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DNA damage-dependent recruitment of nucleotide excision repair and transcription proteins to *Escherichia coli* inner membranes

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ABSTRACT

The entire process of nucleotide excision repair (NER) in *Escherichia coli* has been reconstituted *in vitro* from purified proteins and defined DNA substrates. However, how this system is organized *in vivo* is unclear. We report here the isolation and characterization of macromolecular assemblies containing NER and transcription proteins from *E.coli*. This ensemble consists of at least 17 proteins. They are recruited, as a consequence of DNA damage induced by UV irradiation, to the inner membrane. The UV-induced 6–4 photoproducts are also relocated to the inner membrane following UV-irradiation of the cells. This recruitment process is dependent on the *uvr*A, *uvr*C and *rec*A gene products. These results suggest that at least part of the repair process may associate with the inner membrane and also provide insights into understanding the cellular organization of repair processes.

INTRODUCTION

Nucleotide excision repair (NER), conserved in all organisms, is an ideal repair system responding to a wide spectrum of DNA damage. Its progression consists of five basic steps: damage recognition, incision, excision, resynthesis and ligation. The NER system in *Escherichia coli* has been well studied and is the best characterized DNA repair system (1–3). At least six gene products are involved in these pathways, including UvrA, UvrB, UvrC, UvrD (helicase II), DNA polymerase and ligase. UvrA protein is responsible for recognizing damaged sites (4) and for delivering UvrB to damaged sites (5). It is a DNA-independent ATPase and dimerizes in solution in the absence of ATP (6). The dimeric form of UvrA binds to damaged DNA more efficiently than to DNA that is undamaged (7). UvrB, which does not bind directly to DNA, interacts with UvrA–DNA to generate $UvrA_2B$–DNA complexes. Only when interacted with UvrA does a cryptic ATPase function of UvrB become active (8). This complex functions as a DNA helicase able to displace a short (22mer) complementing strand from a DNA duplex (9) and to supercoil relaxed circular DNA duplexes (10). The translocational capability of the $UvrA_2B$ was proposed to scan the DNA in search of damage (3). Once the damaged site is encountered, UvrB forms a stable complex with damaged DNA (5). UvrC, which has no specific affinity for either duplex DNA or UvrB, binds to UvrB bound to damaged DNA and then induces dual incision (11,12). The incised DNA–UvrBC complex requires the coordinated participation of UvrD and the concomitant gap filling by DNA polymerase I for the release of the damaged fragment and turnover of the Uvr proteins (13). The integrity of the interrupted strand is restored by the action of ligase.

Exposure of *E.coli* to DNA damaging conditions results in the induction of a pleiotropic set of physiological responses termed SOS (14,15). These responses, due to the induction of more than 17 structural genes under the control of the RecA and LexA regulatory proteins, include nucleotide excision repair (*uvr*A, *uvr*B and *uvr*D), UV-induced mutagenesis (*umu*DC), recombination (*rec*A, *rec*N, *rec*Q, *ruv*A and *ruv*B), inhibition of cell division (*sul*A and *sul*B), etc.

DNA repair in *E.coli*, as well as in lower and higher eukaryotes, occurs more rapidly in actively transcribed genes than in non-transcribed ones and is significantly faster in the transcribed strand than in the non-transcribed one (16–18). In *E.coli*, the phenomenon of strand specific repair was found to be dependent on a transcription-repair coupling factor (19) which is a product of the *mfd* gene. This protein presumably removes elongating RNA polymerase complexes stalled at the site of damage and recruits Uvr proteins to these sites through high-affinity interactions with UvrA.

Although the entire progression of NER in *E.coli* has been fully reconstituted *in vitro* with purified proteins and damaged plasmid DNA substrates, it is still unclear whether the *in vitro* system accurately reflects the complex nature that occurs *in vivo*. Our approach therefore, has been to isolate functionally active excision repair complexes from *E.coli* cells and to characterize their structural and functional properties to provide insights into understanding how this system is organized *in vivo*.

In this paper, we present evidence that Uvr proteins and DNA damaged sites are relocated to the inner membrane following UV irradiation of the cells. Further characterization of the DNA–membrane contacts reveals that a number of NER proteins together with proteins from the transcription machinery are recruited to these sites. The study of recruitment processes in different repair-related mutant strains reveals the outline of the NER pathway *in vivo*.

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MATERIALS AND METHODS

Bacterial strains

The following *E.coli* strains were used: wild-type strain MH1 [*araD139*, Δ(*ara,leu*)*7697*, Δ*lacX74*, *galU*, *galK*, *StrA*]; *uvrA* deletion mutant strain MH1 ΔA [*araD139*, Δ(*ara,leu*)*7697*, Δ*lacX74*, *galU*, *galK*, *StrA*, Δ*uvrA*]; *uvrB* deletion mutant strain N364 [w3110 *gal*$^+$, *sup*^{o}, F$^-$, Δ(*att-bio-uvrB*)]; *uvrC* deletion mutant strain N3024 [*uvrC279*::*Tn10*, IN(*rrnD-rrnE*)*1*]; *uvrD* deletion mutant strain SK7772 [*argH1*, *hisG4*, *ilvD188*, *malA1*, *xyl-7*, *str-281*, *supE44*, Δ*uvrD291*]; *recA* deletion mutant strain BNN124 [F$^-$e14$^-$ (mcrA$^-$), *hsd*R514(r_k^- m_k^-), *supE44*, *supF58*, *lacY1*, *galK2*, *galT22*, *metB1*, *trpR55*, Δ*recA*].

