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Phosphorylated and unphosphorylated forms of human single-stranded DNA-binding protein are equally active in simian virus 40 DNA replication and in nucleotide excision repair

(cell cycle regulation/DNA synthesis/DNA repair)

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ABSTRACT **The trimeric human single-stranded DNA-binding protein (HSSB; also called RP-A) plays an essential role in DNA replication, nucleotide excision repair, and homologous DNA recombination. The p34 subunit of HSSB is phosphorylated at the G_1/S boundary of the cell cycle or upon exposure of cells to DNA damage-inducing agents including ionizing and UV radiation. We have previously shown that the phosphorylation of p34 is catalyzed by both cyclin-dependent kinase–cyclin A complex and DNA-dependent protein kinase. In this study, we investigated the effect of phosphorylation of p34 by these kinases on the replication and repair function of HSSB. We observed no significant difference with the unphosphorylated and phosphorylated forms of HSSB in the simian virus 40 DNA replication or nucleotide excision repair systems reconstituted with purified proteins. The phosphorylation status of the p34 subunit of HSSB was unchanged during the reactions. We suggest that the phosphorylated HSSB has no direct effect on the basic mechanism of DNA replication and nucleotide excision repair reactions *in vitro*, although we cannot exclude a role of p34 phosphorylation in modulating HSSB function *in vivo* through a yet poorly understood control pathway in the cellular response to DNA damage and replication.**

The human single-stranded DNA (ssDNA)-binding protein (HSSB; also called RP-A) is essential for DNA replication and nucleotide excision repair and may play important roles in recombination and transcription (1–6). The protein is made up of polypeptides of 70, 34, and 14 kDa; the human genes encoding the three subunits have been cloned, and the complex has been assembled from vectors expressing all three subunits in *Escherichia coli* (7) as well as in baculovirus-infected cells (8). The function of HSSB has been extensively studied in the simian virus 40 (SV40) DNA replication system where it has been shown that all three subunits are essential for replication. The p70 subunit binds ssDNA, whereas the functions of the other subunits are unknown, though antibodies against any one of the subunits have been shown to inhibit SV40 DNA replication (9–11). The subunit structure of HSSB is highly conserved in eukaryotes. In *Saccharomyces cerevisiae* each of the ssDNA-binding protein subunits is encoded by a single essential gene (12).

The p34 subunit of HSSB becomes phosphorylated at the G_1/S boundary of the cell cycle (13) and upon exposure of cells to DNA damage by ionizing (14) or UV (15) radiation. These observations have led to speculation that the phosphorylation state of the protein modulates its function in DNA replication and repair. In support of this model, it has been reported that extracts from UV-irradiated HeLa cells contained almost exclusively the hyperphosphorylated form of HSSB and were incapable of supporting SV40 DNA replication (15) and that the addition of the unphosphorylated form of HSSB restored the replication activity to the cell extract. DNA damage induces the cyclin-dependent kinase (cdk) inhibitor p21 (Cip 1, WAF 1, or Sdi 1), which binds proliferating cell nuclear antigen (PCNA) and inhibits SV40 DNA replication (16, 17) but apparently not nucleotide excision repair (18, 19). These findings suggested the following cellular response to DNA damage. The DNA lesions lead to formation of a PCNA–p21 complex and phosphorylation of HSSB, which inhibits chromosomal DNA replication but allows DNA repair to occur. After repair is completed, sufficient quantities of free PCNA and unphosphorylated HSSB are generated to enable normal progression through the cell cycle. We wished to test this model and, in particular, to investigate whether or not phosphorylation of the p34 subunit of HSSB played a role in coordinating DNA repair with replication during the cell cycle.

