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A method to monitor replication fork progression in mammalian cells: nucleotide excision repair enhances and homologous recombination delays elongation along damaged DNA

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ABSTRACT

The capacity to rescue stalled replication forks (RFs) is important for the maintenance of cell viability and genome integrity. Here, we have developed a novel method for monitoring RF progression and the influence of DNA lesions on this process. The method is based on the principle that each RF is expected to be associated with a pair of single-stranded ends, which can be analyzed by employing strand separation in alkali. This method was applied to examine the rate of RF progression in Chinese hamster cell lines deficient in ERCC1, which is involved in nucleotide excision repair (NER), or in XRCC3, which participates in homologous recombination repair, following irradiation with ultraviolet (UV) light or exposure to benzo(a)-pyrene-7,8-diol-9,10-epoxide (BPDE). The endpoints observed were cell survival, NER activity, formation of double-strand breaks and the rate of RF progression. Subsequently, we attempted to explain our observation that cells deficient in XRCC3 (irs1SF) exhibit enhanced sensitivity to UV radiation and BPDE. irs1SF cells demonstrated a capacity for NER that was comparable with wild-type AA8 cells, but the rate of RF progression was even higher than that for the wild-type AA8 cells. As expected, cells deficient in ERCC1 (UV4) showed no NER activity and were hypersensitive to both UV radiation and BPDE. The observation that cells deficient in NER displayed a pronounced delay in RF progression indicates that NER plays an important role in maintaining fork progression along damaged DNA. The elevated rate of RF progression in XRCC3-deficient cells indicates that this protein is involved in a time-consuming process which resolves stalled RFs.

INTRODUCTION

The mechanism by which replication forks (RFs) stalled as a consequence of DNA damage are rescued in eukaryotic cells has not yet been fully elucidated. In the present study, we describe an assay procedure which can be used to monitor RF progression. Employing this procedure, we have examined the rate of RF progression in repair-deficient cell lines following UV irradiation and treatment with benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE), which gives rise to pyrimidine dimers (1) and N^2-guanine adducts (2), respectively. If not processed by nucleotide excision repair (NER), such DNA lesions block the replication machinery (3), due to the inability of the replicative DNA polymerases to deal with such distortions in DNA.

Bypass of DNA lesions in connection with replication has been suggested to occur either by translesion synthesis (TLS) or by homologous recombination repair (HRR), both of which play important roles in maintaining cell viability (4) and DNA integrity and in avoiding genomic instability (5). TLS involves low-fidelity polymerases that can perform replication across damaged sites (6). *In vitro*, several such polymerases are capable of bypassing different types of DNA lesions with varying specificities.

For example, polymerase eta copies dimers resulting from exposure to UV radiation more rapidly than it copies undamaged DNA (7). Furthermore, this polymerase exhibits higher fidelity than do other DNA polymerases involved in TLS in the presence of most UV-induced adducts (8–11). On the other hand, the presence of BPDE adducts completely blocks the action of polymerase eta (12). In contrast, polymerase kappa can perform relatively error-free replication across BPDE adducts (13–15), but is completely blocked by pyrimidine dimers (16). To date, little is known concerning the role and fidelity of these polymerases with regards to bypass replication of various DNA distortions in intact cells.

HRR is an alternative pathway for rescuing stalled RFs (17–20). It has been proposed that when leading-strand synthesis is blocked due to the presence of DNA damage, lagging-strand synthesis may continue for a short distance along the non-damaged strand, which can then be employed as a template for synthesis of the leading strand. Since replication bypass of unrepaired DNA lesions provides an opportunity for a second round of DNA repair by NER during the next cell cycle, bypass by TLS or HRR is generally considered to represent defenses against toxicity. Consequently, it is

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predicted that cells deficient in either of these mechanisms will demonstrate enhanced sensitivity to the toxic effects of DNA-damaging agents.

In the present investigation, we have examined the role played by NER and HRR in responses to DNA damage caused by UV radiation or BPDE in terms of acute toxicity, formation of double-strand breaks (DSBs) and RF progression using cells deficient in these two repair processes, i.e. the Chinese hamster ovary (CHO) cell lines UV4 and irs1SF, which are deficient in NER (ERCC1) and HR (XRCC3), respectively.

Here, we demonstrate that both NER and HR have important roles during RF progression along DNA, which has been damaged by UV or BPDE.