Bacterial growth, UV irradiation and radioactive labeling conditions

Escherichia coli cells were inoculated from overnight cultures and grown at 30°C in M9 medium at an initial OD_{600} = 0.05. To label chromosomal DNA or membranes, 2 μg/ml thymine and 10 μCi/ml [^{3}H]thymidine (Amersham, 48 Ci/mmol) or 0.2 μCi/ml [^{14}C]glycerol (Amersham, 158 mCi/mmol) were added to the culture, initially. To label proteins, 10 μCi/ml [^{35}S]sulfuric acid (NEN) was added to the culture 40 min before the cells were harvested. Cells were harvested at OD_{600} = 0.5–1.0 by centrifugation and resuspended in cold 0.01 M $MgSO_4$ (OD_{600} = 0.1). Cell suspensions (25 ml) were UV-irradiated (254 nm) by the germicidal lamp (15 W) in a 14 cm diameter Petri dish. Cells were UV-irradiated at 10 or 40 J/m^2 (0.1–0.4 cyclobutane dimers per kb of genome) measured with UV intensity meter (Hoefer). Irradiated cells were collected and resuspended in original M9 medium to continue incubation.

Western blot analysis and antibodies

For determination of the cellular levels of Uvr proteins, wild-type *E.coli* cells (MH1) were UV irradiated at 10 J/m^2. At selected post-UV stages, cells were harvested. Some cells were used to prepare cell extracts, the others were used to determine the number of filamented cells. Cell extracts were prepared as following: cells were pelleted by centrifugation and lysed in boiling loading buffer containing 1% SDS for 10 min. Following centrifugation of total cell lysates, cell debris was pelleted and the supernatant consisted of >95% of the Uvr proteins. The number of filamented cells was determined by serial dilution on agar plates. Cell extracts prepared from 5×10^8 filamented cells were subjected to SDS–PAGE (7.5% acrylamide separating gel). After electrophoresis, proteins were transfered on to a nitrocellulose membrane. The blots were probed with different anti-Uvr protein antibodies and developed using an Amersham ECL detection kit. The same basic procedures were applied for the cell fractionation experiments and for analysis of DM complexes. The following antibodies were used: anti-UvrA and UvrB monoclonal (20), anti-UvrC polyclonal, anti-RNAP subunits monoclonal (R.R.Burgess, University of Wisconsin), anti-DnaK polyclonal (R.McMacken, Johns Hopkins University), anti-RecA polyclonal (C.Radding, Yale University), anti-UvrD polyclonal (P.Modrich, Duke University), anti-DNA pol I monoclonal (S.Linn, University of California/Berkeley), anti-DNA gyrase subunit A and B (M.F.Gellert, NIH), anti-DNA pol III subunits polyclonal (C.McHenry, University of Colorado), anti-DNA topoisomerase I polyclonal (J.C.Wang, Harvard University) and anti-(6–4)-photoproduct (P.T.Strickland, Johns Hopkins University).

Cell fractionation

Wild-type *E.coli* cells (MH1) were UV-irradiated at 10 J/m^2. At selected post-UV stages, cells were harvested and resuspended in cold 0.75 M sucrose, 10 mM Tris–HCl buffer pH 7.8 (OD_{600} = 10) (21). Lysozyme (Sigma) was added to a final concentration of 100 μg/ml and the cells were incubated on ice for 2 min. The suspension was then slowly diluted with 2 vol of cold 1.5 mM EDTA, pH 7.5 to form spheroplasts. To lyse the spheroplasts, the suspension was poured slowly into 4 vol of cold 10 mM spermidine (Sigma) in H_2O and stirred for 10 min at 4°C. The lysates were centrifuged in the cold for 30 min at 10 000 *g* (Rotor SW 28), the supernatant consisted of the cytoplasmic protein fraction. The pellet was further treated with 25 μg/ml of DNase I (BRL) for 4 h. After further centrifugation (10 000 *g*, 30 min, SW 50.1 rotor), the membranes were pelleted and isolated as the membrane protein fraction. The supernatant consisted of the DNA-bound protein fraction.

Dimer excision assay

Dimer excision was measured by thin-layer chromatographic resolution of formic acid hydrolysates of a ^{3}H-labeled cellular DNA. The thymine and thymine dimer regions of TLC plates were counted. This procedure is essentially the same as introduced by Cook and Friedberg (22).

Immunoelectron microscopy

UV-irradiated (10 J/m^2) or unirradiated *E.coli* cells were fixed with 3% paraformaldehyde (Sigma) and 1% glutaraldehyde (Polysciences) in 30 mM phosphate buffer pH 7.0 for 1 h on ice (23). The samples were washed three times with phosphate buffer, once with 0.1 M sodium cacodylate trihydrate and then treated with 1% osmium tetroxide (Polysciences) for 1 h in the cold. The cells were washed in water, dehydrated in a graded ethanol (50%, 60%, 70% and 90%) for 10 min each and embedded in LR white resin (Polysciences) in gelatin capsules at 50°C for 24 h. Sections of 80–100 nm were cut and placed on nickel grids. For immunoreaction, antibodies were diluted in TBST/BSA (10 mM Tris, 500 mM NaCl, 0.05% Tween 20, 2% BSA, pH 7.2). The grids were incubated in 1% sodium metaperiodate in TBS (50 mM Tris, 150 mM NaCl, pH 7.4) for 15 min and then quenched for 15 min in 50 mM NH_4Cl. The grids were blocked with TBST/BSA for 30 min and then incubated with the anti-Uvr protein polyclonal antibodies diluted 1/1000 in TBST/BSA for 1 h at room temperature. For detection of UV-damaged sites, an anti-(6–4)-photoproduct monoclonal antibody (24) was used at 4°C overnight. The grids were washed in TBS and immersed in a 1/40 dilution of 5 nm colloidal gold particles conjugated to goat antibody to rabbit IgG (Amersham) for 1 h. The grids were washed in water and post-stained with 1% uranyl acetate solution and examined by electron microscopy at 60 kV.