We used the *in vitro* SV40 DNA replication system (20–22) to investigate the effect of HSSB phosphorylation on DNA replication and the recently developed reconstituted human nucleotide excision repair system (6) to study its effect on DNA repair. Both replication and excision reactions are absolutely dependent on HSSB. We found that the unphosphorylated and phosphorylated derivatives of HSSB were equally active in supporting replication and repair. While these observations do not exclude a possible role of the phosphorylated HSSB *in vivo*, they do not support a direct link between phosphorylation of HSSB and a coordinated response linking replication inhibition to increased repair activity.

MATERIALS AND METHODS

Preparation of Phosphorylated and Unphosphorylated HSSB. HSSB (0.42 mg), purified from cytosolic extracts of HeLa cells as described (9), was added to a reaction mixture (5.6 ml) containing 40 mM creatine phosphate (pH 7.7), creatine kinase (25 μg/ml), 7 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 4 mM ATP, G_1 extract (21.8 mg), and cyclin A (0.23 mg). The preparation of G_1 extracts from HeLa cells and cyclin A was as described (23). After incubation at 37°C for 1 hr, the mixture was adjusted to 0.2 M NaCl, 20 mM EDTA, 0.5 mM DTT, 1 mM phenylmethanesulfonyl fluoride, antipain (2 μg/ml), leupeptin (1 μg/ml), 1 mM Na_3VO_4, 10 mM NaF, and 0.1 μM okadaic acid and then loaded onto a phosphocellulose

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Abbreviations: ssDNA, single-stranded DNA; HSSB, human ssDNA-binding protein; DNA-PK, DNA-dependent protein kinase; DTT, dithiothreitol; SV40, simian virus 40; PMSF, phenylmethanesulfonyl fluoride; AS, ammonium sulfate; PCNA, proliferating cell nuclear antigen; cdk, cyclin-dependent kinase; T antigen, SV40 large tumor antigen

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column (4 ml) equilibrated with buffer B [25 mM Tris·HCl (pH 8.0), 1 mM EDTA, 0.01% Nonidet P-40, 10% (vol/vol) glycerol, 1 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride, antipain (0.2 μg/ml), and leupeptin (0.1 μg/ml)] plus 0.2 M NaCl. The column was washed with 4 ml of buffer B containing 0.2 M NaCl and phosphatase inhibitors (1 mM Na_3VO_4, 10 mM NaF, and 0.1 μM okadaic acid). The flow-through and wash fractions were pooled, adjusted to 0.5 M NaCl, and then chromatographed on a ssDNA-cellulose column (0.5 ml) equilibrated with buffer B plus 0.5 M NaCl. After washing with 10 ml of buffer B plus 0.7 M NaCl, bound protein was eluted with 2 ml of buffer B plus 2 M NaCl and 45% (vol/vol) ethylene glycol. The eluate was dialyzed against 1 liter of buffer B plus 0.25 M NaCl for 2 hr at 0°C and subsequently concentrated by centrifugation using a Centriflow-25 cone (Amicon). This procedure yielded 64 μg of phosphorylated HSSB (1.6 mg/ml).

As a control for experiments, HSSB was prepared using the same procedure as described above except that ATP, creatine kinase, and $MgCl_2$ were omitted from the reaction mixture. The procedure yielded 53 μg of unphosphorylated protein (1.5 mg/ml). This material is referred to as (mock-treated) unphosphorylated HSSB. HSSB purified from cytosolic extracts of HeLa cells (9) and used for the preparation of phosphorylated HSSB is referred to as untreated HSSB.

***In Vitro* SV40 DNA Replication Assays.** SV40 DNA replication with the monopolymerase system was carried out as described (24). Reaction mixtures (40 μl) contained 40 mM creatine phosphate (pH 7.7), creatine kinase (25 μg/ml), 7 mM $MgCl_2$, 0.5 mM DTT, 4 mM ATP, 200 μM other rNTPs, 20 μM [α-^{32}P]dCTP (≈5000 cpm/pmol), 100 μM other dNTPs, 10 μg of bovine serum albumin, 0.3 μg of pSVΔEP ori$^+$ DNA, 1200 units of topoisomerase I, the DNA polymerase α–primase complex (1.2 and 1.6 units, respectively), 0.6 μg of SV40 large tumor antigen (T antigen), and HSSB in either the unphosphorylated or phosphorylated form, as indicated. After incubation at 37°C for 2 hr, the radioactive, acid-insoluble material was measured.

