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Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable *in vivo* DNA double-strand breaks

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ABSTRACT

Homologous recombination (HR) deficient cells are sensitive to methyl methanesulfonate (MMS). HR is usually involved in the repair of DNA double-strand breaks (DSBs) in *Saccharomyces cerevisiae* implying that MMS somehow induces DSBs *in vivo*. Indeed there is evidence, based on pulsed-field gel electrophoresis (PFGE), that MMS causes DNA fragmentation. However, the mechanism through which MMS induces DSBs has not been demonstrated. Here, we show that DNA fragmentation following MMS treatment, and detected by PFGE is not the consequence of production of cellular DSBs. Instead, DSBs seen following MMS treatment are produced during sample preparation where heat-labile methylated DNA is converted into DSBs. Furthermore, we show that the repair of MMS-induced heat-labile damage requires the base excision repair protein XRCC1, and is independent of HR in both *S.cerevisiae* and mammalian cells. We speculate that the reason for recombination-deficient cells being sensitive to MMS is due to the role of HR in repair of MMS-induced stalled replication forks, rather than for repair of cellular DSBs or heat-labile damage.

INTRODUCTION

The DNA alkylating agent methyl methanesulfonate (MMS) has been used for many years as a DNA damaging agent to induce mutagenesis and in recombination experiments. MMS modifies both guanine (to 7-methylguanine) and adenine (to 3-methlyladenine) to cause base mispairing and replication blocks, respectively (1). DNA damage caused by alkylating agents is predominantly repaired by the base excision repair (BER) pathway and DNA alkyltransferases (2). The sensitivity of cells to MMS also increases significantly when other DNA repair pathways are compromised. For example, in *Saccharomyces cerevisiae* disrupting the homologous recombination (HR) pathway by mutating genes in the *RAD52* epistasis group significantly increases sensitivity to MMS (3). The sensitivity of HR mutant cells to MMS has lead to this agent being called an ionizing radiation mimetic or a DNA double-strand break (DSB) agent, for many years including in some recent publications. More direct evidence that MMS causes DSBs comes from analyses of yeast chromosomes by pulsed-field gel electrophoresis (PFGE), in which treatment with low levels of MMS leads to fragmentation of yeast chromosomes (4,5). Furthermore, MMS induces both inter-chromosomal and intra-chromosomal recombination (6). Other *S.cerevisiae* genes with a wide range of functions are also known to protect the cell from MMS-induced damage. Interestingly, *MMS1* to *MMS5* and *MMS22* were originally identified in a screen for genes affecting MMS sensitivity but not sensitivity to ionizing radiation (7). Thus, the *MMS* genes are unlikely to be required for DSB repair in general. *RAD52* is epistatic to both *MMS1* and *MMS4* for MMS sensitivity, implying that these genes might act in the same pathway (8,9). One possibility is that the *MMS2* and *MMS4* are involved in recognizing a specific subset of DSBs leading to the recruitment of *RAD52*. It has been suggested that DSBs arise after MMS treatment when, during BER, single-strand breaks (SSBs) are encountered by a replication fork. These replication-associated DSBs would

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serve as a substrate for HR (10,11). To check if the role of the HR system in MMS-resistant cells is to repair S-phase-specific DSBs, we analysed DNA samples from mammalian and yeast cells treated with MMS or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG). We find that recently replicated DNA from MMS- or MNNG-treated mammalian cells does not contain DSBs. Further to this, experiments with both mammalian and yeast systems indicate that neither MMS nor MNNG lead to DSBs *in vivo* but, when such DSBs are detected by PFGE, they are an experimentally induced *in vitro* artefact.

MATERIALS AND METHODS

Cell lines

The EM9, irs1SF and V3-3 cell lines all originate from AA8 Chinese hamster ovarian (CHO) cells. EM9 has a mutation in the *XRCC1* gene and has a defect in BER (12,13). The V3-3 is mutated in the *XRCC7* gene resulting in a deficiency in DNA-PKcs and impaired non-homologous end-joining (NHEJ) (14). The irs1SF is defective in the *XRCC3* gene resulting in deficiency in HR (15,16). CXR3 is an XRCC3 corrected irs1SF cell line (17). The HCT116 cell line was obtained from American Type Culture Collection (Manassas, VA). All cell lines were cultured in DMEM, with the addition of 9% fetal calf serum and penicillin–streptomycin (90 U/ml) at 37°C and 5% CO_2 atmosphere.