Isolation of membrane-associated, folded chromosomes

Membrane-associated nucleoids were isolated by a modification of the method of Kornberg *et al*. (25). UV-irradiated cells were immediately harvested and resuspended in a solution containing 0.01 M Tris–HCl pH 8.0, 0.01 M NaCl and 20% sucrose (OD_{600} = 100). A 1/5 vol of lysozyme solution (4 mg/ml lysozyme, 0.12 M Tris–HCl pH 8.0 and 0.05 M EDTA) was added and cells were incubated for 1 min on ice to form spheroplasts. An equal volume of detergent solution (14 mM CHAPS, 10 mM spermidine

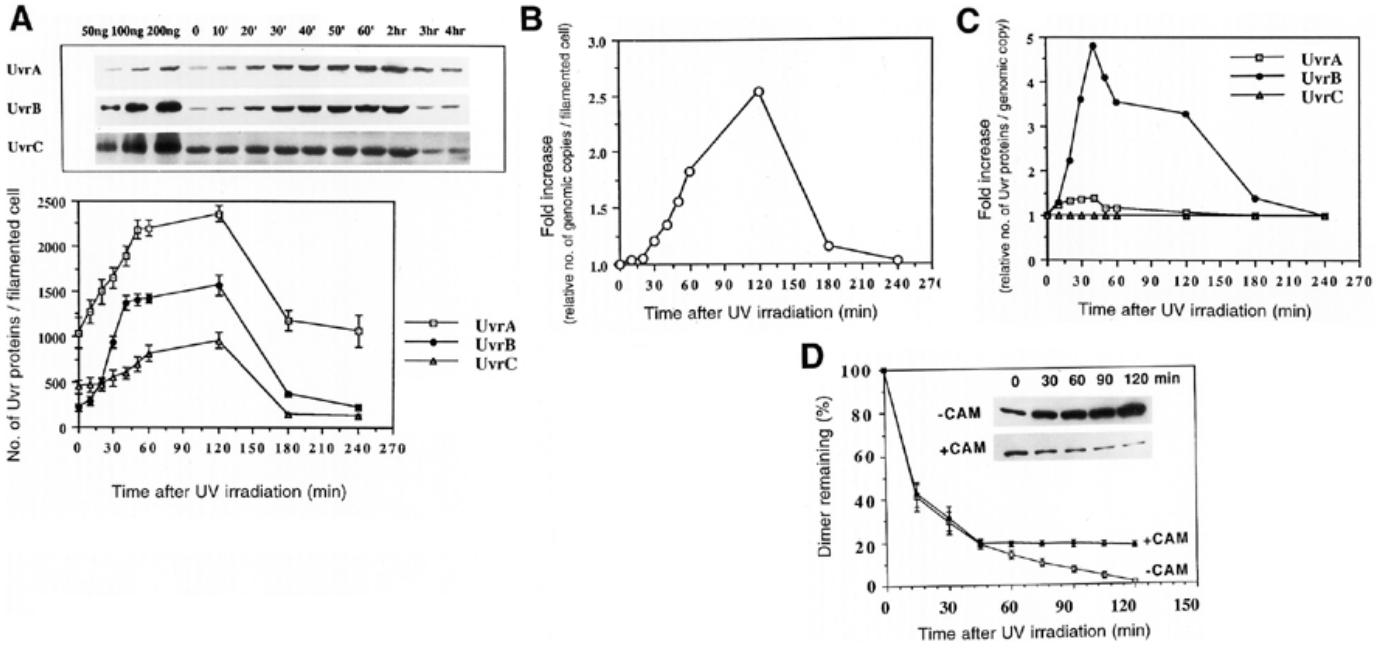

Figure 1. The cellular levels of the Uvr proteins and the removal of UV-damaged sites following UV irradiation. (**A**) Western blots of total cell extracts prepared from 5×10^8 filamented cells at different post-UV (10 J/m^2) intervals. The blots were probed with anti-UvrA, UvrB monoclonal and anti-UvrC polyclonal antibodies. The first three lanes were loaded with increasing concentrations of purified Uvr proteins (4). The levels of Uvr proteins per filamented cell were measured by comparison to known quantities of purified Uvr proteins by using a densitometer (AMBIS). The mean ± SEM was calculated from four independent experiments. (**B**) The relative number of genomic copies per filamented cell were determined by Southern analyses of *uvrA* gene copies. Isolated genomic DNA was digested with *Sma*I and subjected to a 0.7% agarose gel electrophoresis. The gel was dried and hybridized with a ^{32}P-labeled UvrA cDNA probe followed by autoradiography. The results were quantified by use of a densitometer and expressed as fold-increase. (**C**) The levels of Uvr proteins per genomic copy at different post-UV stages. (**D**) The rate of NER activity following UV irradiation was determined by measuring the removal of thymine-containing pyrimidine dimers from genomic DNA in the presence or absence of chloramphenicol (CAM). CAM was added to the medium 15 min prior to harvesting cells for UV irradiation. Inset shows the steady-state levels of UvrA with or without CAM at different post-UV intervals by western blotting with anti-UvrA monoclonal antibody. The mean ± SEM was calculated from four independent experiments. 100% = 367–582 ^{3}H c.p.m.

and 0.01 M EDTA) was then added to solubilized inner membranes at 4°C for 3 min. Lysates were subjected to centrifugation for 10 min at 9000 *g* (Rotor SW 50.1) through a 12–60% sucrose gradient (4.4 ml) which contained 0.01 M Tris–HCl, pH 7.4, 1 mM EDTA, 10 mM NaCl and 5 mM $MgCl_2$. The white, opalescent band containing DNA–membrane complexes (DM) was then removed by syringe.

RESULTS

Biosynthesis of Uvr proteins and their repair activity

As a first step toward the isolation of repair complexes, the scheduled biosynthesis of the Uvr proteins and the removal of damaged sites following UV irradiation of the cells were examined. Wild-type *E.coli* cells were exposed to 10 J/m^2 UV light (>95% of the cells survived), which resulted in the induction of SOS responses. One of the SOS responses is cell filamentation in which cells continue to grow during the post-UV period but fail to septate, generating filaments as a consequence (26). At 2 h post-UV, the level of filamentation reaches a maximum and the cells then start to divide, returning to their normal morphology at ~4 h post-UV (data not shown). The steady-state cellular levels of the Uvr proteins were monitored by the western blotting of total cell extracts prepared from the same numbers of filamented cells. Figure 1A shows that the levels of Uvr proteins per filamented cell increased until division occurred. The filamented cells may contain several genomic copies. To normalize the cellular levels of Uvr protein per genomic copy, the relative number of genomic copies per filamented cell were determined by the Southern analyses of *uvrA* gene copies. Figure 1B shows that the *uvrA* gene copies per filamented cell gradually increased to ~2.6-fold at 2 h post-UV. Therefore, the levels of UvrA and UvrB per genomic copy gradually increased to 1.5- and 4.8-fold, respectively, at 40 min and then started to decline (Fig. 1C). The level of UvrC per genomic copy remains constant over the period, as expected.