SV40 DNA replication, using the ammonium sulfate (AS) fraction-complementation system, was carried out as described (20). Reaction mixtures (40 μl) contained 40 mM creatine phosphate (pH 7.7), creatine kinase (25 μg/ml), 7 mM $MgCl_2$, 0.5 mM DTT, 4 mM ATP, 200 μM other rNTPs, 20 μM [α-^{32}P]dCTP (≈18,000 cpm/pmol), 100 μM other dNTPs, 0.3 μg of pSVΔEP ori$^+$ DNA, 0.6 μg of T antigen, 35–65% AS fraction (152 μg), and HSSB in either the unphosphorylated or phosphorylated form, as indicated. After incubation at 37°C for 1 hr, the radioactive acid-insoluble material was measured.

***In Vitro* Excision Assays.** The excision assay (25) used measured the release of a 28-mer carrying a lesion from a 140-bp duplex containing ^{32}P label at the 54th phosphodiester bond and cholesterol instead of a base at position 70, located 6 nucleotides 3′ to the label. The reaction mixture (50 μl) contained 40 mM Hepes (pH 7.9), 80 mM KCl, 8 mM $MgCl_2$, 2 mM ATP, 20 μM each dNTP, bovine serum albumin (100 μg/ml), 1 mM DTT, 1 nM ^{32}P-labeled substrate, 7% glycerol, fractions I–IV (xeroderma pigmentosum A–G plus TFIIH) as reported previously (6), and the indicated amounts of fraction V (HSSB), either in the unphosphorylated or phosphorylated form. The reaction mixture was incubated at 30°C for the indicated time, and the products were analyzed on a 10% polyacrylamide sequencing gel as described (6). The yield of excision product was quantified using a PhosphorImager (Molecular Dynamics). The excision was expressed as the fraction of total radiolabel migrating in the 25- to 28-nucleotide area.

RESULTS

Analysis of Phosphorylated HSSB. We previously demonstrated that the p34 subunit of HSSB is phosphorylated in cyclin A-activated G_1 extracts by cdk–cyclin A and DNA-dependent protein kinase (DNA-PK; ref. 23). To analyze the effects of phosphorylation of HSSB on SV40 DNA replication and nucleotide excision repair, we isolated the phosphorylated form of HSSB after incubating HSSB with cyclin A-activated G_1 extracts and an ATP-regenerating system, as described in *Materials and Methods*. This procedure quantitatively converted the p34 subunit of HSSB to phosphorylated products (Fig. 1, lane 2) that included at least four different species migrating more slowly through denaturing polyacrylamide gels than the mock-treated HSSB (unphosphorylated form, lane 3) and the HSSB isolated from HeLa cells (untreated, lane 1). The phosphorylated p34 derivatives have been shown to result from the combined action of highly purified cdk–cyclin A and the Ku antigen-stimulated DNA-PK (ref. 23; H. Niu, Z.-Q. P., and J.H., unpublished results).

Effects of Different Forms of HSSB on SV40 DNA Replication. We examined the effects of phosphorylation of HSSB on SV40 DNA replication using two different assays. As previously described (24), SV40 DNA replication can be carried out by the monopolymerase system in which both leading- and lagging-strand DNA synthesis are catalyzed by high levels of the DNA polymerase α–primase complex. In addition to the DNA polymerase α–primase complex, this system requires SV40 ori$^+$ DNA, T antigen, topoisomerase I, and HSSB. We have previously shown that components of the monopolymerase system are both necessary and sufficient to initiate RNA primer synthesis and that HSSB is specifically required for this reaction (26, 27). For these reasons, we compared unphosphorylated and phosphorylated HSSB for their ability to support the monopolymerase system. As shown in Fig. 2*A*, these two forms of HSSB, as well as HSSB isolated from HeLa cells, were equally active except that at low levels (<100 ng), the phosphorylated HSSB was about 2-fold more active than the unphosphorylated form.

