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UV light-induced DNA synthesis arrest in HeLa cells is associated with changes in phosphorylation of human single-stranded DNA-binding protein

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We show that DNA replication activity in extracts of human HeLa cells decreases following UV irradiation. Alterations in replication activity *in vitro* parallel the UV-induced block in cell cycle progression of these cells in culture. UV irradiation also induces specific changes in the pattern of phosphorylation of the 34 kDa subunit of a DNA replication protein, human single-stranded DNA-binding protein (hSSB). The appearance of a hyperphosphorylated form of hSSB correlates with reduced *in vitro* DNA replication activity in extracts of UV-irradiated cells. Replication activity can be restored to these extracts *in vitro* by addition of purified hSSB. These results suggest that UV-induced DNA synthesis arrest may be mediated in part through phosphorylation-related alterations in the activity of hSSB, an essential component of the DNA replication apparatus.

Key words: cell cycle arrest/DNA replication *in vitro*/ phosphorylation/single-stranded DNA-binding protein/UV irradiation

Introduction

DNA damage caused by exposure to genotoxic environmental agents such as UV radiation can lead to cancer through the mutagenic activation of proto-oncogenes or inactivation of tumour suppressor genes (Bishop, 1991; Brash *et al.*, 1991). Cellular DNA repair mechanisms can overcome the mutagenic effects of genotoxic agents by removing pre-mutagenic DNA damage before mutations are fixed during DNA replication (Friedberg, 1985; Rossman and Klein, 1988). The time period available for repair can be prolonged by inducible responses that occur as a result of exposure of cells to genotoxic agents, and which delay the entry of cells into S phase (Denekamp, 1986; Hartwell and Weinert, 1989; Lane, 1992). Despite the possible importance of DNA synthesis arrest in enhancing cell survival and preventing mutagenesis, little is known about the mechanism by which DNA replication is inhibited.

The apparent absence of DNA damage-induced G_1 arrest in certain mutant cells, such as p53-negative cells (Kastan *et al.*, 1991, 1992) and ataxia telangiectasia cells (Painter and Young, 1980), argues that inhibition of DNA replication has a genetic basis, and is distinct from blockage of replication forks by damage in the DNA template. It is possible that inhibition occurs through mechanisms similar to those involved in the normal regulation of cell cycle progression. It has been demonstrated previously that the replication activity of extracts from G_1-phase mammalian cells is greatly reduced compared to extracts of cells in S phase (Roberts and D'Urso, 1988; Virshup *et al.*, 1989; D'Urso *et al.*, 1990). These cell cycle-related changes in DNA replication activity *in vitro* appear to be modulated in part by the phosphorylation state of a protein essential for initiation of SV40 DNA replication *in vitro*: human single-stranded DNA-binding protein (hSSB; Din *et al.*, 1990; Dutta *et al.*, 1991; Dutta and Stillman, 1992).

Here, we describe the use of an *in vitro* SV40 DNA replication system to investigate the mechanisms by which the HeLa cell DNA replication apparatus is altered in response to the genotoxic agent, UV radiation. The replication capacity of cell-free extracts declines in parallel with the UV-induced cessation of cell cycle progression in these cells in culture. We show that UV-induced DNA synthesis arrest in human HeLa cells is associated with biochemical alterations which can be reproduced using an *in vitro* DNA replication system. As in the normal cell cycle, these biochemical alterations include changes in the phosphorylation state of hSSB.

Results

Effect of UV irradiation of HeLa cells on cell cycle progression

The first experiment was designed to demonstrate that UV radiation-induced arrest of cell cycle progression could be observed in HeLa cells in culture. Exponentially growing cells were either treated with 10 J/m^2 UV radiation or mock irradiated, and cells were collected at various times after irradiation. Cells were stained with propidium iodide and DNA content was determined by fluorescence activated cell sorting (FACS) analysis. In the mock-irradiated cells, the proportion of cells in G_1, S and G_2/M phases of the cell cycle, estimated from fluorescence tracings (Figure 1A), was ~70, 18 and 12%, respectively, at the start of the experiment; these proportions did not change appreciably over the 24 h period following mock irradiation (Figure 1B). In contrast, after treatment with 10 J/m^2 UV, the proportion of cells in the various phases of the cell cycle changed dramatically over time. Initially, the proportions were similar to those in the mock-irradiated cells, except that there was a gradual decline in the proportion of cells in G_2/M between 0 and 8 h post-UV. By 12 h after irradiation, the proportion of cells in S phase increased to ~50%. The proportion of cells in G_2/M increased to 40% between 12 and 24 h, as the cells, that had been released into S phase, progressed through the cell cycle. These data show that DNA synthesis is arrested in HeLa cells following irradiation with 10 J/m^2 UV and that DNA replication resumes at ~12 h after irradiation.

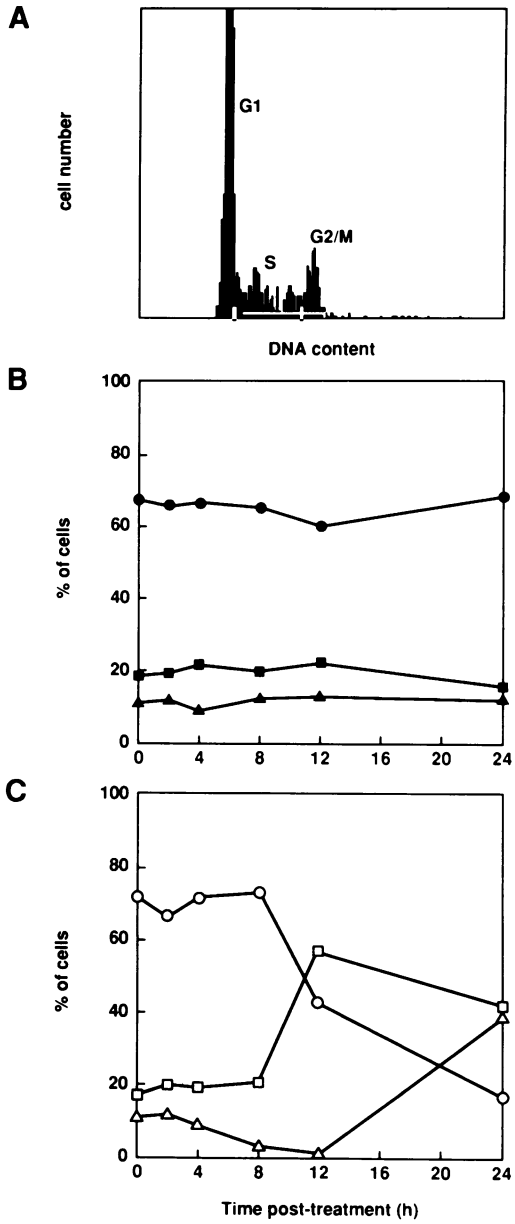

Fig. 1. FACS analysis of cell cycle progression in unirradiated and UV-irradiated HeLa cells. Cells were either mock irradiated or irradiated with 10 J/m^2 UV; after further incubation, the cells were fixed and stained with propidium iodide. (**A**) Profile of cells (2 h after mock irradiation) as a function of DNA content, as determined by measurement of red fluorescence by FACS analysis, following propidium iodide staining. (**B**) The proportion of cells in G_1 (●), S (■) and G_2 (▲) at various times after mock irradiation. (**C**) The proportion of cells in G_1 (○), S (□) and G_2 (△) at various times after irradiation with 10 J/m^2.