Eighty percent of the thymine dimers were removed in the first 30–40 min post-UV (Fig. 1D). The remaining thymine dimers were removed during the later period. When cells were allowed to repair their DNA in the presence of chloramphenicol (CAM) to inhibit SOS responses, only the later stages of repair activity were selectively inhibited. These results are consistent with earlier observations by Cooper (27). The above results led us to choose 10 min and 1 h into the post-UV period as adequate time points for the isolation of repair complexes.

The intracellular location of Uvr proteins

In order to establish procedures for isolating repair complexes, the intracellular localization of Uvr proteins was determined by cell fractionation. Wild-type *E.coli* spheroplasts were gently lysed by osmotic shock in the presence of spermidine. Following centrifugation of total cell lysates, membrane attached chromosomal DNA complexes and free membrane vesicles were pelleted; the supernatant fraction consisted primarily of cytoplasmic proteins. The pellet was then treated with DNase I to digest chromosomal DNA thereby

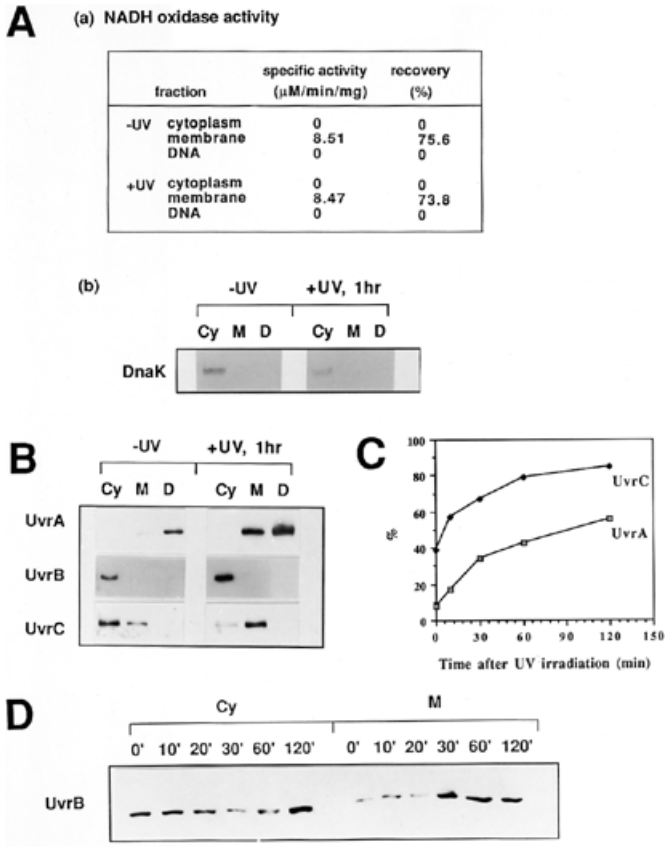

Figure 2. The intracellular location of Uvr proteins by cell fractionation. The cellular proteins were resolved into three major differential centrifugation fractions: cytoplasmic proteins (Cy), membrane proteins (M) and DNA-bound proteins (D). Wild-type *E.coli* cells were UV-irradiated at 10 J/m^2. (**A**) The control experiments: (a) NADH oxidase activity of each cellular protein fractions was measured by the method of Osborn *et al*. (22). (b) The location of DnaK protein in each cellular protein fraction were determined by western blotting with an anti-DnaK polyclonal antibody. The amount of protein loaded in each lane was based on the same number of cells (5×10^8). (**B**) The location of Uvr protein in each fraction was determined by western blotting with anti-Uvr proteins antibodies. (**C**) The percentage of total UvrA and UvrC in the membrane fraction at different post-UV intervals. (**D**) UvrB is found in the membrane fraction when isolated from Δ*uvrD* mutants. Western blot of cytoplasmic fraction (Cy) and membrane fraction (M) isolated from different post-UV stages of the Δ*uvrD* mutant. The blot was probed with an anti-UvrB monoclonal antibody.

releasing DNA-bound proteins. The membranes were then pelleted to yield the membrane protein fraction; the supernatant fraction consisted primarily of DNA-bound proteins. To monitor cross-contamination during this fractionation procedure, two specific markers were employed. NADH oxidase, an inner membrane protein (21), was used to locate the membrane protein fraction. The specific activity of this enzyme was found to locate exclusively in the M fraction (Fig. 2A, a). DnaK, a cytoplasmic heat shock protein (28), was used to identify the cytoplasmic protein fraction and was found primarily in this fraction (Fig. 2A, b).

The distribution of Uvr proteins in each fraction was examined by western blotting (Fig. 2B). In the absence of UV-irradiation, the majority of UvrA is located in the DNA-bound protein fraction and ~10% of UvrA was found in the membrane protein fraction. However, at 1 h post-UV the level of UvrA in the membrane protein fraction increased to ~40% of the total UvrA. UvrB was found only in the cytoplasmic protein fraction irrespective of UV-irradiation. Approximately 60% of the total UvrC was found in the cytoplasmic protein fraction in the absence of UV-irradiation; the remaining UvrC was found in the membrane protein fraction. However, at 1 h post-UV 60–70% of total UvrC is redistributed into the membrane protein fraction. The percentages of total UvrA and UvrC in the membrane protein fraction at different post-UV stages were further examined (Fig. 2C). At 10 min post-UV, UvrA and UvrC were found to increase in the membrane protein fraction to 2- and 1.5-fold, respectively. The extent of relocation of Uvr proteins to the membrane protein fraction increases as a function of post-UV incubation.