The replication activity of HSSB was also tested using an AS fraction–complementation system (20). The 35–65% AS frac-

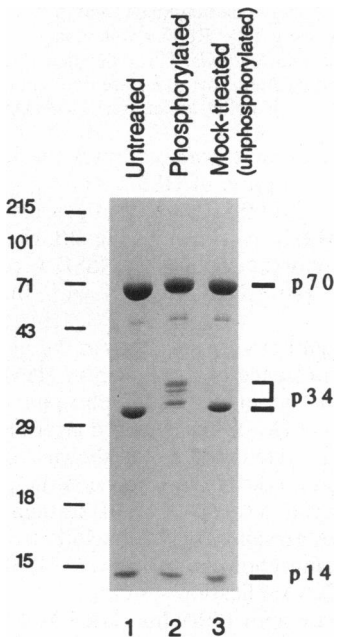

FIG. 1. Denaturing polyacrylamide gel (SDS/PAGE) analysis of unphosphorylated and phosphorylated HSSBs. Purified HSSB (2 μg) either untreated (lane 1) or treated with cyclin A-activated G_1 extracts in the presence (lane 2) or absence (lane 3) of ATP was analyzed by SDS/12.5% PAGE and Coomassie brilliant blue staining. Protein size markers (in kDa) are shown on the left. The bracketed region indicates the slow-migrating phosphorylated forms of the p34 subunit. The protein band migrating between 70 and 43 kDa reacted with anti-p70 antibody and is most likely a degradation product of p70.

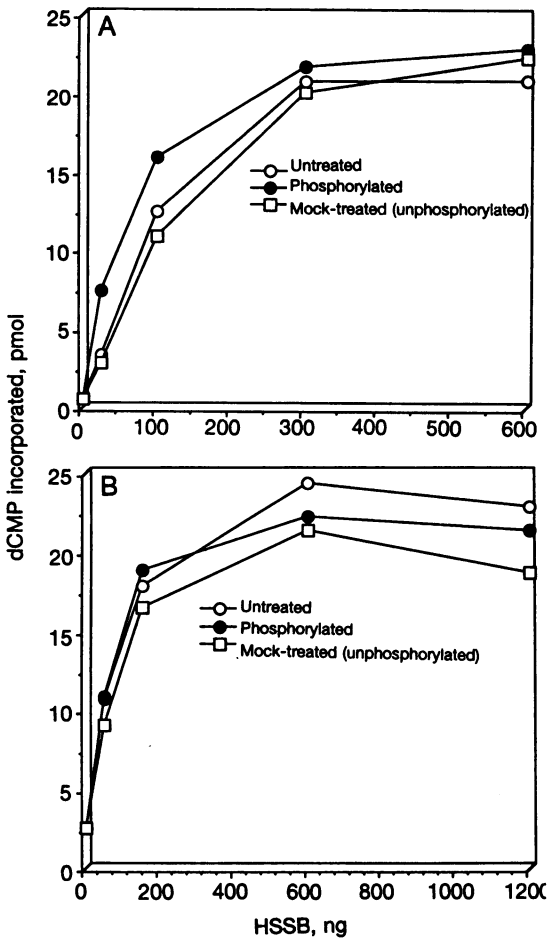

FIG. 2. Effects of HSSB phosphorylation on SV40 DNA replication with the monopolymerase system (*A*) and the 35–65% AS fraction complementation assay (*B*). Replication assay conditions are described in *Materials and Methods*. Three purified HSSB preparations, untreated, phosphorylated, or mock-treated (unphosphorylated), were compared for their ability to support SV40 DNA replication.