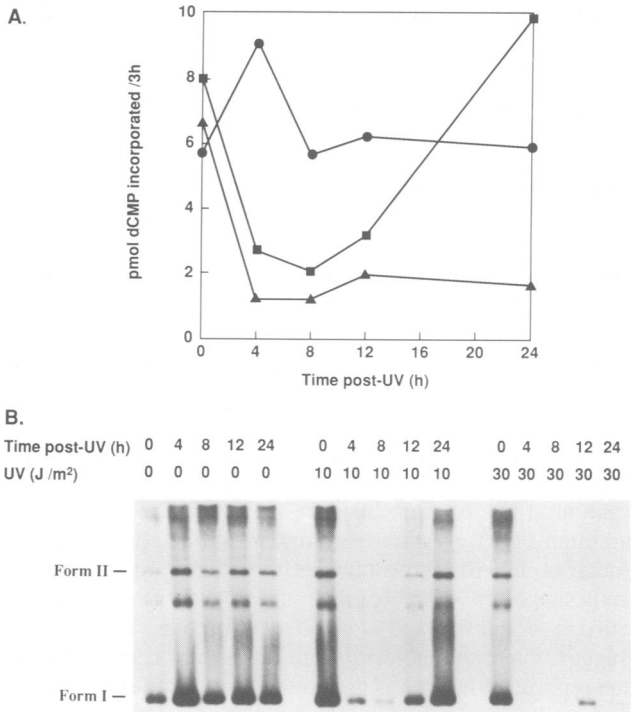

Fig. 2. *In vitro* DNA replication activity of extracts prepared from unirradiated and UV-irradiated HeLa cells. *In vitro* DNA replication reactions were carried out using 60 μg of extract protein, prepared at various times following mock irradiation (●), irradiation of HeLa cells with 10 J/m^2 UV (■) or with 30 J/m^2 UV (▲). Replication reactions were incubated at 37°C for 3 h. (**A**) The extent of incorporation of [^{32}P]dCMP into pZ189 DNA was determined by spotting 3 μl aliquots of the reactions onto 3MM Whatman filter paper discs, and precipitation of the DNA by transferring the filters into cold 10% TCA containing 10 mM sodium pyrophosphate. The filters were washed, dried and counted by liquid scintillation spectrometry. (**B**) The products of *in vitro* DNA replication reactions were purified and analysed by 1% agarose gel electrophoresis in the presence of 5 μg of ethidium bromide/ml. The gel was dried and exposed to X-ray film for autoradiography. The positions of radioactively labelled form I (completely closed circular) and form II (nicked circular) pZ189 DNA were determined by comparison with the mobility of these forms in unlabelled marker pZ189 DNA run in parallel on the same gel.

Effect of UV irradiation of HeLa cells on DNA replication activity in vitro

To examine the regulation of DNA replication activity in UV-irradiated HeLa cells on a biochemical level, we made use of an *in vitro* DNA replication system. Hypotonic extracts, prepared from HeLa cells by the method of Li and Kelly (1984), can replicate SV40 origin-based plasmids in the presence of SV40 large T-antigen (Li and Kelly, 1984; Stillman and Gluzman, 1985; Wobbe *et al.*, 1985). The effect of UV irradiation on the replication activity of these extracts was determined at various times following exposure of the cells to UV radiation by measuring the incorporation of [α-^{32}P]dCTP into acid-precipitable material (Figure 2A). In extracts prepared from mock-irradiated HeLa cells, replication activity using the SV40-based plasmid, pZ189 (Seidman *et al.*, 1985), as template did not change substantially over the period from 0 to 24 h following treatment. In contrast, extracts prepared from cells irradiated with 10 J/m^2 UV showed a time-dependent variation in replication activity; replication activity decreased from 0 to 8 h following UV irradiation, and subsequently recovered to approximately the same level as that at 0 h by 24 h post-UV (Figure 2A). The time course of the changes in *in vitro* DNA replication capacity paralleled the arrest and recovery of cell cycle progression observed *in vivo* (Figure 1C). Extracts prepared from cells irradiated with 30 J/m^2 UV showed an even more marked reduction in DNA replication activity *in vitro* as a function of time after irradiation, and replication activity had not returned to the levels seen in extracts from unirradiated cells by 24 h post-UV. This is consistent with results of FACS analysis of HeLa cells irradiated with 30 J/m^2 UV, indicating that cell cycle

progression had not recovered by 24 h after treatment (data not shown).

Agarose gel electrophoresis of the ^{32}P-labelled products of *in vitro* DNA replication (Figure 2B) revealed that there were no major changes in the electrophoretic mobility of the replication products as a result of UV irradiation of the cells; however, the dramatic decrease in replication activity of the extracts was apparent. These results suggest that UV-induced inhibition of DNA replication in HeLa cells involves alterations in the activity of the DNA replication complex, which can be reproduced *in vitro* using a cell-free replication assay.

That extracts from UV-irradiated cells do not contain an irreversible inhibitor of DNA replication was shown by carrying out mixing experiments. Addition of 6 μg of extract protein, prepared from mock-irradiated cells, stimulated the replication activity of 60 μg of irradiated cell extract (prepared 24 h after 30 J/m^2 UV) by 2.5-fold, while addition of 15 μg stimulated replication activity by 4-fold (data not shown). These amounts of protein from unirradiated cells supported <25% of the normal level of replication *in vitro* observed with the usual 60 μg of protein. These results suggest that only one or a small number of components of the replication apparatus is altered in irradiated cell extracts, and can be supplied *in trans* by extracts of unirradiated cells.

hSSB phosphorylation following UV irradiation

One of the replication components we chose to examine was the hSSB (also called RP-A or RF-A). hSSB, which is composed of three subunits of 70, 34 and 11 kDa, has an essential role in the initiation and elongation stages of DNA replication *in vitro* (Wobbe *et al.*, 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988). The observation that the protein is phosphorylated in a cell cycle-dependent manner in human cells suggests that it could also play an important role in the regulation of DNA replication (Din *et al.*, 1990; Dutta and Stillman, 1992). Cell cycle-related changes in phosphorylation, which occur primarily on the 34 kDa subunit, can be mediated by the *cdc* and *cdk* family of cell cycle-regulated protein kinases (Din *et al.*, 1990). The yeast homologue of this protein (Brill and Stillman, 1989, 1991) also undergoes cell cycle-related changes in phosphorylation state (Din *et al.*, 1990). Therefore, we first examined whether UV-induced arrest of cell cycle progression in human HeLa cells was also associated with changes in the phosphorylation state of this factor. Exponentially growing HeLa cells were irradiated with 10 or 30 J/m^2 UV light, and whole-cell lysates were prepared at various times following treatment. Lysate proteins were separated by denaturing PAGE, and the 34 kDa subunit of hSSB was detected by Western immunoblotting using a specific monoclonal antibody. Under these electrophoretic conditions, phosphorylated forms of this subunit have reduced mobility (Din *et al.*, 1990; Dutta and Stillman, 1992; Fotedar and Roberts, 1992).