The preferential location of UvrB in the cytosolic protein fraction was unexpected based on the formation of $UvrA_2B$–DNA and UvrB–DNA *in vitro* (5,9). This may, however, represent the direction of the steady-state equilibrium determined by the level of repair at that experimental point in time. It has been shown that UvrD, DNA polymerase I and dNTPs are required for turnover of the UvrABC endonuclease from damaged sites *in vitro* (13). Hence, Δ*uvrD* mutants should imitate the same block to excision repair trapping UvrB in DNA in this mutant. Figure 2D shows that UvrB was located in the DNA–membrane protein fraction in Δ*uvrD* mutants.

These results were further confirmed by immunoelectron microscopy with conventional fixation and embedding procedures. Ultrathin *E.coli* cell sections were labeled with polyclonal anti-Uvr antibodies. The location of antibody–Uvr protein complexes was visualized with electron dense colloidal gold particles (Fig. 3A). To verify the specificity of the gold particle signals, the following control experiments were executed: (i) wild-type cell sections were directly incubated with gold labeled secondary antibody in the absence of the primary antibody; (ii) wild-type cell sections were incubated with primary antibody pre-adsorbed to excess antigen and (iii) deletion mutant (Δ*uvrA*, Δ*uvrB* or Δ*uvrC*) cell sections were labeled along with wild-type cell sections. Insignificant levels of gold label were found in these control experiments (data not shown). The quantitative analyses of the distribution of each gold-labelled antibody from the cell membrane to the cellular midline was examined. The change in distribution (%) resulting from UV-irradiation for each gold-labelled antigen is shown in the histograms (Fig. 3B). Consistent with the subcellular fractionation results, anti-UvrA and anti-UvrC gold signals were found to preferentially redistribute into the membrane region in UV-irradiated cell sections.

It is notable that the majority of anti-UvrA gold signals are not associated with the bulk DNA (30) but seem to be localized in an area of the cell where metabolically active DNA is located judging by the presence of RNA polymerase, DNA topoisomerase I and HU (29). In addition, the chromosome conformation in UV irradiated wild-type cell sections is seemingly dispersed with more observable chromosomal DNA–membrane contacts than in non-irradiated cells. This is consistent with the early observation by Kellenberger (30). However, this chromosome conformational change is not observed in UV-irradiated Δ*uvrA* cell sections (data not shown) suggesting that it is a UvrABC specific repair process.

The cellular location of UV-damaged sites in chromosomal DNA was also visualized by labeling with anti-(6–4)-photoproduct antibodies. It was found that anti-(6–4)-photoproduct gold signals are enriched at the membrane region on the UV-irradiated wild-type cell sections (Table 1). This effect was not observed in UV-irradiated Δ*uvrA* cell sections. This is consistent with the observation of the absence of chromosomal dispersion in this mutant following irradiation.

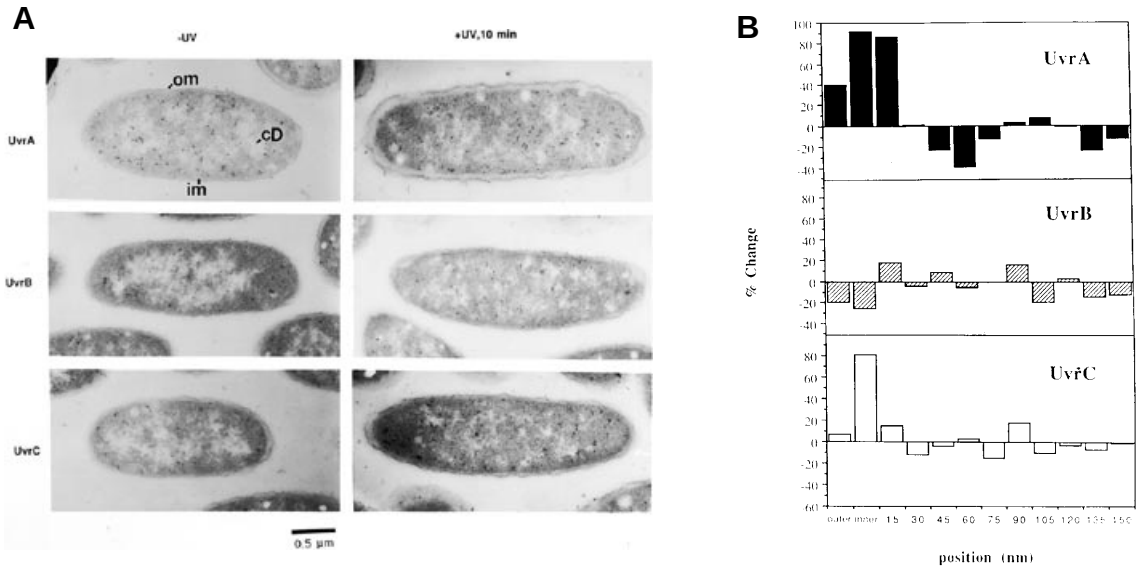

Figure 3. The cellular location of Uvr proteins by immunoelectron microscopy. (**A**) Ultrathin wild-type *E.coli* cell sections, –UV and +UV, were labeled with anti-UvrA, UvrB or UvrC polyclonal antibodies and the location of antibody–Uvr protein complexes was visualized with colloidal gold particles. +UV cell sections were irradiated at 10 J/m^2 and post-UV incubation carried out for 10 min. cD, chromosomal DNA; im, inner membrane; om, outer membrane. (**B**) The percent change in cellular distribution resulting from UV-irradiation for each gold-labeled antibody. EM negative films were magnified by projector (Charles Beseler) on the digitizer (Scriptel) and defined successive 15 nm wide zones from the inner membrane to the approximate midpoint of cell sections. The number of particles in each defined zone and at the membrane was counted. The percent change in mean value was calculated from 30 to 40 cell sections of approximately the same area (–UV, 17 ± 0.6 μm^2; +UV, 21 ± 1.1 μm^2) for each antibody.