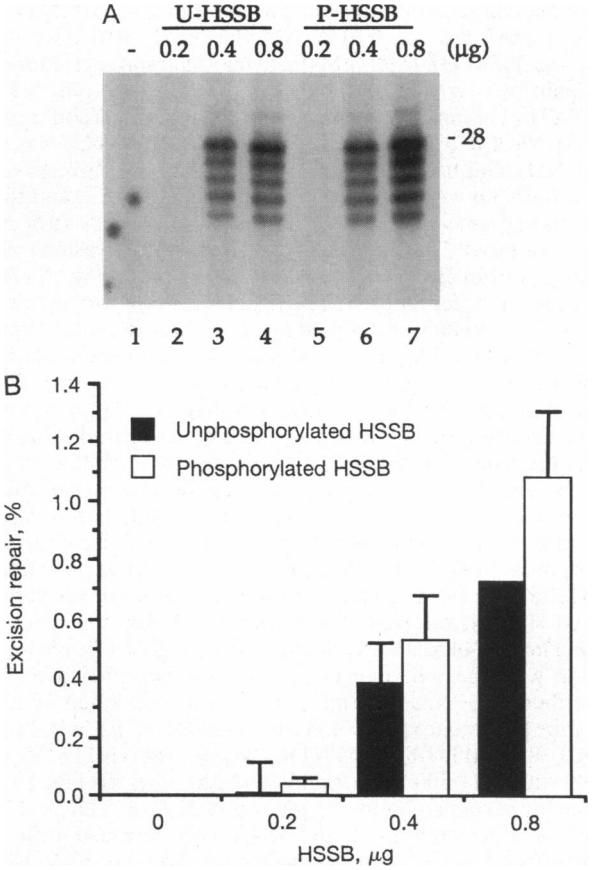

FIG. 3. Excision repair with unphosphorylated (U-HSSB; mock-treated) or phosphorylated (P-HSSB) HSSB in a reconstituted system as a function of HSSB concentration. The indicated amounts of HSSB were added to the reconstituted repair system. The excision reaction was carried out for 45 min at 30°C. (*A*) Autoradiographic analysis of excision products. Lane 1, no addition; lanes 2, 3, and 4, 0.2, 0.4, and 0.8 μg of unphosphorylated (mock-treated) HSSB, respectively; lanes 5, 6, and 7, 0.2, 0.4, and 0.8 μg of phosphorylated HSSB, respectively. The number 28 indicates the nucleotide length of the major excision product. (*B*) Quantitative analysis of excision products. The excision products were analyzed by using a PhosphorImager (Molecular Dynamics).

tion of HeLa cytosolic extract contains the host replication factors with the exception of HSSB. The supplementation of this AS fraction with HSSB and T antigen results in efficient SV40 DNA synthesis. As shown in Fig. 2*B*, both unphosphorylated and phosphorylated forms of HSSB were equally active in supporting SV40 DNA synthesis with the 35–65% AS fraction.

The rate of SV40 DNA replication in the monopolymerase reaction in the presence of the different HSSB preparations was identical, and no change in the phosphorylation state of the p34 subunits of HSSB was detected after incubation in the monopolymerase system (data not shown). Consistent with this, we observed no differences between the phosphorylated and unphosphorylated forms of HSSB in their ability to bind ssDNA (data not presented). We conclude from these experiments that the phosphorylation status of HSSB has no effect on the SV40 DNA replication system.

Excision Repair with Phosphorylated HSSB. Using an *in vitro* system for nucleotide excision repair, we tested the effect of phosphorylation on DNA repair. In this system both excision (6) and repair synthesis (4) are dependent on HSSB. Fig. 3 shows the level of excision of a cholesterol adduct as a function of HSSB concentration. At low HSSB concentration it appears that unphosphorylated HSSB was more active; however, at high concentrations the trend was reversed. These results indicate that phosphorylation either has no or only a moderate effect on excision repair. However, the observed differences in either direction were within experimental variability. Hence we conducted kinetic experiments to assess the effect of phosphorylation on excision.