MATERIALS AND METHODS

Cell lines

The AA8, UV4 and irs1SF cell lines are all obtained from L. Thompson, LLNL (Livermore, CA). The cell lines were cultured in minimum essential medium containing Dulbecco's salt, with the addition of 9% fetal calf serum and 90 U each penicillin and streptomycin per ml (DMEM), at 37°C and under humidified air containing 5% CO_2. The UV4 cell line is deficient in the ERCC1 protein, which together with XPF forms an endonuclease that incises the damaged strand on the 5′ side of DNA lesions (21,22). The irs1SF cell line is deficient in the XRCC3 protein, a Rad51 paralog required for homologous recombination in mammalian cells (23–30).

Determination of cell survival

Cell survival was determined by employing a clonogenic assay. In the case of UV exposure, 10^6 cells were seeded onto 100 mm tissue culture dishes. For exposure to BPDE [(+)-7α,8β-dihydroxy-9α-10α-oxy-7,8,9,10-tetrahydrobenzo[a] pyrene, purity >99% (NCL Chemical Repository, Kansas City, MO)], 5×10^5 cells were seeded into each 25 cm^2 culture flask.

Twenty-four hours after plating, AA8 (wild-type), UV4 (ERCC1) and irs1SF (XRCC3) cells were exposed to different doses of UV or BPDE. The exposure to UV radiation was performed on ice in 5 ml phosphate-buffered saline using a Philips 15W TUV source at a dose-rate of 0.12 J/m^2 sek^{-1}, followed by incubation in DMEM at 37°C under 5% CO_2. The BPDE treatment was carried out in Hank's balanced salt solution for 1 h at 37°C under 5% CO_2. Following these treatments, the cells were harvested by trypsinization and 500 cells were re-seeded in duplicate onto 100 mm dishes. After 16 days of undisturbed incubation in DMEM at 37°C under 5% CO_2, the resulting colonies were fixed, stained with methylene blue, which was dissolved in methanol (4 g/l) and counted.

Alkaline DNA unwinding (ADU)

The extent of strand breakage was examined in cells inoculated previously onto 24 well plates (1×10^5 cells per well), and labeled with ^{3}H-TdR by different procedures. Following this labeling, and with or without exposure to our DNA-damaging conditions, cells were rinsed twice with 0.15 M NaCl prior to adding 0.5 ml ice-cold unwinding solution containing 0.15 M NaCl and 0.03 M NaOH. After 30 min on ice in darkness, the unwinding process was terminated by forceful injection of 1 ml of 0.02 M NaH_2PO_4.

Thereafter, the molecular weight of the DNA fragments was further reduced to ~3 kb by sonication (Branson sonifier B-12, equipped with a micro tip) for 15 s. Following the addition of SDS to give a final concentration of 0.25%, the 24 well plates were stored overnight at −20°C. Subsequently, the separation of single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) was accomplished on hydroxyapatite columns mounted in an aluminum block maintained at 60°C.

For this purpose, the samples were thawed at room temperature and diluted with an equal volume of distilled water. Prior to use, all columns were washed with 2.5 ml of 0.5 M potassium phosphate followed by 4.5 ml of 0.01 M sodium phosphate (pH 6.8) at a flow rate of 0.3 ml/min, in order to remove any DNA remaining from previous samples. Subsequently, 1.5 ml cell lysate was loaded onto each column, followed by two times washing with 4.5 ml 0.01 M sodium phosphate (pH 6.8). ssDNA and dsDNA were then eluted with 4.5 ml of 0.13 M and 0.25 M potassium phosphate (pH 6.8), respectively, and collected in separate vials for quantitation of radioactivity in a liquid scintillation counter.

For the determination of strand breaks in the genome overall, the ratio of ssDNA to dsDNA was used to quantitate the number of single-strand breaks (SSBs) present per cell by comparison with a standard curve obtained using γ-irradiation (31,32). Thus, SSBs/cell = $k_s \times (-\log \text{Fds})$, where k_s is the constant obtained from the standard curve and Fds = fraction of dsDNA = dsDNA/(ssDNA + dsDNA).