Table 1. 6–4 photoproduct lesion labeling experiments

	Wild-type			Δ*uvrA*	
	–UV	+UV,0 min	+UV,10 min[a]	+UV,20 min	+UV,10 min
Total no. of cell examined[b]	133	141	128	117	124
No. of cells containing gold particle	7 (5%)	82 (58%)	51 (40%)	36 (31%)	60 (49%)
Total no. of gold particles	9	117	75	58	79
No. of gold particles located at: inner membrane[c]	2 (22%)	28 (24%)	47 (63%)	40 (69%)	20 (25%)
DNA	7 (78%)	89 (76%)	28 (27%)	18 (31%)	59 (75%)

[a]Cell were UV-irradiated at 10 J/m^2 and incubated for 10 min.
[b]All cells that were in focus on the EM negative films were analysed as described in Figure 3 unless the cell envelope was not detectable.
[c]Gold particles located at the membranes or 15 nm away from either side of the membranes were considered as membrane-associated.

Isolation of membrane-associated, folded chromosomes

The above results suggest that at least part of the repair apparatus and the damaged DNA sites may relocate to the cellular membrane during repair. A routine procedure was established (illustrated in Fig. 4A), as a consequence, to isolate membrane-associated, folded chromosomes. This procedure was modified from that reported by Kornberg *et al.* (25). UV-irradiated *E.coli* spheroplasts were gently lysed by the non-ionic detergent, CHAPS, in the presence of spermidine. It has been reported (31) that outer membranes are resistant to non-ionic detergents, while inner membranes are readily solubilized by them. However, the inner membrane proteins stabilized by the DNA–membrane interaction at chromosomal membrane attachment sites are protected from extraction by non-ionic detergents such as CHAPS (32). Hence, this treatment should preserve the repair protein complexes associated with membrane and folded chromosomal DNA. These DNA–membrane (DM) complexes were further enriched by centrifugation on sucrose gradients in the presence of Mg^{++}. More than 95% of the chromosomal DNA from the lysates was recoverable at very high DNA concentrations. The proteins remaining in the applied lysate include cytoplasmic proteins and CHAPS-soluble inner membrane proteins. A number of control experiments were performed when establishing this isolation procedure. CHAPS has no effect on incision in reconstituted *in vitro* systems with purified Uvr proteins and damaged plasmid DNA, even at CHAPS concentrations as high as 100 mM. The steady state levels in which UvrA and UvrC are associated with DM complexes are consistent with results from cell fractionation studies where CHAPS was not used. Buffer conditions, including pH, ionic strength and type of buffer, were optimized in which the distribution of Uvr proteins was followed by western blotting.

The composition of the DM complexes from wild-type *E.coli* cells was followed in chromosomal DNA with [^{3}H]thymidine, the proteins with [^{35}S]sulfuric acid and the membrane with [^{14}C]glycerol. DM complexes were isolated at varying post-UV stages. It was found that the sedimentation rate of the DM complexes increased as a function of post-UV incubation time reaching a maximum at 2 h. The sedimentation rate then started to decline returning to its original

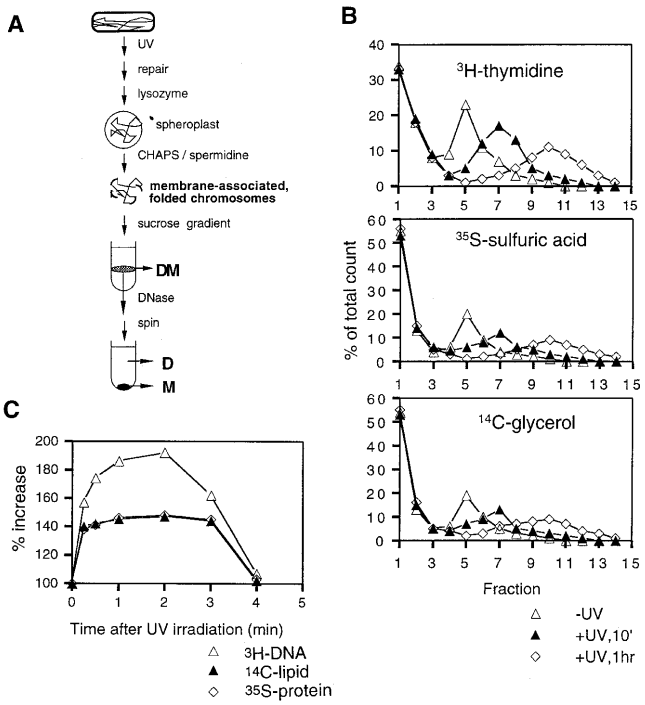

Figure 4. The isolation and characterization of membrane-associated, folded chromosomes. (**A**) The schematic procedure for isolating the membrane-associated, folded chromosomes. DM, DNA–membrane complexes; D, DNA-bound proteins fraction; M, DNA–membrane contact proteins and outer membrane proteins fraction. (**B**) The sedimentation rate of the isolated nucleoids increases as a function of post-UV incubation. The chromosomal DNA of wild-type *E.coli* cells was labeled with [methyl-^{3}H]thymidine, protein with [^{35}S]sulfuric acid and membrane with [^{14}C]glycerol. Cells were UV-irradiated at 10 J/m^2 and incubated in the original medium containing the radioactive substrates. At selected post-UV incubation times, the cells were harvested for isolation of membrane–associated nucleoids. After sucrose gradient centrifugation, 300 µl of each fraction was collected from the sucrose gradient and radioactivity was measured (fraction nos 1–15 from top to bottom of the tube). (**C**) An increase in the DNA–membrane contacts following UV-irradiation. The D fractions and M fractions were isolated as described in Materials and Methods. The percentage increases in ^{3}H-DNA, ^{35}S-protein and ^{14}C-lipid (c.p.m.) in the M fraction were measured as a function of post-UV incubation.

