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A role for the human single-stranded DNA binding protein HSSB/RPA in an early stage of nucleotide excision repair

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ABSTRACT

The human single-stranded DNA binding protein (HSSB/RPA) is involved in several processes that maintain the integrity of the genome including DNA replication, homologous recombination, and nucleotide excision repair of damaged DNA. We report studies that analyze the role of HSSB in DNA repair. Specific protein-protein interactions appear to be involved in the repair function of HSSB, since it cannot be replaced by heterologous single-stranded DNA binding proteins. Anti-HSSB antibodies that inhibit the ability of HSSB to stimulate DNA polymerase α also inhibit repair synthesis mediated by human cell-free extracts. However, antibodies that neutralize DNA polymerase α do not inhibit repair synthesis. Repair is sensitive to aphidicolin, suggesting that DNA polymerase ε or δ participates in nucleotide excision repair by cell extracts. HSSB has a role other than generally stimulating synthesis by DNA polymerases, as it does not enhance the residual damage-dependent background synthesis displayed by repair-deficient extracts from xeroderma pigmentosum cells. Significantly, when damaged DNA is incised by the *Escherichia coli* UvrABC repair enzyme, human cell extracts can carry out repair synthesis even when HSSB has been neutralized with antibodies. This suggests that HSSB functions in an early stage of repair, rather than exclusively in repair synthesis. A model for the role of HSSB in repair is presented.

INTRODUCTION

Mammalian cells use nucleotide excision repair to remove many different mutagenic lesions from DNA. This multiprotein process involves recognition of a lesion, incision of the altered DNA strand, excision of damaged nucleotides, synthesis of a repair patch, and ligation. DNA excision repair can be mediated by mammalian cell extracts, and repair synthesis can be monitored by incubating damaged and nondamaged circular plasmid DNA with extracts in a reaction mixture which includes the four deoxynucleoside triphosphates and [α-^{32}P]dATP (1, 2). Quantification of incorporated radioactive nucleotides gives a measure of DNA repair. Cell lines derived from individuals with the inherited disorder xeroderma pigmentosum (XP) are deficient in DNA repair, and extracts from these and other UV-sensitive cell lines are generally deficient in repair *in vitro* (1, 3, 4).

Most of the gene products represented by UV-sensitive mutants of mammalian cells appear to participate in the early steps of repair in which DNA lesions are recognized and removed. Subsequent formation of the repair patch involves synthesis of a short patch of nucleotides by a DNA polymerase. In addition to the polymerase, evidence is emerging that other factors known to be involved in semiconservative DNA replication are also involved in nucleotide excision repair. One of these is HSSB, the human single-stranded DNA binding protein (5).

HSSB, also called RPA or RF-A (6, 7, 8) is a complex of three tightly-associated polypeptides of 70–76, 32–34 and 11–14 kDa. It is required for the replication of SV40 viral DNA *in vitro* where it assists T antigen and topoisomerase in unwinding the duplex DNA. An additional role in DNA synthesis is suggested by the ability of HSSB to stimulate the activity of DNA polymerases on artificial templates (9, 10, 11, 12). The isolated 70 kDa subunit of HSSB possesses single-stranded DNA binding activity (11, 13) and this subunit can also stimulate homologous pairing of DNA in cell-free systems (14, 15). Less is known of the functions of the other two subunits. The cDNA sequence of the 34 kDa subunit predicts a protein containing two acidic regions (16) which may be involved in interactions with the other HSSB subunits or with proteins involved in replication or repair. The 34 kDa subunit is phosphorylated in a cell cycle-dependent manner (17).

Neutralizing monoclonal antibodies which recognise the 70 and 34 kDa subunits have been used to examine the role of HSSB in SV40 DNA replication *in vitro*. Three antibodies which recognize the 70 kDa subunit (70A, 70B and 70C) and one which recognizes the 34 kDa subunit (34A) were found to inhibit synthesis of SV40 DNA (11). More recently we found that all four antibodies inhibited DNA repair synthesis *in vitro* (5). In the absence of antibody, repair synthesis could be stimulated 2–3 fold by excess HSSB. Analysis of the structure of repair patches produced in DNA damaged at a single specific site showed that

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excess HSSB increased the total number of repair events, rather than the average repair patch size, which remained at approximately 30 nucleotides.

There are several potential roles for HSSB in the excision repair process. HSSB could function early in repair by facilitating recognition of lesions or by participating in the DNA incision reaction in cooperation with other repair enzymes. HSSB might also serve to protect incised or gapped DNA from degradation, and by binding to a gapped single-stranded region it could aid in recycling the incision complex. In addition, the ability of HSSB to interact with DNA polymerases might be utilized in order to recruit a DNA polymerase onto the incised or gapped site generated during the first stage of DNA repair. Here we report experiments which suggest that HSSB is involved in the initial steps of DNA repair, in a role that may be somewhat different from its role in DNA replication.