Assay of NER activity

The capacity of the CHO cell lines to perform NER incisions (which give rise to SSBs) was determined by inhibiting the polymerization step involved in NER, according to Erixon and Ahnstrom (32). Briefly, cells radiolabeled with ^{3}H-TdR (7.4 kBq/ml) for 24 h were chased in media for 1 h, followed by incubation with the inhibitors hydroxyurea (HU, 2 mM) and cytosine arabinocide (AraC, 20 μM) in DMEM for 30 min prior to exposure to different doses of UV or BPDE. Finally, subsequent to a 1 h post-treatment with HU/AraC, the number of SSBs present per cell was measured employing the ADU technique described above.

Determination of the rate of RF progression

Analysis of RF progression was also based on the ADU procedure as described above. The principle involved (illustrated in Figure 3) is that each RF is expected to be associated with a pair of single-stranded ends. A total of 2×10^5 cells were seeded into each well of 24 wells plates and cultured as described above for 24 h prior to pulse labeling. Labeling of RFs was subsequently achieved by incubation with ^{3}H-TdR (37 kBq/ml) in DMEM for 30 min at 37°C under 5% CO_2. Thereafter, exposure to DNA-damaging conditions was performed as described above. As depicted in Figure 3, inhibition of fork progression by the presence of DNA lesions results in a larger fraction of the radioactivity being recovered in the ssDNA. The progress of the fork away from the labeled area will be delayed by the lesions. Bypass or repair of damaged DNA during the elongation process results in more

radioactivity being recovered in dsDNA. The relative levels of labeled ssDNA and dsDNA were determined using the ADU technique described above and expressed as percentage of ssDNA.

Detection of DSBs by pulsed-field gel electrophoresis (PFGE)

Each culture vessel received 4×10^6 cells 24 h prior to treatment with UV irradiation or BPDE. As positive controls treatment with γ-irradiation or HU was employed. At various time points following such treatment, the cells were harvested by trypsinization and 10^6 cells then melted into each agarose insert, as described previously (33).

Subsequently, these agarose inserts were incubated in 0.5 M EDTA containing 1% *N*-laurylsarcosyl, 1 mg/ml proteinase K at 50°C for 48 h, followed by four washes in TE buffer and loading onto 1% agarose (chromosomal grade) gels. The DNA in these samples was then subjected to PFGE for 24 h (120° angle, 60–240 s switch time, 4 V/cm) (Bio-Rad CHEF III). Finally, in order to visualize and quantitate the DNA, these gels were stained with ethidium bromide and analyzed using Image Gauge software (FLA-300; Fujifilm).

RESULTS

The ability to perform both HR and NER is required in order to counteract the lethal effects of bulky DNA lesions

First, we addressed the question as to whether the enhanced sensitivity of cells deficient in XRCC3 to UV irradiation is also observed with other agents that produce bulky adducts. In agreement with the reports of others (34,35), cells deficient in NER (ERCC1) and HRR (XRCC3) were both found here to be hypersensitive to UV irradiation (Figure 1A). In the present investigation, similar results were obtained with regards to treatment with BPDE (Figure 1B). The UV dose required for 37% survival of the wild-type AA8 cells was 7-fold higher than that required to kill the same proportion of NER-deficient UV4 cells; while approximately nine times less BPDE was required for 80% survival of the UV4 cell line. In the case HR-deficient irs1SF cells, the toxicity of UV radiation was elevated 5-fold, while that of BPDE was enhanced 7-fold.

Cells with impaired NER are expected to exhibit severely attenuated survival after treatment with agents that produce DNA damage that is normally repaired by this pathway. However, the increased sensitivity of the XRCC3-deficient cells observed is not fully understood. The sensitivity of XRCC3-deficient cells to UV and BPDE is not as pronounced as that of cells deficient in ERCC1. This enhanced sensitivity of XRCC3-deficient cells to agents producing bulky adducts is in accordance with our previous findings that HR is induced by both UV and BPDE (36,37), and indicates that, in addition to NER, HR is involved in defending against the lethal effects of such agents.

Cells deficient in XRCC3 possess the same level of NER activity as wild-type cells

Since NER plays an important role in protecting cells against the lethal effects of UV radiation and BPDE, we addressed the possibility that XRCC3 deficiency might also be associated with a reduction in the capacity for NER. Wild-type and XRCC3-deficient cells were equally efficient in recognizing and incising DNA lesions resulting from exposure to either UV radiation or BPDE treatment and both cell lines demonstrating saturation at the same level of incisions following a UV dose of 10 J/m^2 or exposure to ~5 μM BPDE (Figure 2). This indicates that XRCC3 plays no direct function in maintaining NER capacity. As expected, the ERCC1-deficient cells were incapable of initiating repair of the DNA lesions induced by either UV light or BPDE. It should be noted that the AA8 and irs1SF cell lines employed here are largely deficient in repair of cyclobutane pyrimidine dimers (CPDs), but active in repairing the (6-4)-photoproduct [(6-4)PPs] (38,39). Thus, the results obtained relate primarily to the incision step involved in NER of the (6-4)PPs, which the UV4 cells are completely incapable of carrying out.