Fig. 4 represents excision kinetics by the excision system with purified proteins. Again, the two forms of HSSB showed essentially the same activity. At later time points (45 and 60 min), the unphosphorylated form appears to be 10–30% more active, an observation we attribute to experimental variation.

Phosphorylation Status of HSSB During and After Repair. The excision repair system we used was reconstituted from highly purified proteins. However, with the exception of xeroderma pigmentosum A, all of the other factors were purified from HeLa cells. It was conceivable that these purified proteins might be contaminated with sufficient quantities of cdk–cyclin A complex and DNA-PK to phosphorylate the nominally unphosphorylated HSSB in our reaction mixtures. Therefore, the phosphorylation status of HSSB was tested after an excision reaction by performing an immunoblot analysis (Fig. 5) or silver staining. No change was seen in the phosphorylation status of either unphosphorylated or phosphorylated HSSB after the repair reaction. Thus, we conclude that the phosphorylation status of HSSB has no bearing in its role in nucleotide excision repair.

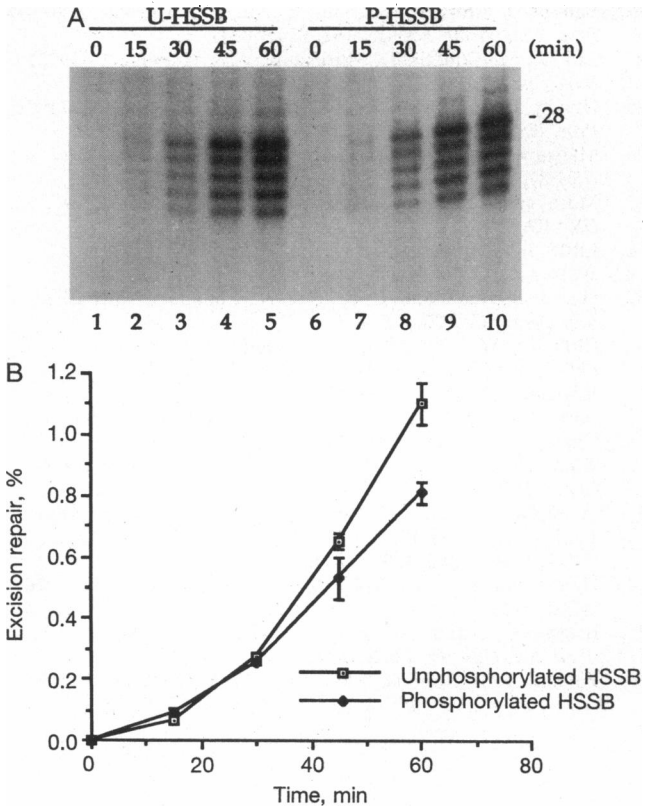

FIG. 4. Kinetics of excision repair with unphosphorylated (mock-treated) or phosphorylated HSSBs in a reconstituted system. Either unphosphorylated (U-HSSB; mock-treated) or phosphorylated (P-HSSB) HSSB (0.4 μg) was added to the reconstituted repair system. The excision reaction was conducted at 30°C as indicated. (*A*) Autoradiographic analysis of excision products. The number 28 indicates the nucleotide length of the major excision product. (*B*) Quantitative analysis of the yield of excision products.