MATERIALS AND METHODS

Cells and extracts

Human lymphoblastoid cell lines were obtained from the Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ) and grown in suspension at 37°C in RPMI 1640 medium supplemented with 2 mM glutamine and 10% fetal bovine serum, under 10% CO_2. Cells were split to a density of approximately 5×10^5 cells/ml every two days or when numbers exceeded 10^6 cells/ml. HeLa cells were grown under identical conditions in medium with 5% fetal bovine serum. Cell extracts were prepared from cultures at densities of $6-8\times10^5$ cells/ml by the method of Manley *et al.* (1, 18) and quick-frozen in 25−100 μl aliquots.

Repair synthesis

Closed-circular plasmid DNA substrates were prepared as described (1, 19). DNA repair synthesis reactions (50 μl) included 100 μg of whole cell extract protein, 300 ng of UV-irradiated (450 J/m^2) pAT153 plasmid DNA (3.7 kb) and 300 ng unirradiated pBR322 plasmid DNA (4.4 kb) in 45 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid-KOH (pH 7.8), 60 mM KCl, 7.5 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 μM each of dGTP, dCTP and dTTP, 8 μM dATP, 74 kBq [α-^{32}P]dATP (110 TBq/mmol), 40 mM phosphocreatine, 2.5 μg creatine phosphokinase (Type I, Sigma), 3.4% glycerol and 18 μg bovine serum albumin. Reactions were incubated for 3 h at 30 °C. DNA was isolated, linearized with EcoRI, and separated by agarose gel electrophoresis. Results were quantified by scintillation counting of excised bands and corrected for DNA recovery by densitometry of photographic negatives (1).

Repair proteins and inhibitors

HSSB was isolated from HeLa cells as described (6, 11) to yield a pure preparation with a specific activity of ~ 400 units/mg, where one unit supports the incorporation of 1 nmol dTMP in 60 min in an SV40 replication assay (11). Where indicated, up to 3 μg of HSSB in not more than 6 μl buffer (20 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0), 0.1 mM EDTA, 250 mM NaCl, 0.01% Nonidet P-40 and 25% glycerol) was incubated with cell extract for 15−30 min at 30°C before addition of repair reaction buffer and plasmid DNA substrates. Monoclonal antibodies against HSSB (11) and DNA polymerase α (20) were purified from hybridoma supernatant on a protein A-Sepharose column and dialyzed against phosphate buffered saline to give final preparations of IgG protein with concentrations of ~ 1 mg/ml. Up to 3 μl was pre-incubated with cell extract for 15−30 min at 30°C before addition of reaction buffer and plasmid DNAs.

Aphidicolin (Sigma) in 2 μl Me_2SO was pre-incubated for 10 min at 30°C with 100 μg cell extract protein. Control reactions contained 2 μl Me_2SO only. Addition of greater than 4 μl of Me_2SO (8% by volume in reaction mixtures) resulted in a marked inhibition of DNA repair synthesis in the absence of aphidicolin.

Polymerase α-primase complex was purified from HeLa cells as previously described (21). For measurements of polymerase activity, reaction mixtures (50 μl) contained 50 mM Tris·HCl (pH 8.0), 7.5 mM $MgCl_2$, 4 mM dithiothreitol, 10 μg bovine serum albumin, 40 μM each dATP, dGTP, and dCTP, 10 μM ^{3}H-dTTP (500 cpm/pmol), 10 μg of DNAse I-activated calf thymus DNA, 0.08 unit of polymerase α, and purified antibody as indicated.

Where indicated, plasmid DNA was incubated with *E.coli* UvrABC proteins (22) which were a kind gift of L.Grossman (The Johns Hopkins University, Baltimore, MD). 300 ng each of unirradiated and UV-irradiated plasmid DNAs was incubated in repair synthesis buffer (in a total volume of 15−20 μl) with ~ 0.4 μg of UvrA and 0.2 μg of UvrB protein for 10 min at 30°C. UvrC protein (22 ng) was added and incubation continued for 10 min. This treatment converted ~ 10% of the UV-irradiated plasmid to the nicked circular form with minimal nicking of the unirradiated plasmid. Cell extracts were then added directly to this mixture of incised DNA and other reaction components.

RESULTS

Inhibition of repair synthesis by anti-HSSB antibodies that disrupt the functional interaction of HSSB with DNA polymerases

To begin exploring the role of HSSB in repair, we considered evidence suggesting that HSSB participates in specific protein-protein interactions with other components of the repair apparatus. Studies of the replication of SV40 DNA *in vitro* have demonstrated that heterologous single-stranded DNA binding proteins such as *E.coli* or yeast SSB cannot efficiently replace HSSB in the overall replication reaction (7, 9, 23), even though these SSBs can readily bind single-stranded regions and remove DNA secondary structure. This has been interpreted as indicating that specific interactions take place between HSSB and other DNA replication proteins. Similarly, we find that heterologous SSBs cannot replace HSSB in cell extract-mediated DNA repair synthesis. Neither *E.coli* SSB nor adenovirus DNA-binding protein could restore the ability of a HeLa cell extract to carry out DNA repair synthesis after endogenous HSSB was neutralized by anti-HSSB antibody (Fig. 1). It therefore seems likely that HSSB interacts in a relatively specific way with other proteins during excision repair. Conceivably, HSSB might associate with factors that recognize lesions and introduce incisions at damaged sites in DNA, or it might interact with a polymerase during the DNA synthesis stage of repair, or both types of interaction could take place.