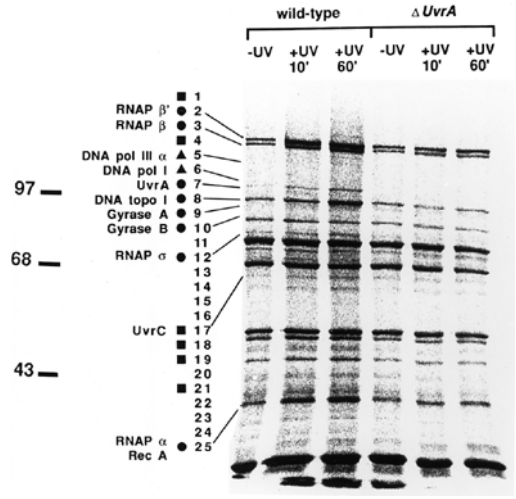

Figure 5. The individual ^{35}S-labeled proteins in the DNA–membrane contacts. Wild-type and Δ*uvrA* mutants of *E.coli* cells with [^{35}S]sulfuric acid-labelled protein were UV-irradiated at 10 J/m^2. UV-irradiated cells were incubated in the original medium containing radioactive substrate. At selected post-UV incubation times, cells were harvested for M fraction isolation. Samples of ^{35}S-labeled M fraction protein (equivalent amounts of radioactivity) were analyzed by SDS–PAGE (7.5% acrylamide separating gel). Individual bands were quantified by a PhosphorImager (Fuji). Twelve proteins identified by western blotting with different antibodies are indicated on the left of panel. Filled circles, proteins increase in the ratio of 1:2.5:5 (–UV:+UV,10 min:+UV,60 min); filled squares, proteins increase in the ratio of 1:1.5:2; filled triangles, proteins are not clearly observed in gel but can be detected by western blotting.

level 4 h post-UV (Fig. 4B shows up to 1 h). This phenomenon coincides kinetically with the SOS induction pathway. However, the increased sedimentation rate as a consequence of UV irradiation was not observed in those DM fractions isolated from Δ*uvrA* mutants. It has been shown that the conformational changes in nucleoid structure are reflected by their rate of sedimentation during centrifugation (33,34). The chromosome conformational changes observed by electron microscopy in wild-type cells are also not evident in Δ*uvrA* mutants. This indicates that the chromosome conformational changes during repair result in an increase in the sedimentation rate through a UvrA-dependent process.

Composition of DNA–membrane contacts

To identify those repair proteins in the isolated DM complexes, the DM complexes were purified and then digested by DNaseI to release the specific DNA-bound proteins. After centrifugation, the membranes were pelleted. Such pellets contain the DNA–membrane contact proteins and outer membrane proteins (M fraction); the supernatant fraction contains the DNA-bound proteins (D fraction) (Fig. 4A). When the amount of ^{3}H-DNA, ^{35}S-proteins and ^{14}C-glycerides were measured in each fraction as a function of post-UV incubation time, they were found to immediately increase in the M fraction, reaching a maximum at ~2 h after which the tracer levels started to decline (Fig. 4C). This profile reflects the SOS induction schedule implying that: (i) there is an increase in DNA–membrane contacts after UV irradiation which is consistent with the EM results; (ii) this increase in DNA–membrane contacts results in chromosome conformational changes reflected in an increase in the sedimentation rate of DM complexes; and (iii) UvrA is one of the factors required for this process to take place.

The individual ^{35}S-labeled proteins in the M fraction were resolved by SDS–PAGE and challenged with specific antibodies (Fig. 5). There are at least 17 proteins recruited to the M fraction as a function of post-UV incubation in a UvrA-dependent manner. They were grouped into three categories based on the degree of recruitment. Group 1 includes at least nine proteins which increase according to the ratio of 1:2.5:5 (–UV:+UV,10 min:+UV,60 min). This group included UvrA, RecA, RNA polymerase subunits α, β, β′ and σ, DNA topoisomerase I and DNA topoisomerase II subunits gyrase A and B. DNA polymerase I was localized in the M fraction only after UV-irradiation. DNA polymerase III subunit α was found in the M fraction only at +UV,60 min. The level of DNA polymerase I and DNA polymerase III subunit α in the M fraction are relatively low (not clearly observed in gel but can be detected by western blotting). Group 2 includes at least six proteins which increase according to the ratio of 1:1.5:2, respectively. One of them was identified as UvrC. Group 3 proteins are non-inducible and may be outer membrane proteins (32). UvrB and UvrD are not found in the M fraction when isolated from wild-type cells.

Table 2. Characterization of DM complexes[a] isolated from different mutants

	Strains wild-type	Δ*uvrA*	Δ*uvrB*	Δ*uvrC*	Δ*uvrD*	Δ*recA*
increase of sedimentation rate	yes	no	yes	no	yes	no
proteins recruited						
UvrA	2×↑	NR	UC	UC	2.5×↑	UC
UvrB	NR	NR	NR	NR	↑	NR
UvrC	1.25×↑	UC	UC	NR	1.5×↑	UC
RNAPα	2×↑	UC	1.5×↓	UC	2.5×↑	UC
RNAPβ	2×↑	UC	1.5×↓	UC	2.5×↑	UC
RNAPβ′	2×↑	UC	1.5×↓	UC	2.5×↑	UC
RNAPσ	2×↑	UC	2.5×↑	UC	2.5×↑	UC
DNA topo I	2×↑	UC	3×↓	1.5×↓	UC	UC
Gyrase A	2×↑	UC	2×↑	UC	UC	UC
RecA	2×↑	UC	UC	UC	2.5×↑	NR

[a] DM complexes were isolated at 10 min post-UV.
UC, present but unchanged; NR, non resident; ↑, fold increase; ↓, fold decrease.

These findings were substantiated in wild-type *E.coli* cells which overexpressed UvrA from an inducible promoter. In such cells there was no accumulation of UvrA in the M fraction following induction in the absence of UV-irradiation suggesting there is no non-specific contamination of the membranes by vesicular entrapment. The levels of recruitment of these proteins into the M fraction are UV dose-dependent up to 60 J/m^2. Furthermore, in the presence of 0.4% phenylethyl alcohol, a membrane-specific drug which dissociates DNA–membrane complexes (35), there is neither protein recruitment nor thymine dimer excision despite normal SOS induction of Uvr protein synthesis.

