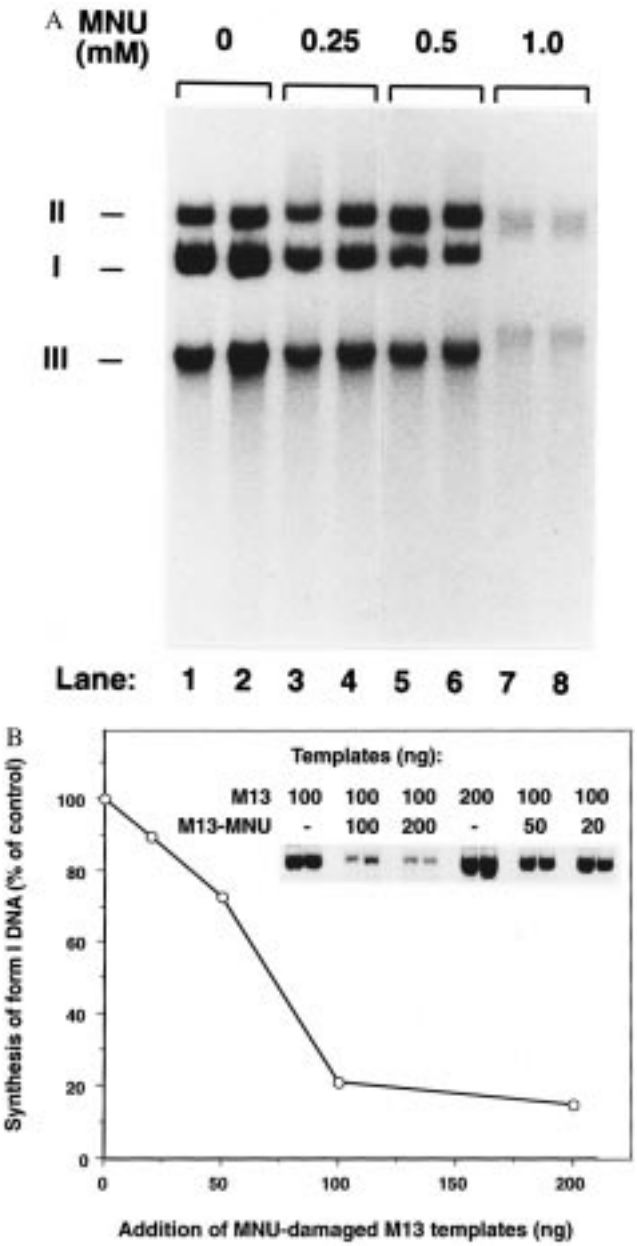

FIGURE 8: Inactivation of complementary strand synthesis by coincubation with MNU-damaged single-stranded DNA. (A) M13 single-stranded DNA was pretreated with MNU at concentrations of 0.25 (lanes 3 and 4), 0.5 (lanes 5 and 6), and 1 mM (lanes 7 and 8), and subsequently purified as outlined under Materials and Methods. These pretreatment mixtures contained dimethyl sulfoxide at a final concentration of 1% (v/v), and control M13 DNA was obtained by incubation with dimethyl sulfoxide only (lanes 1 and 2). The purified templates were incubated in *Xenopus* egg lysate. After 2 h, radiolabeled reaction products were isolated, resolved by agarose gel electrophoresis, and visualized by autoradiography. (B) Undamaged M13 single-stranded DNA (100 ng) was combined with increasing amounts of modified M13 single-stranded DNA that was pretreated with 1 mM MNU, and the mixtures were incubated for 2 h in *Xenopus* egg lysate. Radiolabeled M13 products were resolved by agarose gel electrophoresis and visualized by autoradiography. The level of form I M13 DNA (covalently closed circles) was determined by laser scanning densitometry of the resulting X-ray films and expressed as the percentage of form I M13 DNA obtained in control reactions containing 100 ng of undamaged M13 single-stranded DNA only (mean values of three independent experiments). The inset shows radiolabeled form I replication products resulting from typical reactions containing either undamaged M13 templates alone or undamaged M13 templates with increasing amounts of MNU-modified M13 single-stranded DNA ("M13-MNU"), as indicated.