We considered DNA polymerase α as a candidate for interaction with HSSB during repair. It is known that HSSB can stimulate DNA synthesis by polymerase α, and that heterologous

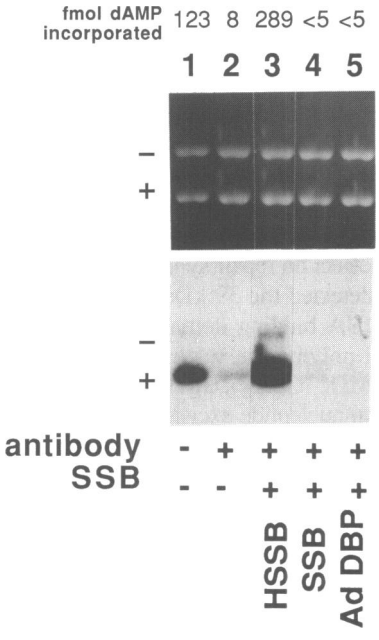

Figure 1. DNA repair synthesis by antibody-inhibited cell extract is not restored by heterologous SSBs. 100 μg of HeLa cell extract protein was pre-incubated with 0.5 μg of anti-HSSB 70C antibody in the presence of 1.5 μg of various SSBs, then tested for repair synthesis activity in UV-irradiated (+) and unirradiated (−) plasmids. Lanes 1 and 2, uninhibited and antibody-inhibited extracts. Lanes 3, 4 and 5, inhibited extract supplemented with HSSB, *E.coli* SSB (Pharmacia) or adenovirus DNA binding protein (Ad DBP; (62) respectively. The net fmol dAMP incorporated by repair synthesis into UV-irradiated plasmid are shown at the top of the figure.

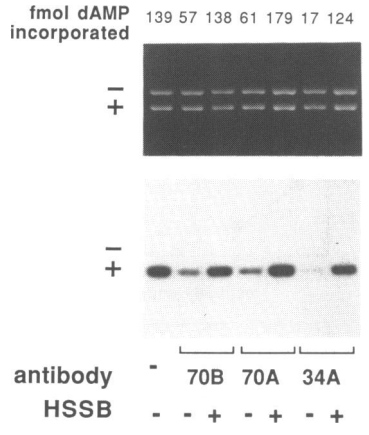

Figure 2. Inhibition of cell extract-mediated repair by anti-HSSB 70A, 70B and 34A antibodies, and reconstitution with purified HSSB. 100 μg of HeLa cell extract protein was pre-incubated for 30 min at 37°C with 1 μg of the indicated antibody and then supplemented with 1 μg of HSSB and incubated for a further 10 min. Reaction buffer and a mixture of UV-irradiated (+) and unirradiated (−) plasmid DNAs were added and incubation continued at 30°C. Top panel, ethidium bromide-stained agarose gel showing linearized plasmid DNAs isolated from the reaction mixture. Bottom panel, DNA synthesis visualized by fluorography. The net fmol dAMP incorporated by repair synthesis into UV-irradiated plasmid (after subtraction of 1−10 fmol incorporated into unirradiated plasmid) are shown at the top of the figure.

SSBs are much less efficient in stimulating this polymerase (9). Protein-protein interactions between HSSB and polymerase α have recently been directly detected (24); M.K. & D.P.L., unpublished data). Moreover, of the four anti-HSSB monoclonal antibodies (70A, 70B, 70C, and 34A) that were previously found to inhibit the repair synthesis activity of human cell extracts (5), three antibodies appear to be able to disrupt the interaction of HSSB with human DNA polymerase α. These antibodies (70A, 70B and 34A) inhibit the ability of HSSB to stimulate *in vitro* synthesis by DNA polymerase α on an artificial template (11). Antibody 70C does not inhibit this activity but instead affects the activity of HSSB in a significantly different way. Of the four antibodies, only 70C inhibits the ability of HSSB to participate in the unwinding of SV40 DNA and inhibits the stimulatory effect of HSSB on DNA polymerase δ. We previously studied this antibody in detail and showed that inhibition of repair synthesis by 70C could be prevented by pre-incubation of the antibody with pure HSSB (5).

In order to demonstrate that the inhibition of DNA repair synthesis caused by 70A, 70B and 34A antibodies was also due to a specific effect on HSSB. 100 μg of HeLa extract protein was preincubated with 1 μg of each antibody and the ability of the extract to carry out repair synthesis was assessed in the presence or absence of additional exogenous HSSB (Fig. 2). As expected, each antibody inhibited repair synthesis, with the 34A antibody causing the greatest suppression of synthesis. Repair activity could be readily restored to the reaction mixture in each case by subsequently adding 1 μg of purified HeLa HSSB. This set of antibodies is known to disrupt the functional interaction

