In vitro analysis of UV-damage-induced inhibition of replication

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We investigated DNA-damage-induced inhibition of replication by using an *in vitro* system, with which both replication and repair can be examined simultaneously. The system contains non-irradiated simian virus 40 (SV40) origin-containing DNA, UV-irradiated circular duplex DNA lacking an SV40 origin, and cell extracts that support both replication and repair activities. Using this system, we show that replication is significantly inhibited in the presence of UV-irradiated, but not non-irradiated, DNA and, to a lesser extent, repair activity is also inhibited by the presence of replication activity. In contrast, replication activity was not affected by UV-damaged DNA when the reactions were carried out with purified replication proteins, suggesting that protein factor(s) in the cell extracts are involved in the inhibition of replication that is triggered by DNA damage. Inhibition was efficiently reversed by the combined actions of proteins involved in both repair and replication, suggesting that the inhibition of replication observed in our system may be caused by the recruitment of replication proteins to damaged DNA sites.

INTRODUCTION

When DNA is damaged by UV irradiation, ionizing radiation or chemicals, it must be repaired to prevent genomic alterations that could otherwise give rise to cancer cells. For this reason, cells invoke various mechanisms to repair their DNA, and to prevent DNA replication until the damage is repaired [1–3]. It is not known how DNA damage induces the inhibition of DNA replication, and yet permits DNA repair; however, proteins such as replication protein A [(RPA), also known as human single-stranded DNA-binding protein (HSSB)], proliferating cell nuclear antigen (PCNA), activator 1 [(A1), also known as replication factor C (RF-C)] and DNA polymerase δ (pol δ) have all been implicated in both processes [4–13].

It has been suggested that when normal cells are exposed to DNA-damaging agents, their levels of the p53 tumour-suppressor protein rise, inducing p21cip1/waf1 [14]. p21cip1/waf1 has two documented functions. First, it inhibits cyclin-dependent kinase, possibly causing cells to arrest at the first gap (G_1) phase of the cell cycle [15–18]; secondly, it interacts with PCNA, inhibiting DNA replication [19,20], without affecting the repair function of PCNA [21]. There is, however, compelling evidence that cells exposed to UV irradiation are inhibited in the synthesis (S) phase, rather than at the G_1–S boundary. Lu and Lane [2] have shown that DNA synthesis in UV-irradiated cells is significantly inhibited while repair occurs. Similarly, the ability of HeLa cell extracts to support replication *in vitro* decreases as the degree of UV-induced S-phase arrest increases [3]. These observations suggest that S-phase arrest upon DNA damage may not simply be due to a block in the replication forks of the damaged DNA template. In this study, we have used an *in vitro* system with which both DNA replication and repair can be examined. When this repair–replication system is established using HeLa cell extracts, simian virus 40 (SV40) DNA replication is significantly inhibited in the presence of UV-damaged DNA that lacks an origin of replication. In contrast, if the system is established using purified replication proteins, but no repair proteins, DNA-damage-induced inhibition of replication is not observed. We suggest that repair of damaged DNA competitively inhibits DNA replication by sequestering proteins that have both repair and replication functions. We discuss how these data influence our current understanding of the regulation of DNA replication upon DNA damage.

EXPERIMENTAL

Cell extracts, proteins and plasmid DNA

HeLa cell cytosolic extracts and whole-cell extracts were prepared as described previously [22,23]. To prepare cytosolic extract, asynchronously grown HeLa cells (5×10^9) were washed twice with PBS, and resuspended in hypotonic buffer [20 mM Hepes/KOH (pH 7.5)/5 mM KCl/1.5 mM $MgCl_2$/1 mM dithiothreitol (DTT)]. Protease inhibitors (0.1 mM PMSF, 0.1 mg/ml leupeptin and 0.2 mg/ml antipain) were then added, and the swollen cells were broken by 15–20 strokes in a Dounce homogenizer. After adjusting the salt concentration to 200 mM, we cleared the extracts by centrifugation and subsequent dialysis against buffer A [50 mM Tris/HCl (pH 7.8)/10 % (v/v) glycerol/1 mM DTT/0.5 mM EDTA], containing 25 mM NaCl. To prepare whole-cell extracts, an equal volume of buffer [50 mM Tris/HCl (pH 8.0)/10 mM $MgCl_2$/2 mM DTT/25 % (w/v) sucrose/50 % glycerol] was added to the Dounce-homogenized extracts, followed by 0.125 vol. of satd. $(NH_4)_2SO_4$ solution (pH 7.5), and the mixture was stirred for 30 min on ice. After centrifugation at 45 000 **g** for 3 h at 2 °C, the supernatant was collected, and proteins were precipitated by the addition of $(NH_4)_2SO_4$ (0.33 g/ml). After

centrifugation, the precipitates were resuspended and dialysed against buffer [25 mM Hepes/KOH (pH 7.9)/0.1 M KCl/12 mM $MgCl_2$/1 mM EDTA/2 mM DTT/17 % (v/v) glycerol].