DISCUSSION

In *E. coli* damage to DNA by a variety of agents elicits the SOS response (28, 29). An essential component of this response is inhibition of the cell division cycle coupled to an increase in DNA repair activity; the net result is a rapid elimination of DNA lesions before resumption of the cell cycle. Recently, several phenomena reminiscent of the SOS response have been described in mammalian cells. (*i*) It has been found that after radiation damage (UV or x-ray) the p34 subunit of HSSB becomes hyperphosphorylated (14, 15), and furthermore it was reported that cell extracts from UV-irradiated cells, which contained mostly phosphorylated HSSB, were unable to support SV40 replication. Thus, it was proposed that phosphorylation of HSSB inhibits replication but may not inhibit repair, creating a status similar to that during the SOS response in *E. coli* (15). (*ii*) It has been found that p21 (Cip 1, WAF 1, or Sdi 1), induced by DNA damage as a result of transcriptional activation by p53 (30, 31), binds to PCNA directly and that this complex of PCNA is inactive in SV40 DNA replication (16, 17) but has normal activity in nucleotide excision repair (18, 19). This response was also proposed to help genomic stability and cell survival by allowing repair to proceed (at a normal rate) while replication is inhibited. (*iii*) Finally, it has also been reported that the growth arrest and DNA damage-inducible Gadd45 protein inhibits cell growth (32) while simultaneously stimulating excision repair by about a factor of 3 (33).

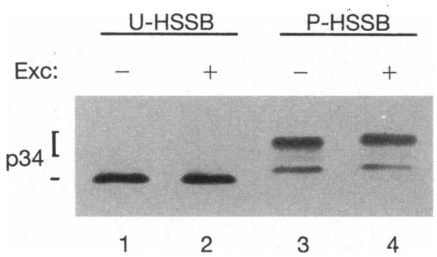

FIG. 5. Effect of excision repair reaction on HSSB phosphorylation. Unphosphorylated (U-HSSB; mock-treated) or phosphorylated (P-HSSB) HSSB was incubated for 45 min at 30°C under the excision (Exc) repair reaction conditions and then analyzed by SDS/PAGE and Western blot with a monoclonal antibody against the 34-kDa subunit of HSSB.

While each of the proposed damage response reactions listed above makes sense from a teleological viewpoint, they must be subjected to rigorous experimental tests before we assume that mammalian cells have an SOS response functionally homologous to the bacterial SOS response. Indeed the experimental results reported in this paper do not support the notion that phosphorylation of HSSB plays a direct role in dissociating repair from replication as a means for cells to maintain genomic stability after sustaining DNA damage.

In light of the results presented here, it might be asked whether phosphorylation of HSSB has any physiological relevance. Three recent studies bear on this question. Pan *et al.* (23) reported that the p34 subunit of HSSB was phosphorylated in two steps: first by cdk–cyclin A and then by DNA-PK. Furthermore, it was found that p21 (Cip 1, WAF 1, or Sdi 1) prevented HSSB p34 phosphorylation entirely in cyclin A-activated G_1 extracts by inhibiting cdk–cyclin A activity, which performed the first phosphorylation reaction. Henricksen and Wold (34) reported that HSSB p34 with serine → alanine substitutions at Ser-23 and Ser-29 (serine cdc2 kinase consensus phosphorylation sites) had normal DNA-binding activity, had normal replication activity in a partially purified system, and became phosphorylated under replication conditions. Finally, Brush *et al.* (35) showed that even though DNA-PK was required for SV40 DNA replication-dependent phosphorylation of HSSB p34 in cell-free extracts (36), depletion of the kinase activity with DNA-PK antibodies had no effect on DNA replication. These studies, which identified some sites and the mechanism of HSSB phosphorylation, do not directly address the issue of whether or not phosphorylated HSSB is active in replication or repair. In contrast, the data presented in this paper provide direct evidence that the phosphorylation status of the HSSB p34 subunit plays no direct role either in SV40 DNA replication or in nucleotide excision repair. It has been suggested that DNA replication intermediates, such as a replication fork, act as a cofactor for DNA-PK to promote phosphorylation of HSSB and that the phosphorylated form of HSSB initiates a signaling pathway that prevents cell cycle progression while replication or repair intermediates exist (35). Further studies are needed to test this model.

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