DISCUSSION

In vitro complementary strand synthesis occurs in a highly effective manner, particularly in *Xenopus laevis* egg lysate

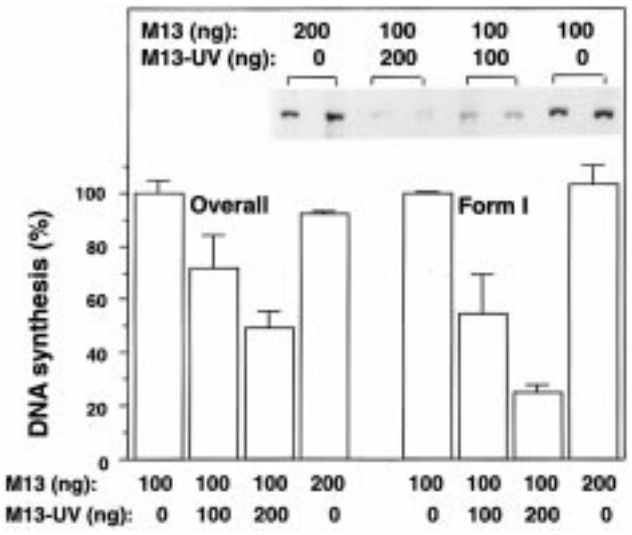

FIGURE 9: Coincubation reactions in HeLa cytosolic lysate. The indicated amounts of undamaged or UV-irradiated (100 J·m^{-2}) M13 single-stranded DNA were coincubated for 2 h in HeLa cell lysate supplemented with all four ribonucleoside triphosphates, ATP and an ATP regenerating system, all four deoxyribonucleoside triphosphates, and [α-^{32}P]dATP. Radiolabeled reaction products were then isolated and resolved by 1% agarose gel electrophoresis, and the resulting gels were autoradiographed. The level of form I, form II, and form III double-stranded M13 DNA was quantified by laser scanning densitometry of the exposed X-ray films. Overall DNA synthesis (all three forms of M13 double-stranded DNA) and the yield of form I M13 DNA were expressed as the percentage of the respective replication products stimulated by 100 ng of undamaged M13 templates (mean values of three independent determinations). The inset shows the levels of form I DNA obtained in a typical experiment with HeLa cell extract.

where circular single-stranded genomes are replicated with 95% recovery (*15*, *16*). Despite this efficiency when operating on undamaged single-stranded templates, the same *Xenopus* egg lysate is essentially unable to promote complementary strand synthesis on damaged templates. For example, we found that incubation with UV-irradiated single-stranded DNA generates only low levels of prematurely aborted replication intermediates. A similar response with strong inhibition of complementary strand synthesis has already been observed before when UV-irradiated single-stranded DNA was injected into *Xenopus laevis* oocytes (*10*). In addition, we found that UV radiation damage abolishes complementary strand synthesis in a HeLa cell lysate, and this result is in agreement with a recent report demonstrating that lysates from HeLa or Chinese hamster ovary cells are unable to promote strand elongation on primed single-stranded templates containing either a cisplatin or an acetylaminofluorene adduct (*38*). Interestingly, a diametrically opposite response was found in this previous study when a replication fork-like substrate rather than a single-stranded template was incubated with the same mammalian cell lysates. In this case, a cisplatin or acetylaminofluorene lesion was efficiently bypassed, and strand elongation was completed in an effective manner (*38*).