We observed that the amount of 34 kDa subunit in the cell lysates did not change appreciably following UV irradiation (Figure 3). However, multiple immunoreactive bands of hSSB p34 were observed. In lysates from unirradiated cells, the majority of the p34 protein was of fast mobility (Figure 3). A smaller fraction of this protein had a slightly slower electrophoretic mobility, and will be referred to below as the intermediate-mobility form. The

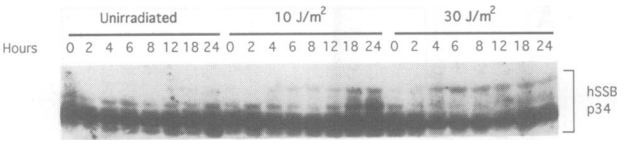

Fig. 3. Western immunoblot of hSSB p34 in HeLa cell lysates. Whole-cell lysates were prepared from HeLa cells at various times following mock irradiation, or irradiation with 10 and 30 J/m^2 UV. Proteins were separated on a 12% denaturing polyacrylamide gel. Following transfer of the proteins to PVDF membrane, hSSB p34 was detected by immunoblotting using a p34-specific monoclonal antibody.

relative intensities of these bands remained constant from 0 to 24 h in mock-irradiated cells. In contrast, lysates of UV-irradiated cells showed the presence of an additional slow-mobility band which was not observed in the lysates of unirradiated cells (Figure 3). The relative intensity of this slow-mobility form increased from 0 to 24 h following irradiation with 10 and 30 J/m^2 UV (Figure 3). The intensity of the intermediate-mobility form decreased slightly from 0 to 8 h post-irradiation with 10 J/m^2 UV, and then increased substantially between 8 and 24 h (Figure 3). Following treatment with 30 J/m^2 UV, the level of the intermediate-mobility form decreased by 2 h post-UV, and did not increase by 24 h after treatment (Figure 3).

Studies in which HeLa cells were arrested in mitosis, by treatment with the drug nocodazole (Din *et al.*, 1990), revealed that the presence of the intermediate-mobility form of p34 hSSB correlates with cells being in the G_2/M phase of the cell cycle (data not shown). Thus, the increase in the intensity of this form between 8 and 24 h following 10 J/m^2 UV would appear to result from the increased proportion of cells in the G_2/M phase at these times, as determined by flow cytometry (Figure 1C). The lack of an increase in the intensity of the intermediate-mobility form following 30 J/m^2 UV is consistent with flow cytometric analysis, which demonstrates that progression through S into mitosis is delayed for >24 h following this dose of UV (data not shown).

We confirmed that the slower-migrating forms of p34 hSSB are phosphorylated by treating cells with 10 J/m^2 UV, and incubating the cells in the presence of [^{32}P]orthophosphate for 24 h to label cellular phosphoproteins. Labelled proteins were immunoprecipitated with anti-hSSB p34, separated on a polyacrylamide gel and the proteins transferred to PVDF membrane. Labelled p34 hSSB was detected by autoradiography of the membrane, while total immunoreactive hSSB protein (labelled and unlabelled) was detected by Western immunoblotting of the same membrane. Immunoprecipitation was specific, as shown by the absence of labelled or immunoreactive bands in the position of hSSB p34 when a control mouse IgG was used to immunoprecipitate cellular proteins (Figure 4A, lanes A–D). The bands observed in the control lanes are the heavy and light chains of IgG. Following immunoprecipitation with anti-hSSB p34, electrophoresis and transfer, four separate labelled forms of p34 hSSB were detectable upon autoradiography of the membrane (Figure 4A, lane E). These labelled forms have been designated 2–5, in order of decreasing electrophoretic mobility (Figure 4A, lane E). Comparison with an immunoblot of the same membrane (Figure 4A, lane F) reveals that these forms correspond to the four more slowly migrating immunoreactive forms of

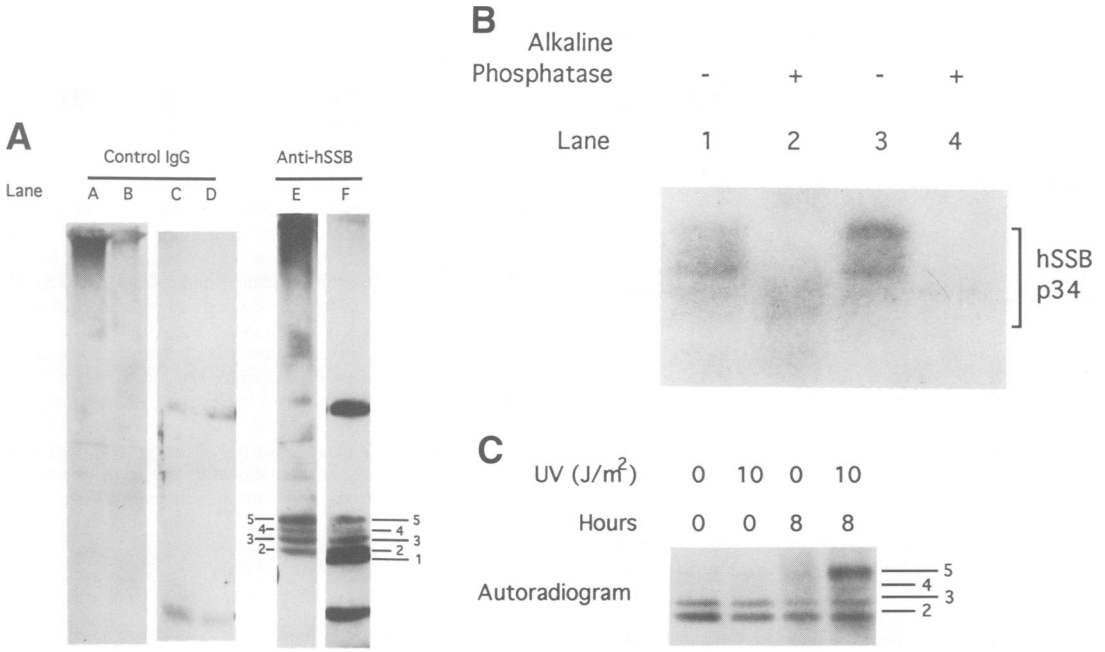

Fig. 4. Phosphorylation of hSSB p34 *in vivo*. (**A**) HeLa cells were labelled with [^{32}P]orthophosphate, and mock irradiated or treated with 10 J/m^2 UV. Twenty-four hours later, whole-cell lysates were prepared, proteins were immunoprecipitated and then separated on a 12% denaturing polyacrylamide gel. Following transfer to PVDF membrane, the membrane was first exposed for autoradiography to detect labelled proteins and subsequently immunoblotted with anti-hSSB p34 antibody. Lanes A and B, autoradiogram of proteins prepared from mock-treated (A) or UV-irradiated (B) cells, immunoprecipitated with control IgG. Lanes C and D, the same lanes immunoblotted with anti-hSSB p34. Lane E, autoradiogram of proteins prepared 24 h after UV irradiation, immunoprecipitated with anti-hSSB p34. Lane F, the same membrane, immunoblotted with anti-hSSB p34. The positions of the five forms of hSSB p34 having different electrophoretic mobilities are indicated by the numbers 1–5. (**B**) Alkaline phosphatase treatment of ^{32}P-labelled proteins. hSSB p34 was immunoprecipitated from HeLa cells 24 h after mock irradiation (lanes 1 and 2), or UV irradiation (lanes 3 and 4). An equal aliquot of the immunoprecipitate was incubated either with calf intestinal alkaline phosphatase buffer (lanes 1 and 3), or with 20 U of enzyme (lanes 2 and 4). Proteins were separated by SDS–PAGE and labelled proteins visualized by autoradiography. (**C**) HeLa cells were labelled with [^{32}P]orthophosphate and either mock irradiated or treated with 10 J/m^2 UV. Whole-cell lysates were either prepared immediately or 8 h after treatment, and hSSB p34 was immunoprecipitated using an anti-p34 monoclonal antibody. Immunoprecipitated proteins were separated by SDS–PAGE and labelled proteins visualized by autoradiography.