HeLa cell cytosolic extracts (20 ml; 350 mg of protein) were $(NH_4)_2SO_4$-fractionated, as described previously [5]. The 35–65 % fraction was dialysed against buffer A containing 150 mM KCl, and loaded on to a phosphocellulose column (15 ml) that was pre-equilibrated with buffer A containing 150 mM KCl. After the column was washed with the same buffer, bound proteins were eluted with buffer A containing 1.0 M KCl. The phospho-cellulose 1.0 M-fraction was pooled and dialysed against buffer A containing 50 mM KCl, before being used as a receptor fraction for the RPA complementation assay in repair and replication.

SV40 T antigen (T-ag), RPA (or HSSB), topoisomerases (topo) I and II, pol α–primase, pol δ, PCNA and A1 (or RF-C) were isolated by the procedures described previously [24,25]. The *Escherichia coli* endonuclease-III-overproducing strain was originally obtained from Dr. J. Cunningham (State University of New York, Albany, NY, U.S.A.), and was purified according to the published procedure [26]. DNA plasmids [pSVLD (10 kb), pSV01ΔEP (2.8 kb) [27] and pBluescript (pBS, 3 kb)] were prepared for the repair and replication assays by two rounds of CsCl gradient centrifugation [28], followed by 5 %–20 % (w/v) sucrose density-gradient centrifugation using at SW28 rotor at 60 000 g for 19 h at 2 °C.

UV-irradiated plasmid DNA preparation

pBS was prepared by two rounds of CsCl centrifugation from the *E. coli* strain JM109 (*recA*), without chloramphenicol amplification. The purified DNA (500 μg) was suspended in 10 ml of TE buffer [10 mM Tris/HCl (pH 8.0)/1 mM EDTA] and placed in a plastic Petri dish (150 mm) with gentle rocking. The Petri dish was then UV-irradiated with germicidal UV light (G15T8) at a fluence rate of 0.9 W/m². UV fluence was measured by using a Black-Ray UV photometer (UVP Inc. Upland, CA, U.S.A.). After UV irradiation, the DNA was treated with *E. coli* Nth protein, and closed circular supercoiled DNA was isolated by CsCl gradient centrifugation followed by sucrose gradient centrifugation, as described previously [23]. Even though the primary source of UV was UV-C light, the final preparation of irradiated plasmid DNA contained more than 90 % supercoiled circular duplex DNA (results not shown).

Nucleotide excision repair and SV40 DNA replication assays *in vitro*

Nucleotide excision repair [23] and SV40 DNA replication *in vitro* [24] were carried out according to the published procedures.

The *in vitro* repair–replication assay

Reaction mixtures (40 μl) contained 200 μg of HeLa cell extract [either cytosolic extracts, or mixture of cytosolic extract (150 μg) with whole-cell extract (50 μg)], 0.9 μg of pSVLD, various amounts (0–0.8 μg) of either non-irradiated or irradiated pBS plasmid, 10 μg of BSA, 1.0 μg of creatine kinase, 40 mM creatine phosphate (di-Tris salt, pH 7.7), 7 mM $MgCl_2$, 0.5 mM DTT, 4 mM ATP and 200 μM each of CTP, UTP and GTP. Where indicated, T-ag (0.8 μg) was added. The established assays with purified replication proteins contained 0.8 μg of SV40 T-ag, pol α–primase (0.1 unit of pol α and 0.05 unit of primase), topo I (500 units), 1.2 μg of topo II, 0.3 μg of RPA, 0.2 μg of PCNA, 1.0 μg of A1 (RF-C) (single-stranded DNA cellulose fraction;

[23]) and 0.06 μg of pol δ. The reaction mixtures were pre-incubated at 30 °C for 30 min before the addition of dNTPs {100 μM each of dCTP, dGTP and dTTP, and 20 μM [α-^{32}P]dATP $(2 \times 10^4$ c.p.m./pmol)}. They were then incubated for 1 h at 37 °C. After this incubation, the DNA products were isolated [29] and analysed by 0.7 % (w/v) agarose gel electrophoresis in $1 \times$ TAE (40 mM Tris/40 mM acetate/2 mM EDTA; pH 8.0) buffer, at 40 V for 15 h. One-tenth of each reaction mixture was retained to measure the trichloroacetic-acid-precipitable radioactivity. Replication activity was measured by subtracting the amount of nucleotide incorporation in the absence of SV40 T-ag from that obtained with the complete coupled system. Repair activity was determined by measuring the level of radioactivity incorporated into the repaired DNA that was excised from the dried gel.