In the present report, we observed that DNA lesions generated by exposure to UV radiation or the alkylating agent MNU not only suppress complementary strand synthesis on the damaged templates but, in addition, also inhibit complementary strand synthesis on undamaged templates added to the same reaction mixtures. For example, the conversion of φX174 single-stranded genomes to double-stranded DNA was nearly completely abolished when the incubation mixtures in *Xenopus* egg lysate were supplemented with UV-irradiated M13 single-stranded DNA. The same intermolecular mode of inhibition also occurred during complementary strand synthesis in HeLa cell lysate. These observations imply that a replicative activity which is indispensable for complementary strand synthesis becomes inactivated upon coincubation with UV- or MNU-treated templates. Substantial inhibition was observed only when undamaged templates were combined with at least equal amounts of damaged single-stranded DNA. This stoichiometric requirement indicates that inactivation is based on quantitative interactions with damaged single-stranded DNA, most likely resulting in sequestration of replication factors at or near sites of template lesions. However, we were unable to rule out an alternative hypothesis of inactivation based on proteolytic processing or other forms of postranslational protein modification following interaction with damaged templates. Also, it is presently unclear whether inactivation reflects only transient inhibition of DNA synthesis or whether this inhibition is irreversible.

Regardless of the precise mechanism of inactivation, the coincubation experiment in which we restored DNA synthesis by supplementing the cell lysate with an exogenous DNA polymerase lends support to the conclusion that a critical replication factor is excluded from its normal function. In fact, bacteriophage T7 DNA polymerase in association with its processivity factor thioredoxin (*39*, *40*) was able to rescue complementary strand synthesis on undamaged templates despite the presence of damaged single-stranded DNA. Interestingly, the T7 DNA polymerase−thioredoxin complex not only restored normal levels of overall DNA synthesis but also reestablished the formation of circular covalently closed replication products. Because T7 DNA polymerase fails to synthesize complementary strands in the absence of intact templates, this rescue experiment argues against another inhibitory mechanism that involves substantial degradation of DNA substrate in response to template damage. We also tested bacteriophage T4 DNA polymerase in the absence of its accessory factors for processivity (*41*, *42*), but, as a solitary enzyme, T4 DNA polymerase was not able to restore DNA synthesis in the presence of UV-irradiated single-stranded DNA.

Inactivation of replication factors may affect one or several steps of complementary strand synthesis, including its initiation by primer formation, the subsequent strand elongation phase, or the final ligation of nicked or linear double-stranded products to form covalently closed circles. The observation that T7 DNA polymerase is capable of restoring both overall DNA synthesis and the yield of covalently closed (form I) products in the presence of damaged single-stranded DNA indicates that neither the initial primer formation nor the final DNA ligation step are principal targets of this inhibitory mechanism. Like all DNA polymerases, T7 DNA polymerase is only active on templates containing preexisting primers, implying that the cell lysate maintains its primer synthesis activity despite the presence of damaged single-stranded DNA. Similarly, the T7 DNA polymerase/thioredoxin complex has no DNA ligase activity, and, hence, the formation of covalently closed replication products when this enzyme was added to the coincubation reactions indicates

that the cell lysate also maintains its DNA ligation capability despite the presence of damaged single-stranded DNA. Therefore, we conclude that inhibition of complementary strand synthesis by coincubation with damaged single-stranded DNA occurs by inactivation of replication factors that are normally implicated in the strand elongation step rather than in early strand initiation or late DNA ligation processes.

Our report is relevant to the question of how DNA damage is converted into intracellular signals that can control cell cycle progression in response to replication blocking lesions. In fact, genetic studies have implicated eukaryotic DNA polymerases and other replication factors in an early step of the S phase checkpoint that delays the rate of ongoing DNA synthesis after exposure to genotoxic lesions (*24*−*27*, *43*). For example, yeast DNA polymerase ϵ contains two separable domains: the N-terminal half of this enzyme has the polymerase active site, whereas its C-terminal half contains a domain that is required for an S phase-specific checkpoint and is characterized by the presence of a zinc finger motif (*24*, *44*). It has been suggested that this C-terminal domain of DNA polymerase ϵ may sense the accumulation of strand termini or single-strand gaps that arise in response to blocks to DNA synthesis. Our present results indicate that a critical effector of DNA synthesis interacts with segments of damaged single-stranded DNA templates. Conceivably, the resulting nucleoprotein complexes may alter the biochemical properties of the eukaryotic replication machinery, such that the targeted subunit undergoes a functional switch from its role in DNA synthesis to an additional activity as a sensor of DNA damage during replication. Whether sequestration itself or the resulting accumulation of single-strand gaps constitutes the signal for checkpoint regulation remains to be assessed.