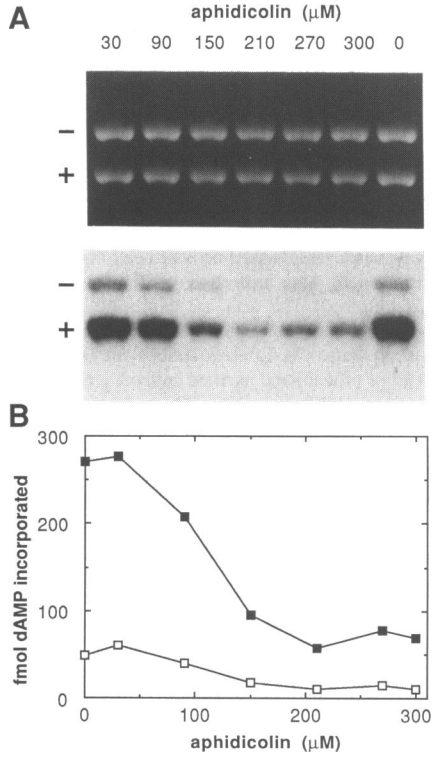

Figure 3. Sensitivity of DNA excision repair synthesis to inhibition by aphidicolin. 100 μg of HeLa cell extract protein was pre-incubated at 30°C for 10 min with aphidicolin, then used in repair synthesis reactions with a mixture of UV-irradiated plasmid DNA (+) and unirradiated control DNA (−). **A.** DNA isolated from repair reaction mixtures was visualized by ethidium bromide staining (top panel). Incorporation of radiolabelled nucleotides into this DNA was visualized by fluorography (lower panel). The final concentrations of aphidicolin in the reaction mixture are indicated. **B.** Repair synthesis plotted as fmol dAMP incorporated in irradiated DNA (■) and unirradiated DNA (□).

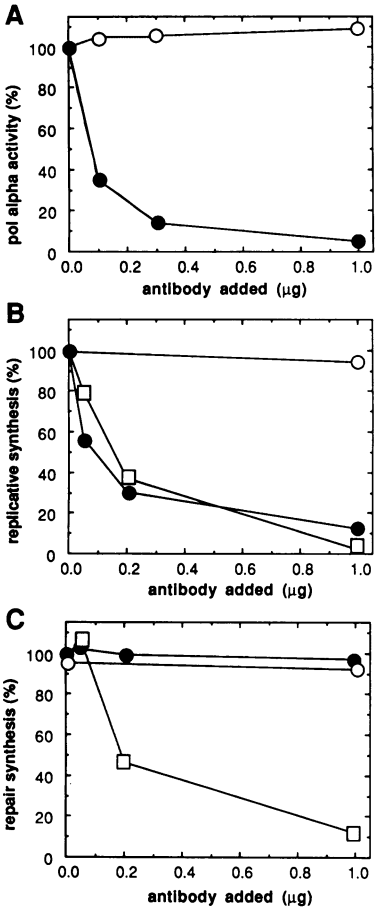

Figure 4. DNA excision repair synthesis does not require polymerase α catalytic activity. **A.** Effect of antibody on polymerase α activity. Reaction mixtures (50 μl) were assembled on ice and included monoclonal antibody SJK 132-20 (●) (directed against DNA polymerase α) or control anti-β galactosidase antibody BG2 (○), DNA polymerase α, DNAse I-activated calf thymus DNA, ^{3}H-dTTP and dNTPs in reaction buffer. After incubation for 20 min at 30°C, acid-insoluble radioactivity was determined. The data are expressed as the percentage of polymerase α activity compared to a reaction where no antibody was added (100% = 56 pmol dTMP incorporated). **B.** Effect of antibody on DNA replication. HeLa S-100 extract (100 μg of protein) and purified antibodies SJK 132-20 (●), BG2 (○), or a mixture of anti-HSSB 70A, 70B, 70C and 34A antibodies (□) were incubated at 4°C for 15 min in a volume of 14 μl. Reaction components for measurement of T antigen-dependent replicative DNA synthesis in pSV01ΔEP DNA were added as described (11) to give a final reaction volume of 30 μl. Following incubation at 37°C for 60 min, acid-insoluble radioactivity was determined. Results are plotted as the percent of synthesis in a reaction with no antibody where 40 pmol dTMP was incorporated. **C.** Effect of antibody on DNA repair. 0.05, 0.2 and 1.0 μg of the same preparations of antibodies SJK 132-20 (●), BG2 (○), or a mixture of anti-HSSB 70A, 70B, 70C and 34A antibodies (□) were pre-incubated with 100 μg of HeLa whole cell extract protein, which was then tested for repair synthesis activity. The amount of repair synthesis in the UV-irradiated plasmid is presented as a percentage of the synthesis obtained in the absence of antibody (100% = 115 fmol dAMP incorporated).

of HSSB with polymerase α and so it was of interest to perform experiments to ask more directly if polymerase α is involved in nucleotide excision repair *in vitro*.