RESULTS

Repair–replication system *in vitro*

UV-induced inhibition of replication *in vivo* may be caused by: (i) blockage of the replication fork by the damaged DNA template; (ii) p53-mediated induction of p21cip1/waf1, which inhibits the replication function of PCNA [19,20]; (iii) an unknown mechanism; or (iv) a combination of all of the above. Extracts prepared from UV-irradiated cells poorly supported SV40 DNA replication, compared with those from non-irradiated cells [3], suggesting that protein factors in the extracts (rather than DNA damage itself) are likely to be responsible for the UV-induced inhibition of replication.

To investigate damage-induced inhibition of replication, we developed an *in vitro* system that allows us to examine the co-ordinated activities of both replication and repair. The system is described in Scheme 1. It contains a non-irradiated SV40 origin-containing plasmid (pSVLD) [26] and a UV-irradiated or non-irradiated plasmid that lacks an SV40 origin (UV irradiation is indicated as small dots on a pBS plasmid). Incubation of both plasmids with cell extracts supported both replication and repair in the presence of SV40 T-ag and UV damage on pBS. HeLa cell cytosolic extracts (see Figure 3) and a mixture of cytosolic and whole cell extracts (3:1) (see Figure 1) supported both activities.
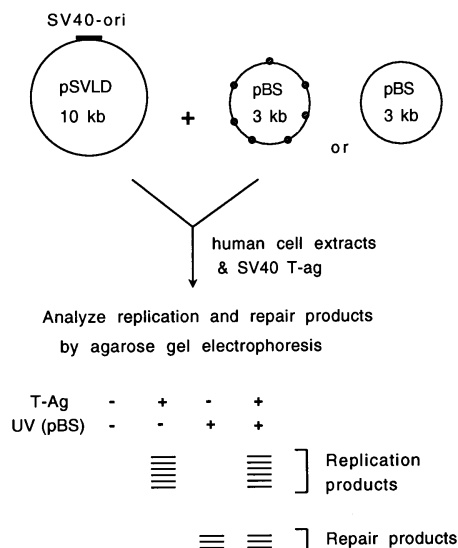
Scheme 1 *In vitro* **analysis of the repair–replication system**
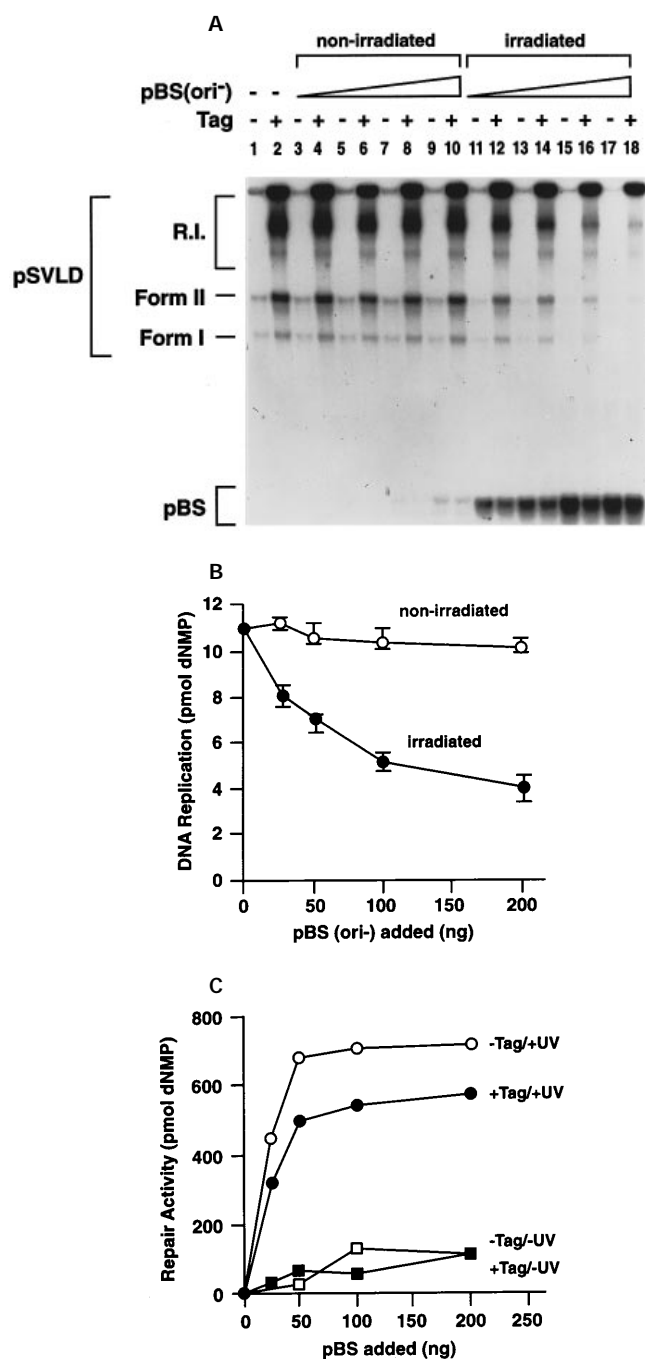
Figure 1   DNA replication and repair activities in the repair–replication system