this subunit, while the fastest-migrating form does not appear to become phosphorylated under these conditions. The fastest-migrating, unlabelled form has been designated form 1 (Figure 4A, lane F). The ^{32}P label incorporated into these protein bands can be removed by incubation of the hSSB protein (immunoprecipitated from unirradiated or UV-irradiated cells) with calf intestinal alkaline phosphatase prior to electrophoresis (Figure 4B). This result indicates that label incorporation is due to phosphorylation, rather than ATP binding, for example.

The ^{32}P-labelled forms of p34 hSSB observed 8 h following irradiation with 10 J/m^2 UV differed from the forms observed 8 h following mock irradiation (Figure 4C). The labelled slow-mobility form of p34 hSSB observed in lysates from irradiated cells was not observed in untreated cells, consistent with the results of the immunoblotting experiments shown in Figure 3. These results demonstrate that UV irradiation of HeLa cells leads to an alteration in the phosphorylation state of hSSB. Specifically, UV irradiation leads to an increase in the relative amount of a ^{32}P-labelled, slow-mobility form of the p34 subunit.

The modification of the slow-mobility form was analysed further using two-dimensional phosphopeptide mapping. hSSB was immunoprecipitated from whole-cell lysates, prepared 8 h after UV irradiation (10 J/m^2) of cells growing in the presence of [^{32}P]orthophosphate. The slow-mobility form, corresponding to that shown in Figure 4C, was excised from a polyacrylamide gel and digested with chymotrypsin. The resulting peptides were analysed by two-dimensional phosphopeptide mapping (Boyle *et al.*, 1991). This analysis revealed multiple phosphorylated peptides, indicating that the protein was phosphorylated on a number of sites (Figure 5A), as previously noted (Dutta and Stillman, 1992). The major labelled phosphopeptide observed may contain the site of UV-induced changes in phosphorylation (Figure 5A); however, further analysis is required to determine the exact identity of all of the sites phosphorylated following UV irradiation. Analysis of the labelled phosphoamino acids present in the hyperphosphorylated form indicated that the phosphorylation occurred primarily on serine residues (Figure 5B).

hSSB phosphorylation in cell extracts in vitro

Since UV irradiation induces changes in the phosphorylation state of hSSB *in vivo* (Figure 3), we examined whether similar changes in hSSB phosphorylation occurred in the hypotonic extracts of UV-irradiated cells used in DNA replication assays *in vitro*. Extract proteins were separated by PAGE, and the 34 and 70 kDa subunits of hSSB were detected using specific monoclonal antibodies. The levels of the 34 kDa subunit (Figure 6) and the 70 kDa subunit (data not shown) in cell extracts were essentially unchanged following UV irradiation. However, in contrast to the observation in whole-cell lysates, the p34 subunit migrated

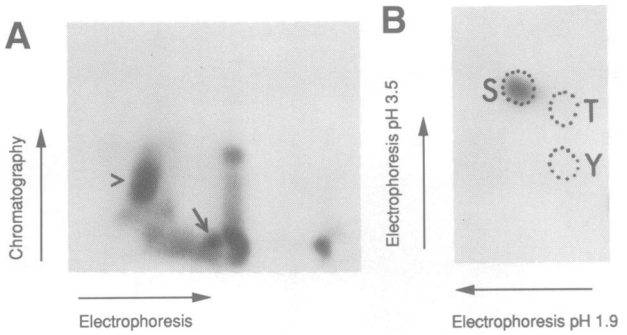

Fig. 5. Analysis of hSSB p34 phosphorylated *in vivo*. HeLa cells were labelled with [^{32}P]orthophosphate, and treated with 10 J/m^2 UV light. Eight hours afer UV treatment, whole-cell lysates were prepared, hSSB p34 was immunoprecipitated and proteins separated by SDS−PAGE. The slow-mobility form, corresponding to band 5 shown in Figure 4C, was excised from the gel. (**A**) Phosphopeptide map generated by chymotryptic digestion of the slow-mobility form. The origin is indicated by an arrow; the major labelled peptide is indicated by an arrowhead. The directions of electrophoresis in the first dimension and of chromatography in the second dimension are shown. Peptides were visualized by autoradiography for 4 days at −70°C. (**B**) Phosphoamino acid analysis of the slow-mobility form of hSSB p34. Phosphoamino acids were separated by electrophoresis in the first dimension at pH 1.9, and in the second dimension at pH 3.5, and visualized by autoradiography. The migration of unlabelled phosphoamino acid markers, run on the same plate and visualized by spraying with ninhydrin, is indicated by the dotted outlines. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

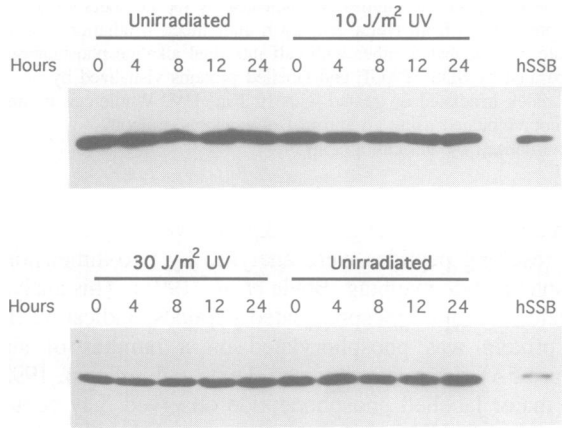

Fig. 6. Western immunoblot of hSSB p34 in hypotonic extracts of HeLa cells. A total of 60 μg of extract protein, prepared at various times following mock irradiation and treatment with 10 J/m^2 (upper panel), or 30 J/m^2 UV (lower panel), was separated by 12% SDS−PAGE and immunoblotted with anti-hSSB p34 monoclonal antibody. The lanes labelled hSSB contain purified hSSB protein from untreated, exponentially growing HeLa cells, run on the same gel as a marker.

as a single band. We then examined the effect of incubation of extracts under DNA replication conditions on the phosphorylation of hSSB *in vitro*, by comparing the gel mobility of hSSB p34 in extracts of unirradiated and UV-irradiated HeLa cells (Figure 7). Extracts were incubated at 37°C, under the conditions for *in vitro* DNA replication, and proteins were subsequently analysed by Western immunoblotting of the 34 kDa subunit of hSSB. The mobility of this subunit was altered following incubation *in vitro*, in both unirradiated and irradiated cell extracts, compared to the form present in the extracts prior to incubation