DNA polymerase α activity is not required for repair synthesis *in vitro*

Of the known nuclear DNA polymerases, α, δ and ε are inhibited by aphidicolin (25), a drug that is thought to act by competing with deoxynucleotide binding sites on a polymerase-DNA complex (26). Nucleotide excision repair synthesis mediated by human cell extracts is sensitive to inhibition by aphidicolin (Fig. 3). Approximately 80% of repair synthesis in damaged DNA is inhibited by the drug. Some synthesis might be effectively aphidicolin-resistant because a polymerase may have a very low K_m for deoxynucleotides when synthesizing a short oligonucleotide from a primer (26). Alternatively, a small fraction of repair synthesis might be carried out by the aphidicolin-resistant DNA polymerase β (27, 28). We tested an anti-DNA polymerase β antibody for an effect on repair synthesis. The polyclonal rabbit antibody readily detected the 39 kDa polymerase and its 8 kDa single-stranded DNA binding domain (29) by immunoblotting (the antibody and polymerase were a gift of Dr. S.H.Wilson, NIH, Bethesda MD, USA). Up to 2 μg antibody had no significant effect on nucleotide excision repair synthesis mediated by 100 μg HeLa cell extract protein. This antibody also did not neutralize the activity of purified polymerase β on a poly (dA) oligo (dT) substrate in a reaction carried out according to Kumar et al. (29) and so we cannot definitively rule out an involvement of pol β in the repair process. The same antibody has, however, been reported to inhibit short-patch mismatch repair by HeLa cell extract (30). The present results nevertheless indicate that most nucleotide excision repair synthesis carried out by cell extracts is mediated by one or more of the aphidicolin-sensitive DNA polymerases α, δ and ε. This is consistent with *in vivo* experiments which show that nucleotide excision repair synthesis in cells is inhibited by aphidicolin (28, 31, 32).

The possible involvement of DNA polymerase α was investigated with the aid of monoclonal antibodies that are known to neutralize the activity of the human enzyme (20). Antibodies SJK 132-20 and SJK 287-38 were utilized and gave similar results. Data with SJK 132-20 are shown in Figure 4. As expected, both antibodies efficiently inhibited the ability of purified DNA polymerase α to carry out synthesis using activated calf thymus DNA as a template (Fig. 4A). More importantly, the antibodies inhibited the *in vitro* synthesis of DNA from the SV40 origin by HeLa cell extract in the presence of T antigen (Fig. 4B), consistent with the known role of polymerase α in this DNA replication reaction. However, similar amounts of the same antibody preparations did not significantly inhibit DNA repair synthesis carried out by HeLa cell extracts (Fig. 4C). This result suggests that the catalytic activity of polymerase α is not necessary for cell extract-mediated DNA excision repair synthesis.

This suggestion is in agreement with data obtained in intact and permeabilized cells, where studies with chemical inhibitors have likewise concluded that DNA polymerase α is not responsible for repair synthesis but instead that DNA polymerase ε or δ is responsible (33, 34, 35, 36). Experiments with one permeabilized human cell system have directly implicated DNA polymerase e in nucleotide excision repair synthesis (34, 37). Recent studies have revealed that the proliferating cell nuclear antigen (PCNA) is required for DNA nucleotide excision repair *in vitro* (38, 39). Since PCNA stimulates the activity of DNA polymerases δ (40, 41) and ε (12, 42), but not α, it seems probable that polymerase ε and/or δ is responsible for nucleotide excision repair synthesis in cell extracts.

Effect of HSSB on repair synthesis in extracts from XP cells

Supplementation of repair-proficient cell extracts with additional HSSB can increase the number of repair events in UV-irradiated DNA by 2–3 fold. This suggests that HSSB is one of the limiting