(**A**) Reaction mixtures contained 200 μg of HeLa cell extracts (3:1 mixture of cytosolic extracts:whole cell extracts), pSVLD (10 kb) for DNA replication, and increasing concentrations of irradiated or non-irradiated pBS (3 kb) (25 ng for lanes 3, 4, 11 and 12; 50 ng for lanes 5, 6, 13 and 14; 100 ng for lanes 7, 8, 15 and 16; and 200 ng for lanes 9, 10, 17 and 18) for nucleotide-excision repair. Other conditions were as described in the Experimental section. After the reaction, DNA was isolated and analysed on a 0.7% agarose gel in TAE buffer (40 V, 15 h). R.I. represents replication intermediates of pSVLD, while Form I (closed circular, supercoiled) and Form II (nicked circular) represent the positions of unlabelled plasmids in the gel. (**B**) The effect of UV-damaged DNA (800 J/m²) on SV40 DNA replication is shown. Replication activity was quantified from the coupled repair–replication reactions (shown in **A**) by measuring the level of radioactivity in the repaired DNA excised from the dried gel. The amount of DNA synthesis is indicated as pmol of dNMP incorporated into the plasmid DNA. (**C**) The effect of replication on the repair of UV-damaged DNA is shown. Repair activities were quantified from the coupled repair–replication reactions (**A**). The values shown in the Figure represent one-quarter of the total reaction mixture from each reaction.

Reactions without T-ag or UV damage on pBS supported only repair or replication activity respectively.

UV-induced inhibition of replication *in vitro*

Using this system, we examined how replication of an SV40 origin-containing plasmid is affected by the presence of UV-damaged DNA that lacks an origin of replication, and vice versa. As illustrated in Figure 1(A), DNA replication was significantly inhibited in the presence of UV-damaged DNA, but not in the presence of non-irradiated DNA. The degree of inhibition of replication by damaged DNA correlated with the level of DNA repair activity. Incorporation of nucleotides into the irradiated pBS was due to nucleotide-excision repair, and not due to non-specific incorporation at the nicked-DNA sites, because the incorporation of nucleotides was dependent on the presence of ATP, a characteristic of nucleotide-excision repair (results not shown). To measure accurately both repair and replication activities, regions representing replication and repair were excised from the gel and quantified. As shown in Figures 1(B) and 1(C), replication activity was inhibited by up to 75% in the presence of 200 ng of irradiated pBS, whereas repair of UV-damaged DNA was inhibited by 20%–25% by the presence of replication activity. It is speculated that two replication complexes exist for each SV40 origin-containing DNA (10 kb), and 20–24 damage sites for each irradiated pBS (3 kb). The number of damaged DNA sites was based on the rough estimate from a previous report [23]. This may partially explain why DNA repair activity was less affected by DNA replication than replication was affected by damaged DNA and its repair (see Discussion for details).

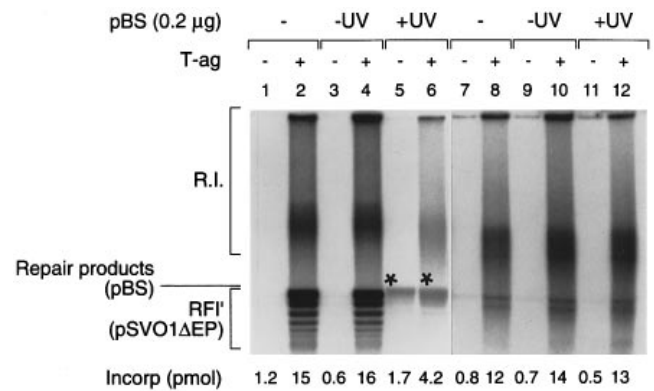## The inhibition of replication correlates with repair activity

To ensure that the observed inhibition of replication was due to the damaged DNA, the amount of UV-induced DNA damage was varied. We then examined the effect of this damaged DNA on the replication of SV40 origin-containing DNA (Figure 2A). With 400 J/m² or less, the inhibition of replication was not completely UV-dose-dependent, particularly in the presence of 100 ng of pBS (Figure 2B). However, the inhibition of replication correlates with UV dosage in the presence of 200 ng of pBS, suggesting that the inhibition of replication not only occurs in a UV-dose-dependent manner, but also depends on the amount of damaged DNA. Similarly, the level of repair activity also corresponded with the UV dosage (Figure 2C).