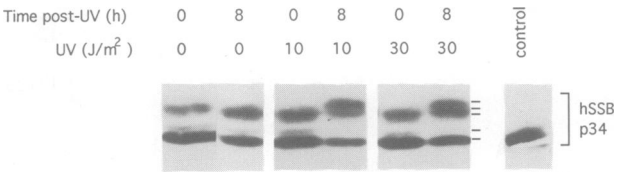

Fig. 7. Electrophoretic mobility of hSSB p34 following incubation of DNA replication reactions *in vitro*. HeLa cell extracts, prepared at zero time, and at 8 h following mock irradiation and irradiation with 10 or 30 J/m^2 UV, were incubated under DNA replication conditions for 2 h at 37°C. Half of the reaction (15 μl) was then analysed by 12% SDS−PAGE. The p34 subunit was visualized by immunoblotting with anti-p34 monoclonal antibody and chemiluminescent detection. The hSSB p34 present in a separate HeLa cell extract (prepared without mock irradiation), which was not incubated under DNA replication conditions, is shown in the control lane. As shown in Figure 6, the mobility of hSSB p34 in the extract prior to incubation *in vitro* was the same as that shown in the control lane. The positions of the five bands having different mobilities following incubation *in vitro* are marked.

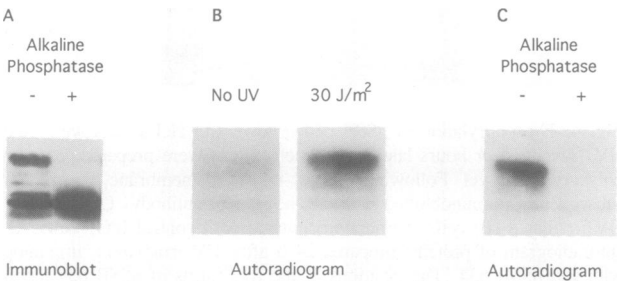

Fig. 8. Phosphorylation of hSSB p34 *in vitro*. (**A**) Hypotonic extract prepared from HeLa cells 8 h after irradiation with 30 J/m^2 UV was incubated under DNA replication conditions for 1 h. hSSB p34 was immunoprecipitated using anti-p34 antibody. Half of the immunoprecipitate was incubated with calf intestinal alkaline phosphatase buffer (−), while half was incubated with 40 U of enzyme (+). Proteins were separated by 12% SDS−PAGE and visualized by immunoblotting. (**B**) Reactions were carried out as in (A), using either extract from unirradiated cells or extract from cells irradiated with 30 J/m^2 UV, except that the reactions also contained [γ-^{32}P]ATP. Following immunoprecipitation, proteins were separated by SDS−PAGE and visualized by autoradiography. (**C**) The immmunoprecipitate from the reaction with irradiated extract shown in panel B was incubated with alkaline phosphatase buffer (−) or 40 U of enzyme (+). The separated proteins were visualized by autoradiography.

(Figure 7). We detected five bands having slightly different electrophoretic mobilities in the 34 kDa range (Figure 7).

The major difference between extracts prepared from unirradiated cells and extracts prepared 8 h after UV irradiation was a shift to the slow-mobility form of p34 hSSB in the irradiated extracts (Figure 7). Incubation of cell extracts with alkaline phosphatase prior to electrophoresis confirmed that these bands represent differently phosphorylated forms of p34 hSSB. This treatment converts the four slower-migrating forms to a single fast-migrating form (Figure 8A). The slowest-mobility form observed in extracts of irradiated cells is in fact hyperphosphorylated, as demonstrated by incubation of extracts from unirradiated or irradiated (30 J/m^2) cells with [γ-^{32}P]ATP, followed by immunoprecipitation of p34 hSSB, electrophoresis and autoradiography. Under these conditions, a highly labelled, slow-mobility form of p34 hSSB is much more pronounced in the irradiated extract than in the extract from unirradiated

cells (Figure 8B). Treatment of the immunoprecipitates with alkaline phosphatase results in loss of detectable label in the p34 hSSB protein, indicating that label incorporation is a result of phosphorylation occurring *in vitro* (Figure 8C). Two-dimensional phosphopeptide mapping of the ^{32}P-labelled, slow-mobility form of hSSB p34 confirmed this conclusion, and indicated that a number of peptides became phosphorylated in extracts of irradiated cells *in vitro* (data not shown). Thus, the UV-induced hyperphosphorylation of hSSB p34 observed in HeLa cells *in vivo* (Figure 2) appears to be reproduced in hypotonic extracts of these cells, used for DNA replication *in vitro*. The occurrence of these reactions *in vitro* should facilitate the identification of the enzymes involved in modifying hSSB following UV irradiation.

Addition of purified HeLa cell hSSB stimulates replication activity in extracts of UV-irradiated cells

The hSSB protein plays an essential role in the initiation of replication of SV40-based plasmids *in vitro* (Fairman and Stillman, 1988; Kenny *et al.*, 1989; Wold *et al.*, 1989); therefore, any change in hSSB activity could have profound effects on the replication activity of cell extracts. To test if the loss of replication activity *in vitro* might be mediated by a reduction in the activity of hSSB in replication, we examined the effect of addition of purified hSSB on DNA replication in extracts from UV-irradiated cells. Titration of increasing amounts of purified hSSB, from 40 to 400 ng, into extracts of irradiated HeLa cells led to a dose-dependent activation of *in vitro* DNA replication activity. The magnitude of the effect increased from 2-fold up to 14-fold when 400 ng of hSSB were added (data not shown). Figure 9 shows the effect of the addition of 200 ng of purified hSSB on the DNA replication activity of extracts prepared at various times following mock irradiation or UV irradiation. Addition of 200 ng of purified hSSB to extracts from irradiated cells (30 J/m^2) stimulated [^{32}P]dCTP incorporation into plasmid DNA by >10-fold, as determined by counting the radioactivity in the replicated plasmid DNA loaded on the agarose gel (Figure 9C). A similar effect was obtained when hSSB was added to extracts prepared from cells 4 and 8 h after irradiation with 10 J/m^2 UV, except that the extent of stimulation was ~5- to 6-fold (Figure 9B). As previously reported (Kenny *et al.*, 1990), replication with extracts of unirradiated cells was stimulated by an average of 3-fold in the presence of added hSSB (Figure 9A), indicating that this component may be limiting in these extracts. Two lines of evidence indicate that the increase in label incorporation is a result of increased DNA replication. First, the increase is dependent on the presence of large T antigen in the reaction. Second, the labelled DNA products are resistant to digestion by the restriction enzyme *Dpn*I, which specifically degrades unreplicated plasmid DNA (data not shown). One interpretation of these results is that the DNA replication activity of hSSB, present in extracts of UV-irradiated cells, is decreased.