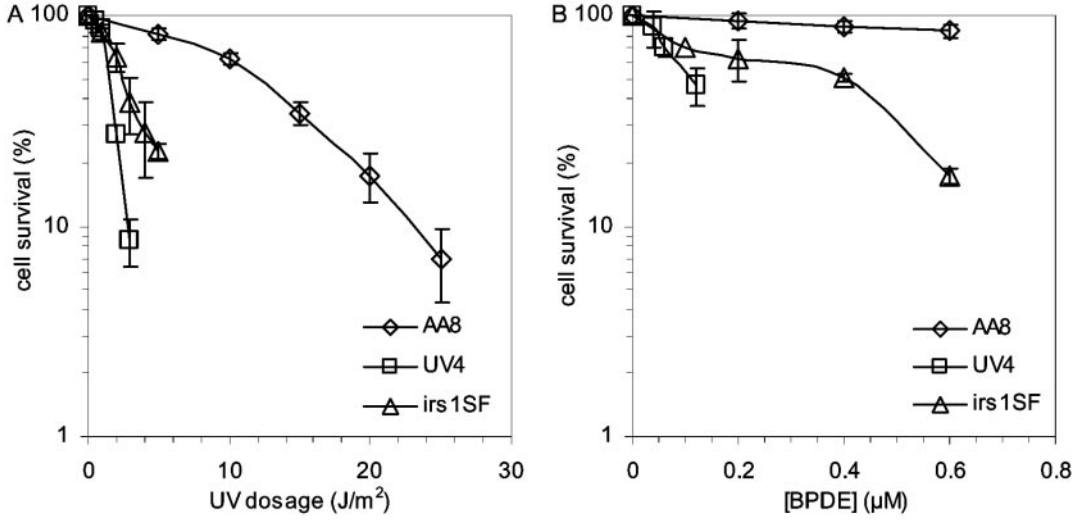

Figure 1. Survival following (**A**) UV irradiation or (**B**) exposure to BPDE of the cell lines AA8 (open diamonds), UV4 (open squares) and irs1SF (open triangles). Survival was plotted as the log of the percentage of living cells and the standard errors indicated are based on 2–4 independent experiments.

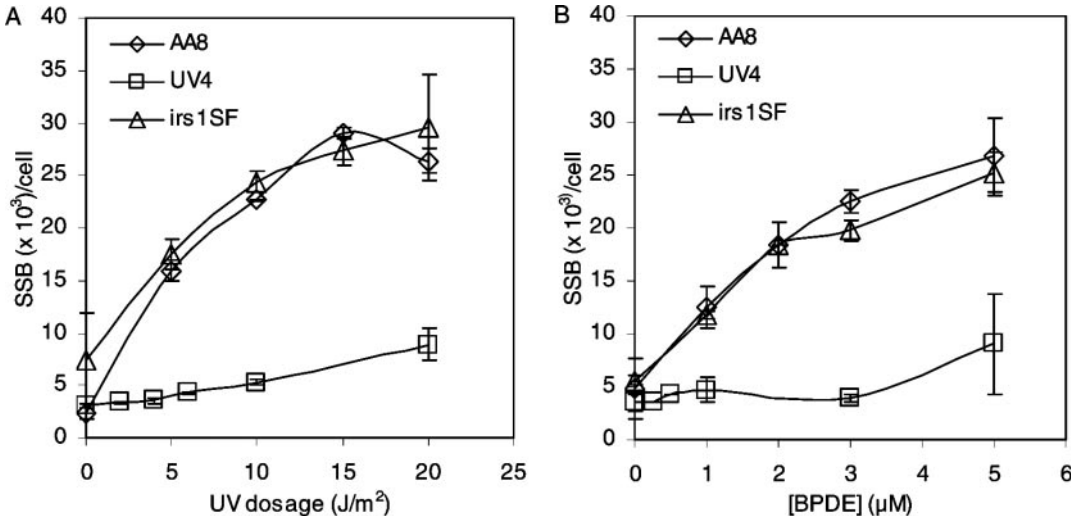

Figure 2. The effect of UV irradiation (**A**) and exposure to BPDE (**B**) on the rate of incision step involved in NER in the cell lines AA8 (open diamonds), UV4 (open squares) and irs1SF (open triangles). The standard errors indicated are based on two independent experiments.