To further examine the relationship between the inhibition of replication and repair activity, we asked whether UV-induced inhibition of replication can be established by using purified replication proteins, i.e. generating a dipolymerase system that contains SV40 T-ag, DNA pol α–primase complex, topo I and II, RPA, pol δ, PCNA and A1 (RF-C) (Figure 3). Instead of pSVLD (10 kb), pSV01ΔEP (2.8 kb) was used as a source of origin-containing DNA because replication efficiency was better with the smaller DNA template in the dipolymerase replication system. The reconstituted system lacks repair proteins, therefore no repair activity was anticipated (compare lane 5 with lane 11 in Figure 3). In fact, compared with the repair–replication system with HeLa cell cytosolic extracts (Figure 3, lanes 5 and 6), we saw no repair activity in the reactions with purified replication proteins (Figure 3, lanes 11 and 12). Furthermore, UV-damaged DNA did not inhibit replication in the repair–replication re-actions with purified replication proteins (Figure 3, lane 12). Taken together, our results strongly suggest that the inhibition of replication correlates with the repair of UV-damaged DNA in this repair–replication system.

A

B

C
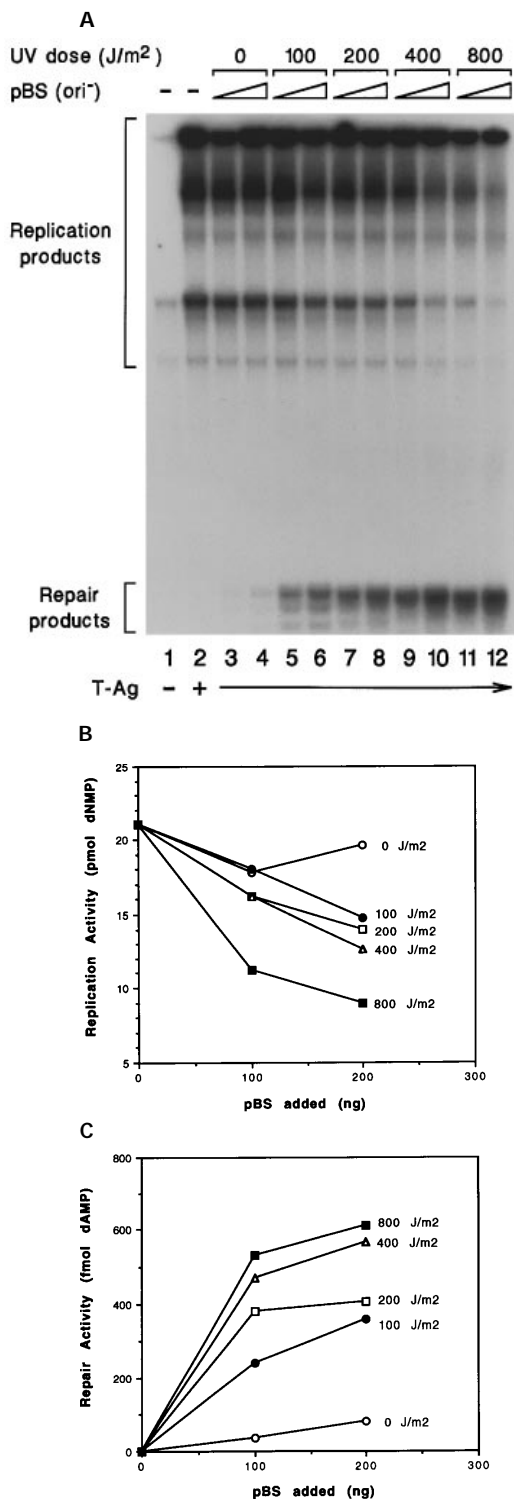
Figure 2   Effect of UV dosage on the inhibition of DNA replication

(**A**) Correlation between UV dosage and the inhibition of replication. Where indicated, increasing amounts (100 and 200 ng) of irradiated pBS [0 J/m^2 (lanes 3 and 4); 100 J/m^2 (lanes 5 and 6); 200 J/m^2 (lanes 7 and 8); 400 J/m^2 (lanes 9 and 10); and 800 J/m^2 (lanes 11 and 12)] were included in the coupled repair–replication assays. Other reaction conditions were the same as those described in the legend to Figure 1(**A**). Replication and repair products are indicated to the left of Figure 2(**A**). The quantification of replication (**B**) and repair (**C**) activities is derived from the experiment described in (**A**).