Purified hSSB becomes phosphorylated in cell extracts in vitro

We examined what changes in the phosphorylation state of hSSB were associated with stimulation of *in vitro* DNA replication activity by addition of purified protein. Increasing amounts, from 10 to 200 ng, of purified HeLa cell hSSB

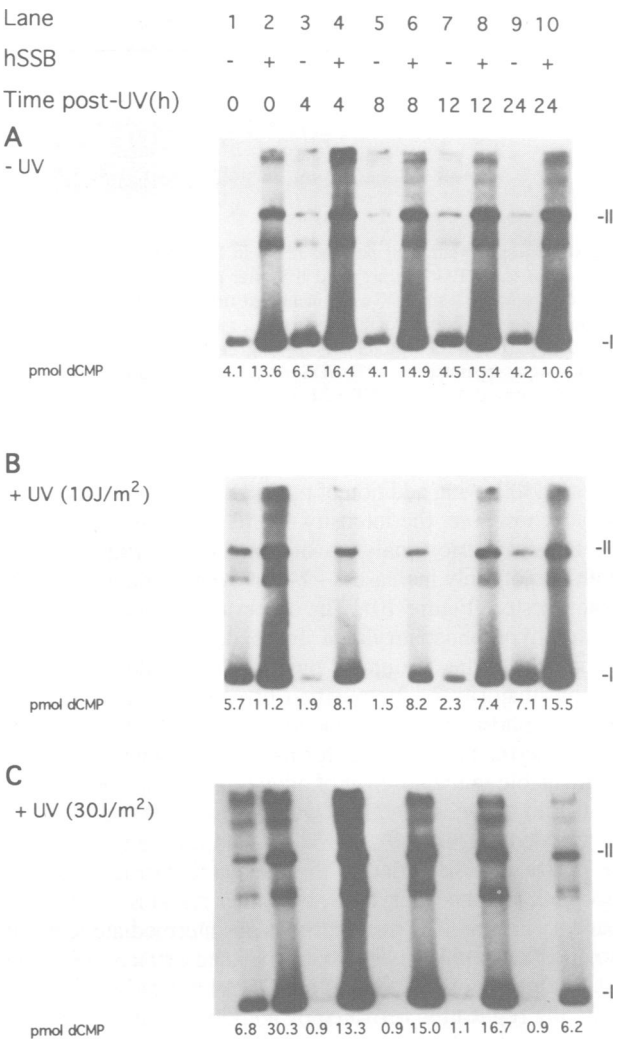

Fig. 9. Effect of purified HeLa hSSB on the *in vitro* DNA replication activity of extracts from unirradiated and UV-irradiated HeLa cells. DNA replication reactions, containing 60 μg of extract protein prepared at various times following mock irradiation (panel **A**), irradiation with 10 (**B**) or 30 J/m^2 (**C**) of UV light, were incubated in the absence (lanes 1, 3, 5, 7 and 9) or presence (lanes 2, 4, 6, 8 and 10) of 200 ng purified hSSB for 3 h at 37°C. The ^{32}P-labelled pZ189 DNA products were analysed by agarose gel electrophoresis and visualized by autoradiography of the dried gel. The extent of DNA synthesis was also determined, as described in the legend to Figure 2, and is shown as pmol dCMP incorporated in each reaction.

were added to extracts prepared 8 h after mock irradiation or after treatment with 30 J/m^2 UV light. As described above, addition of hSSB leads to a dose-dependent increase in replication activity. Following incubation under replication conditions *in vitro*, the phosphorylation state of the 34 kDa subunit of hSSB was examined by immunoblotting. The hSSB preparation used, which was purified from exponentially growing HeLa cells (Kenny *et al.*, 1990), has gel mobility corresponding to the fast-mobility form observed in HeLa extracts (see Figure 6). The protein becomes phosphorylated in unirradiated extracts during incubation *in vitro* (Figure 10); however, little hyperphosphorylated form is detected (band 5, Figure 10). In the irradiated extract, the hyperphosphorylated form is the major modified form of hSSB p34 detected in the absence of added hSSB (Figure 10). The hyperphosphorylated form of p34 is also

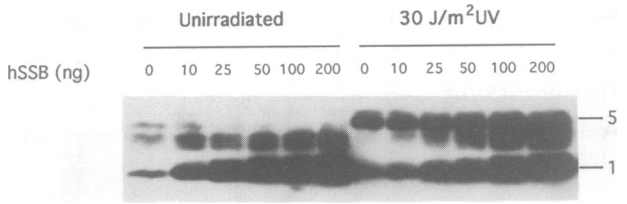

Fig. 10. Phosphorylation of purified hSSB in DNA replication reactions *in vitro*. Extracts prepared 8 h after mock irradiation or after irradiation with 30 J/m^2 UV were incubated under DNA replication conditions, in the absence of hSSB (0 ng), or in the presence of from 10 to 200 ng of purified protein. Following incubation for 1 h, proteins were separated by 12% SDS–PAGE and visualized by immunoblotting using anti-hSSB p34 monoclonal antibody. The positions of the fast-mobility form (1) and the slow-mobility form (5) of hSSB are shown.

observed following addition of purified hSSB to the irradiated extract; however, the intensity of this band (as determined by densitometric analysis of an X-ray film of the immunoblot) only increases ~2-fold over the range of hSSB protein tested (Figure 10). The observation that the intensity of the hyperphosphorylated form does not increase in proportion to the amount of purified hSSB added suggests that the majority of the purified protein does not undergo the hyperphosphorylation reaction. In contrast, the intensity of the intermediate-mobility forms of this subunit, which are undetectable in the absence of added hSSB, increases by up to 10-fold between reactions where 25 and 200 ng of hSSB were added (Figure 10). These results indicate that most of the purified hSSB added to the irradiated extract does not become hyperphosphorylated, but either remains unmodified (band 1, Figure 10), or undergoes an intermediate level of modification similiar to that in unirradiated extracts. We have consistently observed that active DNA replication *in vitro*, including replication activated by addition of purified hSSB, is associated with an increase in the relative amount of the intermediate-mobility forms, whether or not the slow-mobility form is also present. While further analysis of the biochemical activity and the phosphorylation state of each of the individual forms detectable by gel electrophoresis is required to directly address the function of these forms, the present results suggest that the hyperphosphorylated form of hSSB is not active in DNA replication *in vitro*, but this form is not inhibitory. This interpretation would also be consistent with the appearance of this form *in vivo* under conditions where DNA synthesis is arrested (Figures 3 and 4C).

Discussion

We have shown that the DNA replication capacity of extracts prepared from UV-irradiated HeLa cells decreases as a function of time after irradiation. At lower UV doses (10 J/m^2), normal replication activity is restored within 24 h following irradiation, whereas at 30 J/m^2, replication activity remains low. This loss of replication capacity of cell extracts parallels the inhibition of cellular DNA replication that is observed following UV irradiation of these cells in culture. The present results provide evidence that UV-induced arrest of cellular DNA replication may involve biochemical alterations in the DNA replication apparatus similar to the type that occur during normal cell cycle regulation. One of these changes involves the post-translational modification of a replication protein, hSSB, by phosphorylation. hSSB, which undergoes a reversible cycle of phosphorylation and dephosphorylation during normal cell cycle progression (Din *et al.*, 1990; Dutta and Stillman, 1992), becomes hyperphosphorylated following exposure of HeLa cells to UV irradiation. An apparently similar hyperphosphorylation reaction occurs *in vitro* in hypotonic extracts of UV-irradiated cells. A role for hSSB in the loss of *in vitro* DNA replication activity in extracts of UV-irradiated cells is supported by the observation that such inactive extracts can be rescued by the addition of purified HeLa hSSB. Restoration of replication activity by the addition of purified hSSB appears to correlate with the occurrence of particular hSSB phosphorylation events *in vitro*.