Alkylation

Mammalian cells. MMS and MNNG was dissolved in phosphate-buffered saline and dimethyl sulfoxide just before use (DMSO; the treatment dose of MNNG did not exceed 0.2% of DMSO), respectively. Treatments with MMS and MNNG were performed in Hank's balanced salt solution (HBSS, GIBCO), unless otherwise indicated. All solutions were prepared just before treatment. γ-irradiation was performed in a ^{137}Cs chamber (10.6 Gy/min).

Yeast. Fresh MMS (Sigma, 100% purity) was added directly to yeast cultures in standard YEPD to a final concentration of 0.05 or 0.1%. Cells were shaken at 30°C until sampling when they were washed twice in fresh YEPD before use in assays described below.

Yeast recombination assay

Recombination was measured between *arg4-nsp* and *arg4-bgl* alleles by measuring the frequency of *ARG4* cells in treated (0.05% MMS for 30 min) and untreated populations of SK1 diploid cells [construct described fully in (18,19)]. Cells were sonicated briefly to break down clumps, and plated onto both rich medium and arginine dropout plates immediately after exposure to MMS. The recombination frequency is the fraction of colony forming units on arginine dropout plates compared to rich medium. The frequency of *ARG4* is a conservative estimate of the number of gene conversion events as it does not account for gene conversion to the opposite mutant allele or the double mutant allele *arg4-nsp,bgl*.

Viability estimates in wild-type and mutant yeast

Viability was estimated by plating serial dilutions wild-type, *rad52::KanMX* and *apn1::KanMX* haploid BY4741 cells from the Euroscarf deletion collection on YEPD solid medium following exposure to 0.05% MMS for 0–90 min.

Toxicity assays in mammalian cells

In the toxicity assay, 500 cells were plated onto a Petri dish (∅ 100 mm) 24 h prior to a 0.5 h treatment with MNNG or MMS. Following treatment, plates were rinsed three times with 10 ml HBSS (GIBCO) and 10 ml medium was added. After 7–12 days, when colonies were observed, the plates were harvested and the colonies were fixed and stained using methylene blue in methanol (4 g/l). Colonies containing >50 cells were counted.

Pulsed-field gel electrophoresis

Mammalian cells. Flasks were inoculated with 4×10^6 cells for 4 h prior to a 24 h treatment with hydroxyurea or etoposide, or 28 h prior to a 0.5 h treatment with MMS or MNNG. After treatment, the cells were released from the flask by trypsinization and 1×10^6 cells were set into each agarose plug (75 µl, 1% InCert Agarose, BMA). γ-irradiation (^{137}Cs, 10.6 Gy/min) was performed after cells had been set into the agarose plugs. Inserts were incubated in 0.5 M EDTA, 1% *N*-laurylsarcosyl and proteinase K (1 mg/ml) at 50 or 20°C for 48 h, and thereafter washed four times in TE-buffer prior to loading onto an agarose separation gel (1% Chromosomal grade agarose, Bio-Rad). Separation was performed on a CHEF DR III equipment (BioRad; 120° field angle, 240 s switch time, 4 V/cm, 14°C) for 18 h. The gel was stained with ethidium bromide overnight and subsequently analysed by a scanning fluorescence reader (FLA-3000, Fujifilm) using Image Gauge software.

In the ^{14}C-thymidine labelling experiments, flasks were inoculated with 2×10^6 cells 27.5 h prior to a 30 min incubation with ^{14}C-thymidine (4.39 µM, 9.25 kBq/ml) or with 2×10^6 cells 4 h prior to ^{14}C-thymidine labelling (0.439 µM, 0.925 kBq/ml) for 24 h. Flasks were rinsed twice with 10 ml HBSS (GIBCO) before initiation of a 30 min treatment with MMS or MNNG or a 24 h treatment with hydroxyurea. After treatment, cells were released by trypsinization, melted into agarose inserts as described above and separated on PFGE for 24 h (BioRad; 120° field angle, 60–240 s switch time, 4 V/cm, 14°C). After separation, the DNA was transferred from the gel to a nylon membrane according to the manufacturer's protocol (Hybond-N, Amersham Pharmacia Biotech). The membrane was then dried for 2 h at 80°C and exposed onto a phosphoimager plate (FujiFilm) for 18 h before quantification employing Image Gauge software (FLA-3000, Fujifilm).

When treating isolated DNA with MMS, untreated AA8 cells were set in agarose plugs and incubated in 0.5 M EDTA, 1% *N*-laurylsarcosyl and proteinase K (1 mg/ml) at 50°C for 48 h and thereafter washed as described above. The agarose plugs with naked DNA was then treated with MMS 1 mM in HBSS for 30 min before incubation in 0.5 M EDTA, 1% *N*-laurylsarcosyl and proteinase K (1 mg/ml) at 50 or 20°C for 48 h. Samples were rinsed and separated by PFGE for 18 h as described above.