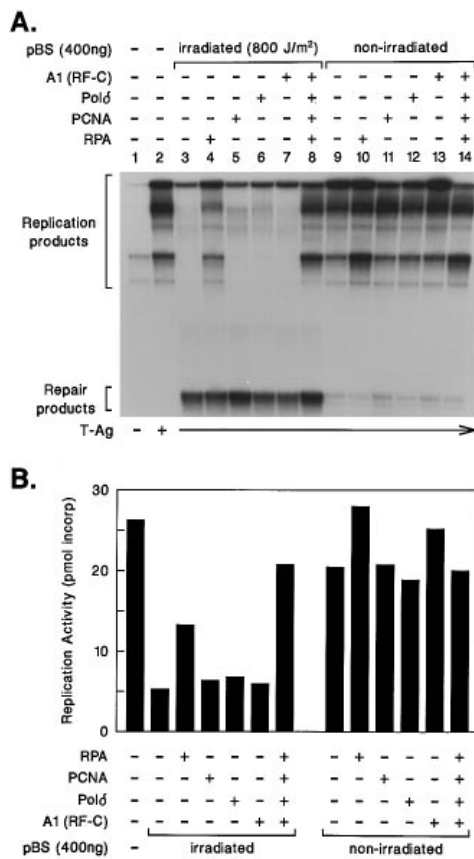**Figure 3   Inhibition of replication correlates with repair activity in the repair–replication system**

The repair–replication assay was carried out in the presence of 200 μg of HeLa cytosolic extract (lanes 1–6) or in the presence of purified replication proteins (see the Experimental section for details) (lanes 7–12). The $-$UV and $+$UV headings on the top of the Figure indicate pBS DNA with 0 and 450 J/m^2 irradiation respectively. Where indicated, 0.8 μg of SV40 T-ag was included. pSV01ΔEP (2.8 kb) was used instead of pSVLD (10 kb) as a source of SV40 DNA in this reaction. After the reactions, the products were analysed by 1% agarose gel electrophoresis. The * in lanes 5 and 6 indicates the position of pBS. Other reaction conditions are as for those described in the legend to Figure 1. Abbreviations: R.I., replication intermediates of pSV01ΔEP; RFI′, relaxed pSV01ΔEP.

Inhibition is reversed by proteins with both replication and repair functions

DNA replication was inhibited by both the presence and repair of UV-damaged DNA. To a lesser extent, repair activity was also inhibited by DNA replication in the repair–replication system. DNA repair and replication processes share several proteins, and the inhibition of replication may be due to competition in the repair–replication system for these shared proteins. If this is the case, we would expect the inhibition to be reversed by the addition of such proteins to the repair–replication system. RPA, PCNA, pol δ and A1 (RF-C) are all known to be involved in both replication and repair [10–13]. Inhibition of replication was not reversed by the addition of either PCNA or pol δ, but was partially reversed by RPA addition (Figure 4). Furthermore, when RPA, PCNA, pol δ and A1 (RF-C) were collectively added to the system, the inhibition was reversed by up to 80%. The stimulatory effect of RPA, or the combination of RPA, PCNA, pol δ and A1 (RF-C), was due to the reversal of DNA-damage-induced inhibition, since this stimulatory effect was significantly reduced in the presence of non-irradiated DNA (Figure 4, lanes 9–14). This result suggests that the inhibition of replication by the presence of damaged DNA may involve proteins (particularly RPA) that are involved in both repair and replication activities. On the other hand, repair activity was stimulated in the reaction containing additional PCNA but not additional RPA, suggesting that inhibition of repair during replication (observed in Figure 1C) may involve PCNA.

## Differential requirement for RPA in replication and repair

RPA is a key factor in damage-induced inhibition of replication *in vitro* because it is required for both replication and repair. To assess whether there is any difference in the RPA requirement of these two metabolic events, RPA was carefully titrated in both SV40 DNA replication and nucleotide-excision repair assays *in vitro*. HeLa cytosolic extracts, from which RPA had been

Figure 4 Effect of RPA, PCNA, pol δ and A1 (RF-C) on DNA-damage-induced inhibition of replication

(**A**) Reaction mixtures contained 200 μg of HeLa cell extracts (cytosolic extracts:whole cell extracts = 3:1) and all other conditions were the same as those described in the legend to Figure 3. Where indicated, 0.8 μg of RPA, 0.4 μg of PCNA, 0.2 unit of pol δ (0.2 μg), 0.2 μg of A1 (RF-C) and 0.8 μg of SV40 T-ag were added. After the reaction, repair and replication products were analysed by 0.7% agarose gel electrophoresis.(**B**) Quantification of the replication products from the reactions in (**A**). The regions representing the replication products were excised from the gel and measured.