The arrest of DNA replication that follows exposure of cultured mammalian cells to DNA damaging agents appears to involve not only a block in the entry of cells into S phase from G_1, but also a cessation of initiation at new origins of replication in cells that are already in S phase (Painter and Young, 1975; Kaufmann *et al.*, 1980). Since G_1 arrest is absent in cells lacking the normal AT or p53 genes (Painter and Young, 1980; Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992), these blocks to replication would appear to require active regulatory events that are separable from blockage of replication fork progression on damaged templates. In addition, a number of yeast mutants have been identified in which these responses are lacking (Weinert and Hartwell, 1988; Murray, 1992). Our observation that the capacity of extracts from UV-irradiated cells to replicate plasmid DNA, in the absence of damage to the template, is lost in parallel with the arrest of cell cycle progression argues for a direct effect on the replication apparatus *in vivo*.

The ability of a single purified protein, hSSB, required for SV40 DNA replication *in vitro*, to restore replication activity to irradiated cell extracts suggests that this protein is one of the cellular mediators of UV-induced DNA synthesis arrest. The correlation of hSSB phosphorylation with the DNA replication activity of extracts *in vitro* suggests that phosphorylation may be involved in the regulation of hSSB activity. A previous report has demonstrated a difference in the pattern of phosphorylation of hSSB in extracts from G_1 phase Manca cells compared with that in exponentially growing cells, which was correlated with the capacity to form a functional replication initiation complex *in vitro* (Fotedar and Roberts, 1992). However, there is as yet no direct demonstration of a role for hSSB phosphorylation in either DNA unwinding at the SV40 origin of replication, or in the elongation step of DNA replication *in vitro*. The functions of the individual phosphorylated forms of hSSB in the initiation or elongation steps of DNA replication can be further investigated by using a replication system reconstituted from purified cellular proteins (Lee *et al.*, 1989; Tsurimoto and Stillman, 1990; Weinberg *et al.*, 1990). The three-subunit hSSB isolated from extracts of UV-irradiated HeLa cells, using single-stranded DNA cellulose chromatography (Kenny *et al.*, 1990), contains a mixture of the unphosphorylated and hyperphosphorylated forms of the 34 kDa subunit (M.P.Carty, unpublished). Experiments in progress to resolve these forms of hSSB should allow the function of each in DNA replication *in vitro* to be tested. hSSB is also an essential component in the repair of UV-

damaged DNA *in vitro*, where it is required in the incision step of the repair reaction (Coverley *et al.*, 1991; Shivji *et al.*, 1992). While the effect of the phosphorylation state of the protein in controlling its activities in repair relative to replication is not known at this time, hyperphosphorylation *in vivo* may be related to a role for this protein in the repair of UV-damaged cellular DNA.

The fact that purified hSSB, added to previously inactive extracts, undergoes the pattern of phosphorylation characteristic of active extracts suggests that the added hSSB differs in some way from the hSSB already present in these extracts. It is possible that the endogenous hSSB has been modified in such a way that it is incapable of undergoing the appropriate changes in phosphorylation characteristic of the active protein. For example, the endogenous hSSB activity may be negatively regulated by certain phosphorylation steps (such as those generating band 5, Figure 4) due either to additional kinase activity or to the lack of an appropriate phosphatase activity. Another possibility, not directly addressed here, is that direct protein−protein interactions between hSSB and other factors (including the p53 tumour suppressor gene product) may influence hSSB function in DNA replication (He *et al.*, 1993; Li and Botchan, 1993). Such interactions could affect, and be affected by, the phosphorylation state of the hSSB protein. Additional experimentation will be required to distinguish these possibilities.

The identity of the protein kinases and phosphatases involved in modulating hSSB phosphorylation following UV irradiation remains to be elucidated. hSSB p34 can be phosphorylated *in vitro*, on Ser 23 and Ser 29, by the cell cycle-regulated *cdc* and *cdk* families of kinases, including the $p34^{cdc2}$ kinase (Dutta and Stillman, 1992). This provides a potential link between regulation of kinase activity, cell cycle progression and hSSB phosphorylation (Dutta and Stillman, 1992). The relationship of $p34^{cdc2}$ kinase-mediated phosphorylation of hSSB to the phosphorylation-related changes in gel mobility of p34 is not yet clear. The hyperphosphorylated form of hSSB induced by UV irradiation is phosphorylated primarily on serine residues, indicating a role for serine kinase(s) in this response. Additional phosphorylation events, independent of $p34^{cdc2}$ kinase, which lead to an altered mobility of the 34 kDa subunit of hSSB on PAGE, have also been found to occur in cell extracts *in vitro* (Fotedar and Roberts, 1992). It has been suggested that $p34^{cdc2}$ kinase-mediated phosphorylation is a prerequisite for further phosphorylation-related changes in the p34 subunit of hSSB (Dutta and Stillman, 1992), which occur during *in vitro* DNA replication and alter the gel mobility of this subunit. UV irradiation of mammalian cells has been reported to alter the activity of protein kinases, including the mitogen-activated (MAP-2) kinase and Raf-1 kinase (Radler-Pohl *et al.*, 1993). However, these kinases may be involved in the signal transduction pathway leading to hSSB phosphorylation, rather than directly modifying this protein (Radler-Pohl *et al.*, 1993). Changes in the activity of phosphatases which normally dephosphorylate the p34 subunit of hSSB may be equally important in controlling the phosphorylation state of this protein. The ability of extracts of UV-irradiated cells to carry out phosphorylation events apparently similar to those occurring *in vivo* should allow the identification and further characterization of these enzymes.

Inhibition of DNA synthesis following treatment of cells with DNA damaging agents has been reported in a number of cell types (Painter and Young, 1975, 1980; Denekamp, 1986). This inhibition may be important in limiting the deleterious consequences of DNA damage, such as the induction of mutations and rearrangements in the genome (Lane, 1992), by allowing time for repair of the damage in the genome before replication proceeds. Recent studies indicate that the tumour suppressor gene p53 may play a role in the induction of G_1 arrest following irradiation of certain cell types (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992). In addition, ataxia telangiectasia cells, which have an X-ray-sensitive phenotype (Gatti *et al.*, 1991), are defective in the activation of this pathway (Kastan *et al.*, 1992). The details of the signal transduction pathway, which leads to the biochemical changes in the DNA replication apparatus described here, remain to be elucidated. However, it is likely that the changes that occur at the level of DNA replication are linked to the complex changes in gene expression that are triggered by UV radiation and other DNA damaging agents (reviewed in Herrlich *et al.*, 1992). Presumably, many of these processes are aimed at enhancing the survival of cells exposed to environmental insult. Here we have provided evidence that mechanisms, similar to those involved in the regulation of cell cycle progression, may operate to bring about the arrest of DNA replication that follows UV irradiation of cells. It remains to be seen whether these mechanisms are the same in all respects. However, our demonstration that DNA synthesis arrest-related changes in DNA replication capacity can be observed *in vitro* should facilitate further investigation of the biochemistry of the cellular response to UV irradiation.