Cells deficient in ERCC1 have prolonged RF progression whereas cells deficient in XRCC3 have enhanced RF progression after DNA damage

The results concerning NER described above raised the question as to whether a deficiency in replication bypass, rather than in repair of damaged DNA, could explain the enhanced sensitivity of XRCC3-deficient cells to UV radiation and BPDE. In order to address this question, we examined RF progression using a modification of the ADU approach (32,40–43). The principle underlying this procedure is that each RF provides a pair of single-stranded ends which function as starting points for DNA unwinding (Figure 3). Cells pulse-labeled with tritiated thymidine were exposed immediately thereafter to our DNA-damaging conditions and the kinetics of RF progression monitored as a reduction in the percentage of ssDNA (Figure 4). This approach measures fork elongation only in replicons that have already initiated. As expected, all three cell lines indicated the same kinetics for conversion of ssDNA to dsDNA along undamaged DNA.

Our previous studies allow calculations, suggesting (32) that the alkaline treatment employed resulted in unwinding of ~60 kb of DNA. When the progression of such RFs was analyzed after different periods of time, it could be estimated that ~60 kb of DNA is synthesized during 30 min of pulse-labeling in agreement with other studies (44,45). This was thus the fork elongation distance that could be measured here.

Exposure to UV at an intensity of 15 J/m^2 inhibits replication to different extent in the cell lines examined. Under these conditions, wild-type AA8 cells required 3 h to reach halfway the background level of dsDNA, whereas the corresponding value for UV4 was >6 h and in the case of irs1SF cells <2 h. A highly similar pattern was obtained after these three cell lines were treated with BPDE. When untreated, all the cells displayed the same rate of RF progression of about 30 min.

The severe delay observed in RF progression in treated NER-deficient cells (Figure 4) suggests that this process plays an important role in maintaining fork progression. On the other hand, RF progression in XRCC3-deficient cells exposed to UV irradiation or BPDE was more rapid than in the corresponding wild-type cells (Figure 4), suggesting that HRR is a relatively time-consuming process, in agreement with the findings by others (46). In the case of UV radiation, the difference in the delay of RF progression in AA8 and UV4 cells reflects repair of the (6-4)PPs. Together with the observation that three times as many CPDs as (6-4)PPs are induced by such treatment (47), this suggests that bypassing CPDs occurs considerably more rapid than bypass of (6-4)PP.

Exposure to agents that produce bulky adducts does not lead to the formation of DSBs

In connection with HR, three possible substrates have been proposed, i.e. DSB, collapsed RFs or stalled RFs (48). Here, the formation of DSBs was investigated after the treatment of our cell lines with UV radiation or BPDE, also employing treatment with HU or γ-irradiation as positive controls. HU was chosen because it induces DSBs only during replication, whereas γ-irradiation induces DSBs at all stages of the cell cycle (49).

As shown in Figure 5, DSBs could be detected in all three of our cell lines following treatment with HU or γ-irradiation, in the latter case immediately after exposure and were later repaired. On the other hand, replication was, as expected, required in order to generate DSBs for HU. This was also predicted to be the case for any formation of DSBs induced by UV or BPDE. However, as also shown in Figure 5, no DSBs were found over background level in any of the cell lines at any time point after exposure to UV radiation or BPDE. At a dose of 15 J/m^2, each RF is expected to encounter a dimer. If these events all produced a DSB, then several thousand DSBs per cell would occur. The detection limit of the PFGE method used here is approximately 100 DSB/cell (33,49). Thus, <5% of blocked forks form DSBs. We have demonstrated earlier that HR can restore RFs stalled by treatment with thymidine without the formation of DSBs (49), which thus may also be the situation with regards to bulky lesions encountered during replication.