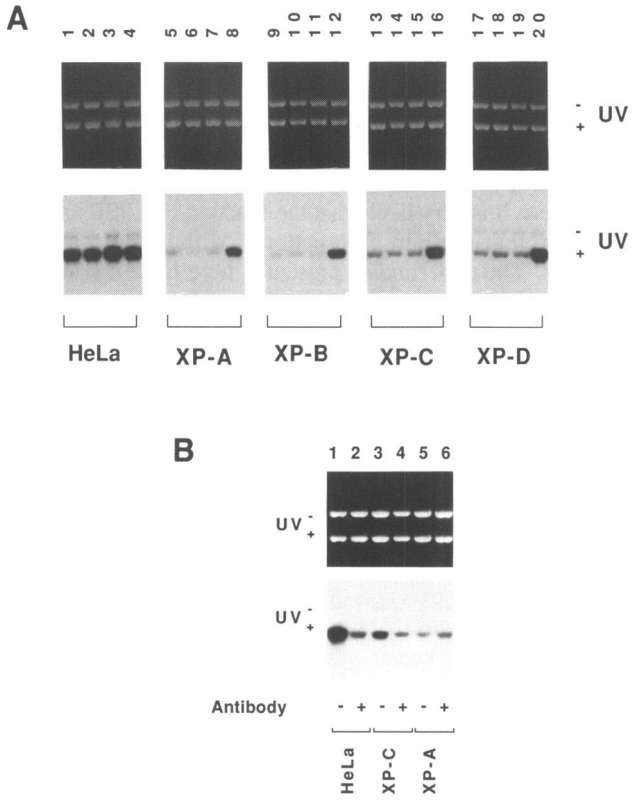

Figure 5. A. Effect of HSSB on XP cell extracts. 100 μg whole cell extract protein from HeLa cells or XP cell lines of complementation groups A (GM2345), B (GM2252), C (GM2249), D (GM2253) was supplemented with HSSB or *Micrococcus luteus* UV endonuclease before addition of plasmid DNA mixture and reaction buffer. Top panel, agarose gel of linearized plasmids. Bottom panel, DNA synthesis visualized by fluorography. Lanes 1,5,9,13,17: extracts alone. Lanes 2,6,10,14,18: extracts preincubated with 50 ng HSSB for 30 min at 37°C. Lanes 3,7,11,15,19: extracts preincubated with 1 μg HSSB for 30 min at 37°C. Lanes 4,8,12,16,20: extracts supplemented with 0.2 μg of *Micrococcus luteus* pyrimidine dimer-DNA glycosylase/AP lyase (Applied Genetics Inc., Freeport, NY). Addition of *M. luteus* enzyme completely bypasses the normal human incision system, and serves as a positive control for the presence of DNA synthesis activity in the extracts. **B.** Residual damage-dependent DNA synthesis resistant to inhibition by anti-HSSB antibody. 100 μg HeLa cell extract protein or extract protein from cells of XP complementation groups A (GM2345) and C (GM2249) was pre-incubated with 2 μg of anti-HSSB 70C antibody and then assayed for remaining repair synthesis activity.

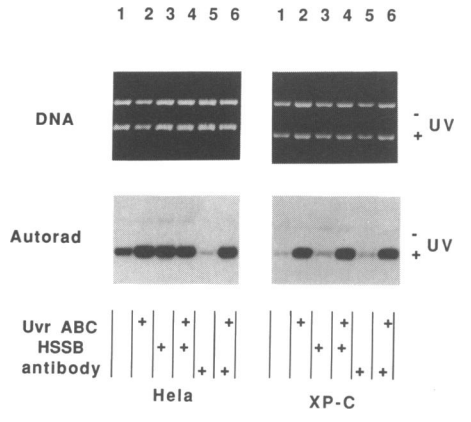

Figure 6. Repair of UV-irradiated DNA pre-incised by *E.coli* UvrABC does not require HSSB. 100 μg of cell extract protein prepared from HeLa or XP-C cells was pre-incubated with 1 μg HSSB and/or 2 μg 70C antibody, then tested for repair activity using UV-irradiated substrate DNA or this DNA pre-incised by *E.coli* UvrABC excision nuclease. Lanes 1, extract only with normal DNA mixture. Lanes 2, extract tested using UvrABC incised DNA mixture. Lanes 3, extract supplemented with HSSB. Lanes 4, extract supplemented with HSSB and tested using UvrABC incised DNA mixture. Lanes 5, extract pre-incubated with 70C antibody. Lanes 6, as lane 5 but tested using UvrABC incised DNA mixture.

components of the repair apparatus in normal cell extracts (5). We previously noted that HSSB did not stimulate repair synthesis in extracts from cells of XP complementation groups A, B, C, D, F, and G, indicating that the reduced repair in these groups is not caused by a defect in HSSB. Such experiments reveal a further relevant point. XP extracts show a small amount of synthesis that is greater in irradiated plasmid DNA than in non-irradiated DNA (Fig. 5A, lane 1 in each panel). This residual damage-dependent synthesis is not enhanced by the addition of amounts of HSSB that increase synthesis by a repair-proficient cell extract (Fig. 5A, lanes 3). What is the nature of the residual damage-dependent synthesis in XP extracts? If the XP mutations in these cell lines are leaky and allow a small amount of incision to take place, some of the residual synthesis could represent a low level of true excision repair. For XP-C and XP-D extracts this is a plausible explanation, since XP-C and XP-D cell lines generally exhibit >10% of normal repair synthesis by *in vivo* assays (43). However, the mutant alleles present in the XP-A and XP-B cell lines used here are examples of very tight mutations, with gene alterations that inactivate the protein function so that excision repair is undetectable *in vivo* (44, 45).

A more plausible reason for the lack of stimulation of synthesis in XP extracts by HSSB is that the residual damage-dependent DNA synthesis arises from an aberrant process that is not true nucleotide excision repair and is independent of HSSB. To test this idea, extracts from repair-proficient HeLa cells and from XP cells of complementation groups A and C were incubated with a saturating amount of neutralizing anti-HSSB antibody and then assessed for repair synthesis. This treatment reduced DNA repair synthesis by HeLa cell extract by 80%, from 380 to 80 fmol dAMP incorporated into UV-irradiated DNA (Fig. 5B, lanes 1 & 2). The XP-C extract incorporated 130 fmol dAMP in the absence of antibody, and 60 fmol with antibody (lanes 3 & 4), and no reduction in synthesis was seen with the XP-A extract (50 fmol dAMP with and without antibody, lanes 5 & 6). The level of antibody-resistant DNA synthesis was approximately equal for the normal and the two XP extracts. Thus, much of the residual damage-dependent repair synthesis carried out by XP extracts appears to arise by a mechanism that is independent of HSSB. One possibility is that a small amount of repair is initiated by an S1-type nuclease than can adventitiously nick UV-irradiated DNA.