Characterization of DM complex isolated from different repair-related mutants

The increase in the nucleoid sedimentation rate as a consequence of UV irradiation reflects an increase in repair-dependent DNA–membrane contacts. It was found that there is no increase in the nucleoid sedimentation rate as well as protein recruitment in Δ*uvrA*, Δ*uvrC* or Δ*recA* mutants (Table 2), suggesting that the formation of the repair-dependent DNA–membrane contacts is dependent on these gene products. In the absence of UvrB (Δ*uvrB* mutant), the ability of the damaged DNA to relocate to the membrane is unaffected. This is based on the increase in the nucleoid sedimentation rate. It is possible that under these circumstances DNA–membrane contact complexes cannot be formed properly. The functional role of UvrD in NER appears to be the turnover of the UvrABC incision complex. In the absence of UvrD (Δ*uvrD* mutant), there is a greater than normal nucleoid sedimentation rate as well as protein recruitment. Importantly, some UvrB is localized in the DNA–membrane contacts.

DISCUSSION

The experiments presented in this paper provide insights into the overall process of *E.coli* NER *in vivo*. The data established the following points. First, there is an increase in DNA–membrane contacts following UV irradiation of the *E.coli* cells. Second, repair proteins together with proteins from the transcription machinery are recruited to the DNA–membrane contact points. Third, UV-induced 6–4-photoproducts are also relocated to the membrane, Fourth, this recruitment is dependent on the *uvrA*, *uvrC* and *recA* gene products.

A comparison of steady-state levels of Uvr proteins with the kinetics of nucleotide excision repair (Fig. 1) revealed that 80% of UV-induced thymine dimers are removed rapidly from genomic DNA by constitutive Uvr proteins. The remaining thymine dimers are belatedly excised by the nascent Uvr proteins induced as part of the SOS response. Early repair seems to be the short patch repair type which occurs immediately after irradiation by constitutive repair systems (36). Late SOS-induced repair seems to be the long patch repair type that is controlled by the RecA–LexA regulatory circuit (27). The cellular levels of Uvr proteins were determined by quantitative western blotting. In contrast to a previous report (2), we found the cellular level of UvrA in the uninduced state is significantly higher than that of UvrB and that the induction rate of UvrB is higher than that of UvrA (Fig. 1A and C). However, the method used to determine the data in the literature has not been published nor confirmed (37). The method we used here is a direct immunological one. The results of western blotting are also supported by immuno-electron microscopy studies (Fig. 3A).

From the results of subcellular fractionation (Fig. 2) and immunoelectron microscopy (Fig. 3 and Table 1) studies, it appears that Uvr proteins and the damaged portions of DNA are relocated to the inner membrane during the repair period. The dispersion of chromosomal DNA during repair (Fig. 3A) was also observed when cells are treated with other DNA damaging agents including alkylating agents and ionizing radiation (38–40). This indicates that it is a repair process and not a radiation-induced artifact. Furthermore, the chromosome conformational change and the relocation of damaged DNA to the membrane are not observed in Δ*uvrA* cell sections. This indicates that it is a UvrA-dependent repair process and not an artifact generated during the cell fixation process.

The procedure employed for isolating *E.coli* membrane-associated nucleoids (Fig. 4A) was modified from the procedure established and characterized by Worcel and co-workers (25,41). The sedimentation rate of the isolated nucleoid on sucrose gradients was found to increase as a function of post-UV incubation time (Fig. 4B). This reflects the conformational changes in nucleoid structures (34) during the repair period. This is consistent with the EM observations (Fig. 3A). The increase in ^{3}H-DNA, ^{35}S-protein and ^{14}C-lipid in the M fraction after UV

irradiation (Fig. 4C) reflects an increase in the repair-dependent DNA–membrane contact points during the repair period.

Further characterization of the DNA–membrane contacts (M fraction) reveals that at least 17 proteins are recruited to these sites (Fig. 5). Unexpectedly, the four subunits of the RNA polymerase (β, β′, α and σ) are recruited to this repair-dependent DNA–membrane contact point. The cellular levels of RNA polymerase are not under SOS control. These and other findings from this laboratory support the notion of a direct molecular coupling between DNA repair and transcription. The recruitment of RecA to the DNA–membrane contact point is consistent with the earlier findings that RecA relocates to the membrane during SOS in *E.coli* (42,43). That the yeast RecA protein, HsRad51 protein, is recruited to the nuclear membrane as a consequence of damage suggests that this may be a general process (44). The *mfd* gene product was identified as a transcription-repair coupling factor (21). Lacking anti-Mfd antibodies does not allow for identification of Mfd in the M fraction although one band with a similar mass of Mfd (130 kDa) is detected in the complex. That DNA polymerase I was found in the M fraction after UV irradiation suggests that the repair resynthesis step may also occur on the cellular membrane.

Translocation of the $UvrA_2B$ complex along the DNA in search of damage has been suggested from *in vitro* studies (3). The damage recognition step has been shown to be the rate-limiting step *in vivo* (45). Therefore, we would expect to see some UvrB localized on the chromosomal DNA. However, UvrB was limited to the cytoplasmic fraction when isolated from the wild-type cells. This probably reflects the equilibrium state of UvrB in the repair process. UvrB was found in DNA–membrane contact points (M fraction) but not in the DNA fraction when isolated from Δ*uvrD* mutants. This suggests that the incision step may occur in the inner membrane. In support of this finding, Todo and Yonei (46) showed that phenylethyl alcohol, which dissociates DNA–membrane complexes, inhibits the incision step.

Why should the repair complexes fasten to the membrane? One speculation is that during early repair two events occur on the chromosomal DNA, excision repair and transcription. Damaged DNA is recognized by coupling to transcription and then relocates to the membrane where repair occurs. However, some specific genes or DNA regions may be selected for repair by the SOS-induced long-patch repair pathways. During the SOS period many events occur on the chromosomal DNA including excision repair, recombination repair, transcription, replication and mutagenesis. These individual events may have to cooperate with each other in order to function. The fluid property of the cell membrane provides an excellent matrix allowing these events to be accomplished. In teleological terms, recruitment of many different proteins involved in a common process such as repair linked to both transcription and replication provides for a localization of proteins in a cellular system with no specific organelles.

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