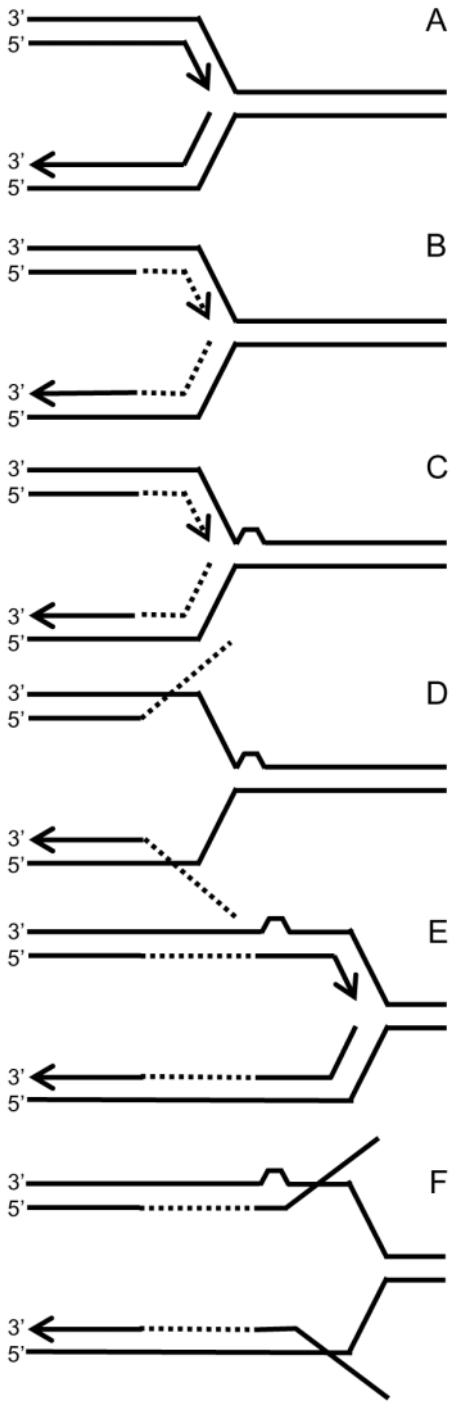

Figure 3. Schematic illustration of the principle involved in analysis of the rate of RF progression using the ADU procedure. This procedure is based on determination of interruptions (SSBs) in cellular DNA employing strand separation in alkali. The level of strand breaks is expressed as the ratio of ssDNA to dsDNA (see the legend to Figure 2). (**A**) The method applied to study RF progression, where each RF is expected to provide two single-stranded ends. (**B**) Labeling of forks with a 30 min pulse treatment with ^{3}H-TdR. (**C**) Exposure to DNA damaging conditions which may cause a block in DNA replication. (**D**) Inhibition of fork progression by DNA lesions results in recovery of most of the radiolabeled DNA in the single-strand fraction. (**E**) After different periods of delay, the fork may progress, resulting in movement of the radiolabeled segment away from the fork. (**F**) Enhanced levels of radiolabeled dsDNA are expected to result from bypass or repair of damaged DNA during the elongation process.

DISCUSSION

Both UV- and BPDE- induced DNA lesions are candidates for repair by NER (50–52). Since this pathway plays an important role in protecting cells from the lethal effects of UV radiation and BPDE, we have addressed here the possibility that XRCC3 deficiency might also be associated with a reduced capacity for NER. However, XRCC3-deficient cells exhibit the same capacity for NER incision as wild-type cells (Figure 2), indicating that this protein is not directly involved in the NER process.

The presence of stalled RFs may explain why XRCC3-deficient cells demonstrate enhanced susceptibility to the lethal effects of UV radiation and BPDE in comparison with wild-type cells (Figure 1), especially in light of our previous findings that both of these agents induce enhanced levels of HR in hamster cells (36,37). However, cells deficient in HR carry out RF progression at a higher rate than wild-type cells (Figure 4), suggesting that the presence of a functional XRCC3 slows down fork progression. From this result, it is not possible to conclude what mechanism is causing the enhanced rate of RF progression along damaged DNA when XRCC3-deficient cells are observed. In speculation, one intriguing possibility is that XRCC3 is involved in the reversal of the fork and formation of a 'chicken foot' structure, which is time consuming.

Furthermore, our data support recent findings that cells deficient in XRCC3 demonstrate enhanced fork progression following UV radiation or cisplatin, indicating that HRR is an active process that requires a certain amount of time (46). The space and time required for the NER complex to process damaged DNA during replication might be provided by an XRCC3-dependant regression of the RF. A similar model, which proposes that recombination proteins maintain the integrity of the RF such that lesions can be repaired by NER, has been proposed for replication repair in *Escherichia coli* (53). Recruitment of NER in front of forks is further supported by the observation that repair of lesions induced by UV light in non-active genes is enhanced during late S-phase (54).