The requirement for HSSB in excision repair can be bypassed by incising DNA with *E.coli* UvrABC enzyme

The data presented in Fig. 5 indicate that HSSB does not have an aspecific, general activity that can stimulate all types of repair synthesis. Further, we have noted that although HSSB can stimulate repair synthesis in normal cell extracts, it does so by increasing the number of repair events, while the repair patch size remains unchanged (5). Taken together, the data are consistent with the idea that instead of directly promoting DNA polymerization during repair, HSSB might be involved in the

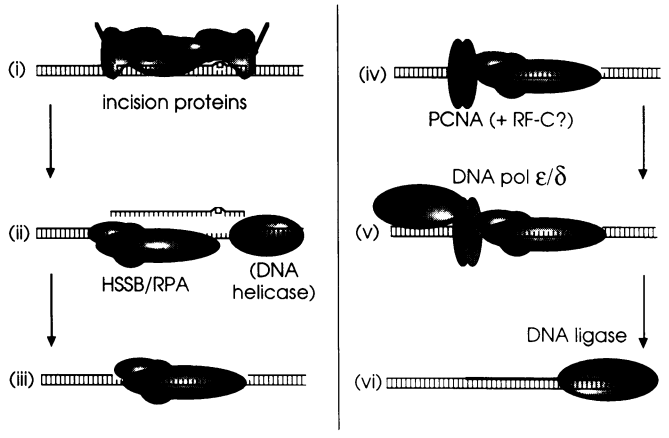

Figure 7. Model for nucleotide excision repair in mammalian cells. The model makes use of the data reported here and other recent information, and is revised and updated from that presented by Shivji *et al.* (38). (i) Incisions are introduced about 29 nucleotides apart (58) by an unknown number of proteins; one of them is probably the XP-A polypeptide (59). (ii) The oligonucleotide containing DNA damage, and the incision proteins are displaced. HSSB may aid in this process, possibly in concert with a DNA helicase. (iii) HSSB could protect the gapped region and the termini from being degraded. (iv) PCNA (possibly in conjunction with the RF-C protein) binds to an incision site on the 5′ side of the gap, perhaps mediated by specific protein-protein interactions with HSSB. (v) Controlled repair synthesis is carried out by DNA polymerase ε or δ. (vi) The repair patch is completed and sealed by a DNA ligase.

generation or stabilization of incisions in damaged DNA. In order to examine this possibility, we performed experiments to ask if HSSB is still required when the normal incision pathway is bypassed by an exogenously added prokaryotic repair enzyme.

The *E.coli* UvrABC enzyme has a well-characterized ability to incise damaged DNA by introducing two nicks flanking the damaged site, 12−13 nucleotides apart (46, 47, 48). We previously showed that when damaged plasmid DNA is incised by the cooperative action of the purified UvrA, B, and C proteins, human cell extracts (including XP extracts) can carry out DNA synthesis to complete the repair process (3). The repair synthesis initiated by these incisions results in patches that are confined to the region surrounding the DNA damage (49); J. Hansson and R.D. Wood, unpublished experiments). In Fig. 6, HeLa cell extract (left) or XP-C cell extract (right) was added to a mixture of damaged and nondamaged DNAs that had been previously incubated with UvrABC. As expected, damage-dependent DNA synthesis by both cell extracts was promoted by incising damaged DNA with UvrABC (lanes 1 and 2). In the absence of UvrABC, pre-incubation of HeLa cell extract with the antibody 70C neutralized HSSB in the extract and caused a marked reduction in DNA repair synthesis, as anticipated (Fig. 6, left, lane 5). However, a striking result was obtained in experiments where DNA was first incised by UvrABC and then added to extracts in which HSSB had been neutralized. Repair synthesis took place in damaged DNA, and the amount of synthesis was unaffected by the presence of antibody (Fig. 7, lanes 6). Thus the requirement for HSSB in excision repair was bypassed by first incising damaged DNA with UvrABC. This result suggests that HSSB normally functions in mammalian excision repair by participating in some aspect of the process responsible for controlled incision of damaged DNA. We propose several specific possibilities for this function below.

DISCUSSION

Any model for the role of HSSB in nucleotide excision repair should take into account the affinity of HSSB for single-stranded DNA (8, 13). However, the ability to bind single-stranded DNA is clearly not sufficient to fully explain the function, since two heterologous SSBs cannot substitute for HSSB in the overall repair process. It is very likely that the function of HSSB involves specific protein-protein interactions with other cellular components. The experiments presented here have investigated two general areas: interaction with DNA polymerases, and the generation of incisions in DNA.