Collectively, this study and previous reports suggest that the eukaryotic replication system has adopted a dual strategy to minimize the potentially deleterious effects of replication blocking lesions. In fact, eukaryotic cells display a remarkable ability to circumvent blocks to DNA synthesis and complete replication of their genomes even in the presence of persisting sites of damage (*45*−*49*). As mentioned before, efficient translesion of DNA synthesis across bulky base adducts was observed only when mammalian cell extracts were incubated with fork-like substrates, whereas no bypass of DNA damage was observed when the same cell extracts were incubated with primed single-stranded templates (*38*). Similarly, other authors observed that replication forks operating on SV40 genomes are capable of bypassing cyclobutane pyrimidine dimers (*14*, *50*, *51*). To accommodate the observation that mammalian cell extracts support translesion synthesis on fork-like but not on single-stranded templates, it has been proposed that the former may attract different components of the eukaryotic replication machinery, implying that efficient translesion synthesis is strictly dependent on the activity of enzymes and accessory factors that are normally encountered at the replication fork (*38*). Thus, as indicated in Figure 10, the replication fork complex appears to bypass most types of DNA lesions during replication, thereby generating two branches of double-stranded replication products despite the presence of non-instructional sites. Only occasionally, the replication fork is blocked by highly obstructive DNA lesions, and, in this case, DNA synthesis may proceed in an asymmetric manner generating single-stranded intermediates (*11*−*14*, *52*), which attract a different subset of replication factors (Figure 10). In contrast to the enzymes and accessory proteins at the replication fork, those factors that operate preferentially on single-stranded DNA are exquisitely sensitive to template lesions and, hence, fail to promote any bypass of DNA damage by translesion synthesis (*10*). The present study indicates that a possible mechanism underlying this sensitivity involves inactivation of replication factors, presumably by sequestration on damaged single-stranded DNA or, possibly, additional biochemical changes. In any case, interaction of a replicative factor with damaged single-stranded DNA molecules may constitute an early step in the pathway that leads to S phase-specific cell cycle checkpoints in response to genotoxic insults.

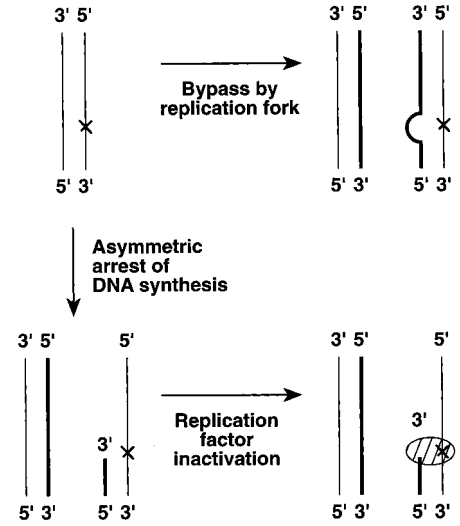

FIGURE 10: Scheme illustrating the dual strategy of eukaryotic replication complexes in response to DNA damage. The replication fork operates on duplex templates and frequently bypasses DNA damage by translesion synthesis. DNA lesions which impede replication fork progression result in the formation of blocked intermediates containing regions of single-stranded DNA. A different subset of replication factors operates on such aborted single-stranded DNA intermediates to convert them to double-stranded products. In contrast to the enzymes and accessory proteins that function at replication forks, these latter factors are inactivated upon encountering replication blocking lesions during complementary strand synthesis. Their exquisite sensitivity to template lesions may be exploited to monitor DNA damage and stimulate S phase arrest in response to genotoxic insults. Thus, the eukaryotic replication machinery may counteract genotoxic lesions by a dual strategy of translesion synthesis (using the replication fork) and DNA damage monitoring (during complementary strand synthesis). Thin lines, parental DNA; thick lines, newly synthesized daughter strands.

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