Materials and methods

Cell culture and UV irradiation

Monolayer cultures of HeLa cells (~8 × 10^6 cells/150 mm dish) were grown in Dulbecco's Minimal Essential Medium containing 10% fetal bovine serum (FBS), at 37°C and 10% CO_2. Cells were irradiated in phosphate-buffered saline (PBS) with 254 nm UV light from a low-pressure mercury lamp. Following irradiation, the cells were washed twice with PBS and incubated for the indicated times following irradiation with the growth medium that had been removed from the dishes prior to irradiation. Mock-irradiated cells were washed in the same way, but were not exposed to UV light. For preparation of zero time samples, cells were harvested immediately after UV irradiation or mock treatment.

Cell cycle analysis

Cells (2 × 10^6/100 mm dish) were exposed to UV light or mock irradiated as described. Following incubation for the indicated length of time, cells were washed twice in PBS, trypsinized, washed again in PBS and fixed in −20°C 70% ethanol. Cells were treated with 1 mg of RNase A, and then stained for DNA content with propidium iodide (10 μg/ml). Red fluorescence from excitation at 488 nm was measured using a Coulter Epics Profile Analyser flow cytometer.

Preparation of cell extracts and in vitro DNA replication

Hypotonic cell extracts were prepared (at 0−4°C) as described previously (Li and Kelly, 1984; Carty *et al.*, 1993). Briefly, the cells were washed with PBS, and then extracted in 20 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 5 mM KCl and 1 mM dithiothreitol (DTT), followed by clarification by centrifugation at 10 000 r.p.m. in an Eppendorf model 5402 centrifuge for 10 min. The protein concentration of the cell extracts was estimated by the method of Bradford (1976), using a Bio-Rad kit (Bio-Rad, Richmond, CA) with bovine serum albumin as a standard. DNA replication assays were carried out as described previously (Carty *et al.*, 1993), using 60 μg of extract protein in each assay. The SV40-based plasmid, pZ189 (Seidman *et al.*, 1985), was used as the DNA template in these assays. Reactions contained (in a final volume of 30 μl) 30 mM HEPES (pH 7.5); 7 mM

$MgCl_2$; 0.5 mM DTT; 100 μM each dATP, dGTP and dTTP; 50 μM dCTP; 10 μCi [α-^{32}P]dCTP; 200 μM each GTP, UTP and CTP; 4 mM ATP; 40 mM creatine phosphate; 10 μg creatine kinase; 25 ng of pZ189 DNA; and 3 μg of SV40 T antigen (purified from SV40-infected cells by immunoaffinity chromatography and purchased from Molecular Biology Resources, Madison, WI). Where indicated, hSSB, purified from exponentially growing HeLa cells (Wobbe *et al.*, 1987; Kenny *et al.*, 1990; a generous gift of Dr J.Hurwitz, Sloan Kettering Cancer Center, New York) was added to the reaction. In these experiments, the same volume (1 μl) of single-stranded DNA-binding protein buffer [20 mM HEPES (pH 7.5), 25% glycerol, 0.1 mM EDTA, 0.01% NP40, 1 mM DTT, 250 mM NaCl] was added to parallel control reactions. Replication reactions were incubated at 37°C for 3 h unless indicated otherwise, and terminated by addition of EDTA to 15 mM and proteinase K to 1 μg/ml, followed by incubation at 37°C for 30 min. Incorporation of [^{32}P]dCTP into acid-insoluble material was measured by trichloroacetic acid (TCA) precipitation. DNA was purified from replication reactions by the caesium chloride method as described previously (Hauser *et al.*, 1988).

Agarose gel electrophoresis

The products of DNA replication *in vitro* were analysed by electrophoresis on a 1% agarose gel in the presence of 5 μg/ml ethidium bromide, in buffer containing 0.04 M Tris–acetate (pH 7.8) and 1 mM EDTA. Following electrophoresis, the gels were dried and exposed to X-ray film (Kodak) for autoradiography.

Cell labelling, immunoprecipitation and phosphopeptide mapping

To label cellular proteins with ^{32}P, medium was removed from exponentially growing HeLa cells (2 × 10^6/100 mm dish), and replaced by phosphate-free medium (Gibco-BRL) containing 10% dialysed FBS (Gibco-BRL), for 1 h. Following the addition of 2–5 mCi [^{32}P]orthophosphate, the cells were incubated for a further 1 h. Cells were mock irradiated or treated with UV light, and incubated in the presence of [^{32}P]orthophosphate-containing medium for another 8 or 24 h. Cells were lysed in 4 ml PBSTDS (58 mM Na_2HPO_4, 17 mM $NaHPO_4$, 68 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS), containing 0.1 mM PMSF, 1 μg/ml leupeptin, 5 mM NaF and 1 mM Na vanadate. For immunoprecipitation of hSSB p34, whole-cell lysates (1 ml) were mixed with anti-p34 monoclonal antibody 34A (a generous gift of Dr J.Hurwitz, Sloan-Kettering Cancer Center, New York) and 20 μl of protein G–agarose (Gibco-BRL) overnight at 4°C. The immunoprecipitates were washed four times in PBSTDS buffer, resupended in 1 × Laemmli gel loading buffer (Laemmli, 1970), and the proteins separated by electrophoresis on a 12% SDS–polyacrylamide gel, using the Laemmli buffer system (Laemmli, 1970). Labelled proteins were visualized by autoradiography of the dried gel. Phosphopeptide mapping and phosphoamino acid analysis were carried out by the method of Boyle *et al.* (1991). The appropriate labelled protein bands were excised from the gel and digested with chymotrypsin (Boehringer-Mannheim). Peptides were separated electrophoretically at pH 1.9, followed by ascending chromatography, and phosphorylated peptides were visualized by autoradiography.

Western immunoblotting of hSSB

Extract proteins (60 μg) were adjusted to 1 × Laemmli gel loading buffer (Laemmli, 1970), boiled for 5 min and separated by SDS–PAGE as described. Proteins were transferred to Immobilon-P polyvinyl-divinyl fluoride transfer membranes (Millipore Corp., Bedford, MA) using a semi-dry transfer apparatus (Bio-Rad), following the manufacturers' recommendations. The blots were washed four times with TTBS [100 mM Tris–HCl (pH 7.5) 0.9% NaCl, 0.1% Tween 20] and blocked with the same solution. Blots were probed for the 34 kDa subunit of hSSB using a 1:50 dilution of monoclonal antibody SSB34A raised against this subunit (Kenny *et al.*, 1990), and for the 70 kDa subunit using a 1:50 dilution of monoclonal antibody 70A (Kenny *et al.*, 1990; both antibodies were gifts of Dr J.Hurwitz). Blots were then washed four times with TTBS, and the proteins were visualized by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG second antibody and the chemiluminescent substrate, disodium 3-(4-methoxyspiro (1,2-dioxetane-3,2′-tricyclo-[3.3.1.1^{3,7}]decan)-4-yl) phenyl phosphate (AMPPD; obtained from Oncogene Science, New York), followed by exposure to X-ray film.

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Note added in proof

A recent report has also described that both ionizing radiation and UV light induce phosphorylation of RPA p34 in cultured human cells [Liu,V.F. and Weaver,D.T. (1993) *Mol. Cell. Biol.*, **13**, 7222–7231].