removed by biochemical fractionation, was used as a receptor fraction for RPA complementation in the replication and repair assays. Differences in total incorporation (150 fmol in repair, versus 30 pmol in replication; see Figure 5) can be explained by the size differences of the DNA per event (3 kb in replication versus 35 nts in repair patch), and also by the fact that repair occurs only on one strand, whereas DNA replication involves both DNA strands. Virtually no replication activity was observed in a reaction containing less than 60 ng of RPA (Figure 5A). On the other hand, 70% repair activity was observed in the presence of 30 ng of RPA, as compared with that observed with a saturating amount of RPA (Figure 5B). This is somewhat surprising, because the number of damaged DNA sites in the repair reaction was at least ten times greater than the number of replication origins in the replication reaction. It is possible that a large number of RPA molecules are required to unwind and stabilize the unwound DNA in the replication reaction, whereas only one or two molecules of RPA may be required for each repair reaction. It should be pointed out that this assumption is on the basis of a theoretical estimate of the number of DNA repair sites. This result further supports our data, described in Figure 1, which indicate that replication is more susceptible to
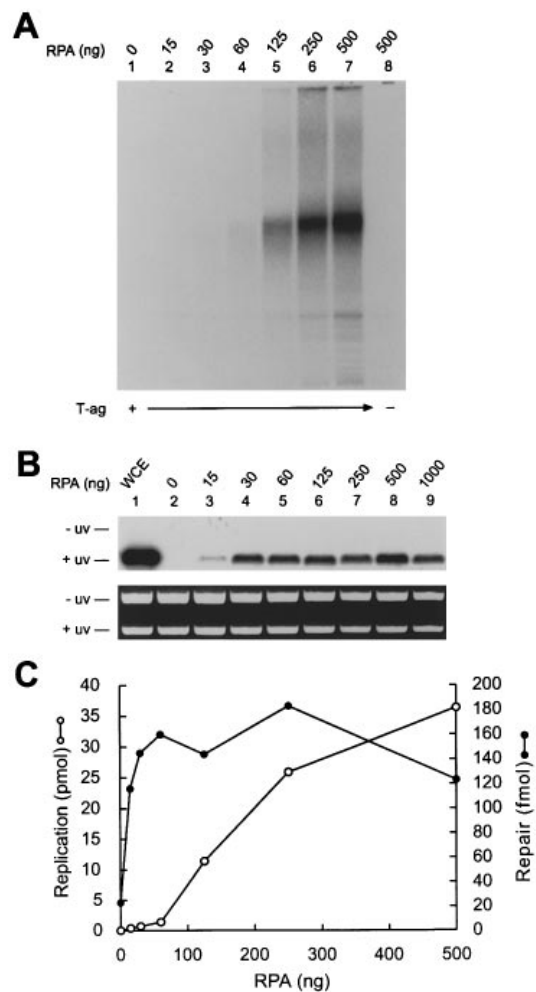
Figure 5 Replication and repair reactions require different amounts of RPA for their activities

Phosphocellulose column fractionated HeLa cell cytosolic extracts lacking RPA (PC-1.0 M fraction, see the Experimental section) were used for both replication (**A**) and repair (**B**) assays. (**A**) The titration of RPA in SV40 replication *in vitro* is shown. Reaction mixtures (40 μl) contained 0.2 μg of pSV01ΔEP, 150 μg of PC-1.0M fraction, 0.4 μg of PCNA and the indicated amounts of RPA. Where indicated, 0.8 μg of SV40 T-ag was added. After incubation at 37 °C for 1 h, DNA was isolated and analysed by 1.0% agarose gel electrophoresis.(**B**) An *in vitro* nucleotide excision repair assay is shown. Reaction mixtures (50 μl) contained 0.2 μg of UV-irradiated pBS (450 J/m²; 3 kb), 0.2 μg of non-irradiated p5A (4.5 kb), 150 μg of PC-1.0 M fraction, and (with the exception of lane 2) 0.4 μg of PCNA. Lane 1 contained 200 μg of HeLa whole-cell extracts (WCE) instead of the PC-1.0 M fraction. After the reactions, the DNA products were analysed by 1.0% agarose gel electrophoresis. The top and bottom panels depict an autoradiogram and an ethidium-bromide-stained gel containing the DNA products respectively. (**C**) Replication and repair activities were quantified from (**A**) and (**B**) respectively.

inhibition by the presence of damaged DNA than repair activity is susceptible to inhibition by replication activity (see Discussion for details).

DISCUSSION

Using an *in vitro* DNA replication and repair system, we examined UV damage-induced inhibition of replication. This system, which contains a non-irradiated SV40 origin-containing circular duplex DNA and a UV-irradiated circular duplex DNA that lacks an SV40 origin, has allowed us to show that

DNA replication can be inhibited *in vitro* in the presence of damaged DNA. It has also allowed us to show that repair activity is inhibited by DNA replication, albeit to a lesser extent.