Alternatively, the elevated sensitivity of XRCC3-deficient cells to UV radiation and BPDE could be due to an accumulation of toxic intermediates at stalled RFs as a consequence of abortive attempts to perform HRR. However, no DSBs were detected in irs1SF cells following exposure to these agents. When the RF is stalled, a Holliday junction (HJ) may be formed (20,55), which leads to fork regression and a 'chicken foot' structure (56), with participation of XRCC3 or any of the other Rad51 paralogs.

Recent evidence indicates that XRCC3 not only facilitates loading of the Rad51 protein onto DNA (57–59), but also participates in the late stages of resolution of the HJ (60). However, such a situation may generate DSBs, which were not observed here. This leads us to speculate in the possibility that initiation of HR might facilitate performance of NER in front of the fork. Indeed, interplay between HR and ERCC1, in the absence of any formation of DSBs, has been observed in connection with repair of DNA interstrand cross-links during replication (61,62). In addition, exposure to thymidine induces HR without the formation of DSBs (49). Thus, taken together, the evidence suggests that repair or bypass involving HR during replication does not necessarily involve the formation of DSBs.

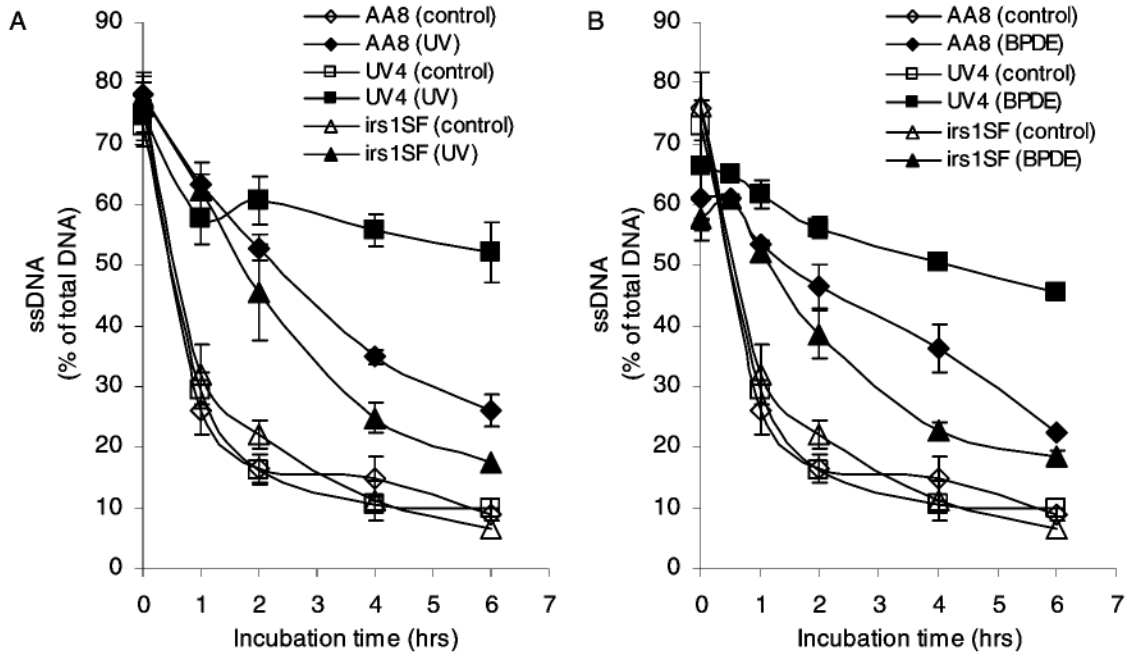

Figure 4. Time-course of the effect of (**A**) UV irradiation (15 J/m^2) or (**B**) exposure to BPDE (0.6 μM) on RF progression in the cell lines AA8 (diamonds), UV4 (squares) and irs1SF (triangles). Control and treated cells are indicated by the open and closed symbols, respectively. The standard errors indicated are based on two or three independent experiments.