Interaction of HSSB with DNA polymerases

The anti-HSSB antibodies 70A, 70B and 34A inhibit both SV40 viral DNA replication (11) and nucleotide excision repair synthesis *in vitro* (Fig. 4). In principle, the effect on DNA replication is adequately explained by the observation that these antibodies suppress the ability of HSSB to stimulate DNA polymerase α (9, 11). However, this does not appear to be the case for nucleotide excision repair of DNA, since the data presented here indicates that the catalytic activity of polymerase α is not required for repair synthesis *in vitro*. Thus, the anti-HSSB antibodies appears to affect some other aspect of HSSB function. HSSB can also stimulate the activity of polymerases δ and ε (9, 10, 11, 12), and we have summarized evidence suggesting that one or both of these polymerases carries out nucleotide excision repair synthesis in mammalian cells. Thus HSSB could interact with pol ε or δ during repair. One distinct possibility is that HSSB could bind to and stabilize the single-stranded region created by excision of nucleotides, and subsequently facilitate loading of the polymerase at the start of gap-filling synthesis. This could occur in cooperation with other protein factors, by analogy with the reactions that take place during SV40 DNA replication *in vitro*. In the replication reaction, HSSB cooperates with PCNA and RF-C to promote recognition of primed sites for leading strand synthesis by polymerase δ (50, 51). As noted above, PCNA is required for nucleotide excision repair synthesis *in vitro*.

Role of HSSB in lesion recognition or incision

When DNA was first incised by the *E.coli* UvrABC proteins, human cell extracts were able to perform repair synthesis even when HSSB in the extract had been neutralized (Fig. 6). This ability of UvrABC to bypass the requirement for HSSB in repair suggests that UvrABC has replaced one or more of the major functions of HSSB in repair. The UvrABC proteins are multifunctional and cooperate to recognize and bind to lesions, to introduce incisions flanking the damaged site, and to protect the nicks thereby generated until gap-filling synthesis can take place (46, 52, 53). Our data therefore suggest that HSSB might normally have a role in the generation or stabilization of incisions at damaged sites in DNA. In support of this, we recently found that stable incised intermediates can be generated with fractionated cell extract protein in the absence of PCNA, and that accumulation of these intermediates requires HSSB (38).

How might HSSB participate in the generation or stabilization of incised intermediates? It is conceivable that HSSB could aid in recognition of DNA damage by binding to short regions of single-stranded DNA caused by lesions that distort the double helix. Such a role has been suggested for the T4 gene 32 single-stranded DNA binding protein, where biochemical and genetic

evidence suggests that the gene 32 product participates in excision repair in bacteriophage-infected *E.coli* (54, 55). We have so far been unable to detect a difference in the binding of purified HSSB to UV-irradiated *vs.* unirradiated double-stranded closed circular plasmid DNA by a nitrocellulose filter binding assay (data not shown). However, HSSB might cooperate with other factors to recognize DNA lesions or to put a damaged region of DNA into a conformation suitable for incision. Incision at damaged sites may require unwinding of DNA around the lesion (46). HSSB is known to participate in an unwinding reaction in conjunction with the DNA helicase activity of T antigen during the initiation of SV40 DNA replication (9, 23, 56). Proteins with DNA helicase activity are strongly implicated in nucleotide excision repair in mammalian cells (45). Indeed, a DNA helicase which is stimulated by HSSB has recently been purified from human cell extracts (57). Nevertheless, there is no direct evidence for a role of HSSB in an unwinding reaction during DNA repair.

It seems likely that one function of HSSB in repair could be to bind to the single-stranded gap created by the mammalian excision system (58). By binding to DNA after incision, HSSB could facilitate recycling of the incision proteins, aided by specific protein-protein interactions. This could effectively increase the incision rate. Additionally, HSSB may protect the single-stranded region and the DNA termini generated by incision/excision proteins from degradation by other DNA processing enzymes. Bypassing the normal incision process by introducing UvrABC would obviate both the putative recycling and protective functions of HSSB, as the incised site could remain bound and protected by Uvr proteins until displaced by polymerase and helicase activities during the creation of a short repair patch. A model incorporating these features is shown in Fig. 7. HSSB is depicted as having a role in an early stage of repair, and polymerase δ or ϵ (but not α) is shown to be involved in the polymerization step. In addition, the model makes use of recent information from other sources which is consistent with the data presented here. The XP-A protein, which binds DNA (59) is likely to be one of the factors involved in lesion recognition or incision. We also incorporate the observation that repair patches mediated by human cell extracts are ~30 nucleotides long (5, 38, 49), and the recent experiments of Huang et al. demonstrating that the human nucleotide excision repair nuclease incises at the ~22nd phosphodiester bond 5′ to a cyclobutane thymine dimer and the ~6th phosphodiester bond 3′ to the dimer (58). Recently, HSSB/RP-A has been shown to have a binding site size on single stranded DNA of approximately 30 nucleotides (60), and so one heterotrimeric molecule of HSSB is depicted as bound to an excision gap. As demonstrated by Shivji et al. (38), the DNA polymerase accessory protein PCNA is involved in the synthesis step, possibly in association with RF-C. It seems likely that a multimeric form of PCNA is wrapped around the DNA during synthesis, by analogy with the functionally similar β-subunit of *E.coli* DNA polymerase III (61). Protein-protein interactions between PCNA, RF-C, HSSB, and a DNA polymerase (10, 11, 12) may then allow controlled gap-filling DNA synthesis followed by ligation to complete the repair process.

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