What causes the inhibition of replication in this *in vitro* repair–replication system? We argue that the answer lies in the regulation of proteins that are involved in both replication and repair. Our reasoning is based on three lines of evidence: (i) the level of inhibition of SV40 DNA replication by irradiated pBS correlates with the level of DNA repair activity; (ii) the repair–replication system that contained purified replication proteins [8,9,30] did not support repair activities, and failed to inhibit replication (Figure 4); and (iii) RPA, PCNA, A1 (RF-C) and pol δ, which are all involved in both replication and repair [4–13], can collectively reverse the DNA damage-induced inhibition of replication.

Because repair activity influences replication activity, and vice versa, it seems likely that proteins implicated in both processes would have a central role in the co-ordinated regulation of replication and repair. It is interesting to point out, however, that whereas replication activity was significantly inhibited (up to 75 % in the presence of 200 ng of UV-damaged plasmid) by the presence of damaged DNA (and its repair), repair activity was only mildly (20–25 %) affected by replication (Figure 1). This can be explained the fact that the number of repair complexes in the reaction is 15–20 times higher than the number of replication complexes; as a result, repair activity was less affected by the presence of replication activity. Furthermore, the amount of the shared protein RPA required for replication is much greater than that for repair (Figure 5). Consequently, the competition for shared proteins is more likely to affect DNA replication than repair in the repair–replication system, and subsequently to contribute to the preferential inhibition of replication by UV-damaged DNA.

Upon UV irradiation, cells are dominantly arrested at S phase, with little or no DNA replication occurring while they undergo active DNA repair [2,3]. Similarly, when UV-irradiated HeLa cells were examined by immunofluorescence with a monoclonal antibody specific for the RPA p34 subunit, the S-phase nuclei exhibited small granular structures (results not shown), which were quite distinct from the large dot-like distribution of RPA observed in non-irradiated cells [31–33]. We believe that the small granular structure shown in the irradiated cells is likely to be due to the involvement of RPA in small patch repair. These observations *in vivo* suggest that RPA and other shared proteins are recruited to the damaged DNA sites for their repair functions, in preference to their replication activities.

Among the shared proteins involved in repair and replication, RPA is the only one that participates in the initiation stages of both processes [5–7,34]. All the other shared proteins [pol δ, PCNA and A1 (RF-C)] are involved in both the elongation stage of replication and in the later stage of repair [35]. Our initial observation indicated that initiation of replication is affected primarily by UV-damaged DNA in our repair–replication system, suggesting that RPA is the key factor involved in UV-damage-induced inhibition of replication. Both replication and repair are likely to compete for RPA and, in this event, increasing amounts of UV-damaged DNA would quantitatively inhibit DNA replication. Recent studies have shown that RPA interacts with two known repair proteins: (i) *Xeroderma pigmentosum* group-A-complementing protein (XPA) [36,37], a protein that recognizes damaged DNA [38]; and (ii) *Xeroderma pigmentosum* group-G-complementing protein [36], an endonuclease that cleaves the 3′-side of damaged DNA lesions [39]. When RPA interacts with XPA, RPA's replication function is inhibited [40], whereas XPA's ability to bind UV-damaged DNA is stimulated [36,41]. Because

RPA interacts with SV40 T-ag and the pol α–primase complex, it is possible that the replication protein and the repair protein compete with each other to interact with RPA. This idea is supported by the finding that the C-terminal domain of the 34 kDa subunit of RPA is involved in RPA's interactions with both SV40 T-ag [42] and XPA (E. Stigger and S.-H. Lee, unpublished work).

An alternative explanation for the involvement of RPA in the inhibition of replication would be if it underwent UV-damage-induced phosphorylation. It has been suggested that the UV-damage-induced inhibition of replication observed *in vivo* might be related to RPA phosphorylation [3]. However, it is unlikely that phosphorylation of RPA by the presence of damaged DNA caused the inhibition of replication in our repair–replication system, because: (i) inhibition of replication by the presence of UV-damaged DNA was also observed when wild-type RPA was replaced by a non-phosphorylatable RPA mutant (results not shown); and (ii) extracts prepared from human glioblastoma cells (M059J) that lack DNA-dependent protein kinase [43] also showed UV-induced inhibition of replication under the condition used in this experiment (results not shown). DNA-dependent protein kinase is known to phosphorylate RPA p34 upon DNA damage [3,44]. It should be noted, however, that RPA phosphorylation may be involved in the regulation of chromo-somal DNA replication *in vivo* which is deficient in SV40 replication *in vitro*. Alternatively, the *in vitro* system described here may lack some of the regulatory factors that are induced upon UV damage *in vivo*. Our future studies will include analysing the function of RPA phosphorylation *in vivo* by introducing mutant RPA into cells.

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