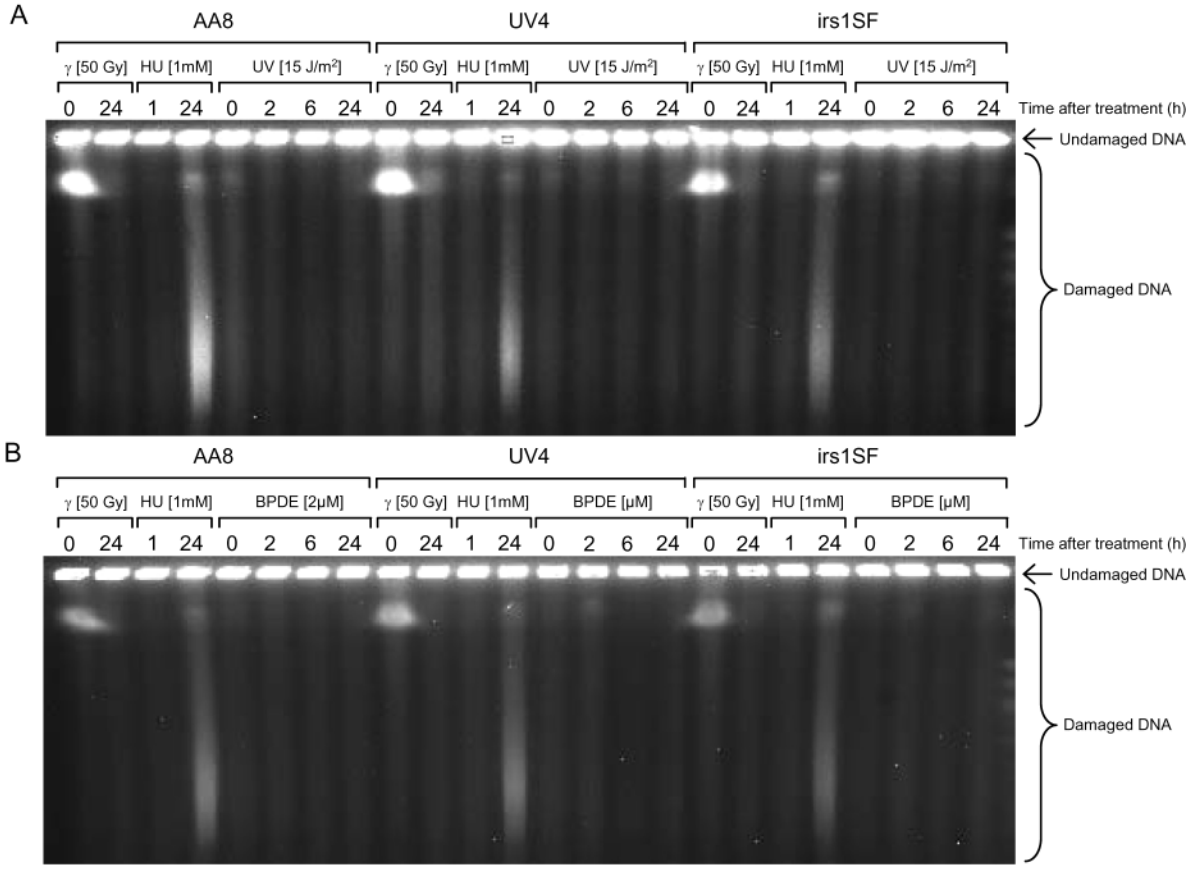

Figure 5. Lack of formation of DSBs, in AA8, UV4 and irs1SF cells, as a consequence of exposure to (**A**) UV radiation and (**B**) BPDE treatment. DSBs were analyzed by PFGE and HU and γ-irradiation used as positive controls, resulting in S-phase-dependent and S-phase-independent formation of DSBs, respectively.

In conclusion, we have developed the ADU procedure here in such a manner as to allow measurement of RF progression in repair-deficient cell lines. Employing this method, we have provided independent support for the findings that cells deficient in HR exhibit an enhanced rate of replication across lesions other than those produced by UV radiation (46); we demonstrate the same effect with BPDE-induced lesions suggesting that the presence of bulky adducts is a relevant parameter for the effect on RF progression.

Finally, it should be strongly emphasized that although our present observations in mammalian cells can be explained by the model proposed by Courcelle and Hanawalt (53) for bacteria, further studies on the interplay between HR and NER in both prokaryotes and eukaryotes are required to confirm the suggested model.

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