When studying repair of heat-labile sites, 1×10^6 cells were melted into each agarose plug (75 µl, 1% InCert Agarose, BMA) and treatment was performed by transferring the agarose plugs to $HBSS^{++}$ containing 1 mM MMS for 30 min at 37°C. Following treatment, agarose plugs washed three times in ice-cold $HBSS^{++}$ and then transferred to DMEM and kept on a turning table at 37°C. At given time points, agarose plugs were incubated in 0.5 M EDTA, 1% *N*-laurylsarcosyl and proteinase K (1 mg/ml) at 50°C for 48 h, treated as described above and separated on PFGE for 18 h as described above.

Yeast cells. A single colony of haploid yeast was used to inoculate a 5 ml YEPD liquid culture grown overnight at 30°C. The whole culture was transferred to 250 ml of YEPD and grown overnight at 30°C. MMS (0.05 or 0.1%) was added for the times indicated in the figures, with shaking at 30°C followed by washing the cells twice in 250 ml of fresh YEPD. The cells assayed for repair of heat-labile damage were maintained at high density in stationary phase and sampled for PFGE at regular intervals. Plugs for PFGE were prepared as soon as the cells were sampled and stored in 50% glycerol/1× TE at −80°C until the final time point was sampled. All plugs were subsequently treated with Proteinase K (1 mg/ml) at 55 or 30°C for 24 h. Samples were rinsed and subjected to PFGE (BioRad; 120° field angle, 6 V/cm, initial switch time of 19 s, final switch time of 160 s for 26 h at 11°C).

Southern hybridization

Southern transfer from PFGE was undertaken in standard denaturing conditions (0.4 M NaOH) after soaking the gel in 0.25 M HCl for 15 min using ZetaProbe membrane (Biorad). The DNA probe specific to the *SPO11* open reading frame on chromosome *VIII* was prepared from a plasmid (pAG191) by restriction enzyme digestion and gel purification. The probe was labelled with ^{32}P using the HighPrime random primer labelling kit (Roche) as the manufacturer recommends.

Computer simulations

Under the assumption of a Poisson process for the formation of alkylations, the positions of the alkylations were uniformly distributed along chromosomes. For each chromosome, a random number was generated for each alkylation event to determine its position. Our own in-house developed random number generator was used to undertake this. It was assumed that half of the total number of alkylation events occurred on each DNA strand. The number of times that the breaks on the two strands were separated by 14 bp or less was recorded using a purpose-written C programme. A total of 1000 replicates were simulated for each of four conditions. Under two conditions, the alkylation rate for MMS was used and it was assumed that either 100 or 10% of modified bases would be converted to an SSB *in vitro*. The other two conditions were set for the alkylation rate for MNNG with the same efficiencies of conversion to SSBs. The mean number of DSBs for each of the four cases is shown in Table 1.

Table 1. Alkylation grade and expected and found number of DNA DSBs following conversion of heat-labile sites to SSBs, produced following MMS and MNNG treatments

	MMS	MNNG
DNA reactivity (nmol/gDNA and mM h) (35–38)	900	84 000
Dose (mM h)	1.5	0.005
Alkylation grade (nmol/g DNA)	1350	420
Alkylation grade (% alkylated bases)	0.044 (1/2, 254)	0.014 (1/7, 245)
No. of alkylations in human genome	2.7×10^6	8.3×10^5
Expected number of DSBs		
100% conversion of alkylated bases to SSBs	17 364 ± 136.0	1653 ± 45.6
10% conversion of alkylated bases to SSBs	173.4 ± 13.6	17.3 ± 4.0
Observed DSBs	8000	8000
Minimal distance between events required to obtain 8000 DSBs, assuming random distribution of alkylation events		
100% conversion of alkylated bases to SSBs (bp)	6	70
10% conversion of alkylated bases to SSBs (bp)	700	8800

RESULTS

HR-deficient mammalian cells are hypersensitive to alkylating agents

It is well established that HR-deficient *S.cerevisiae* strains are hypersensitive to alkylating agents such as MMS (3). To test if HR-deficient mammalian cells are also hypersensitive to alkylating agents, we investigated cell survival in a wild-type (AA8) and a HR-deficient Chinese hamster cell line [irs1SF (16,17)] to MMS and MNNG. We also treated XRCC1-deficient EM9 cells (20), that are partially BER deficient (21), and DNA-PKcs-deficient V3-3 cells (14). We found that irs1SF cells were 7-fold more sensitive to MMS than wild-type cells and 9-fold more sensitive to MNNG (Figure 1). To ensure that this difference in sensitivity is due to deficiency in HR, we used an irs1SF cell line that is complemented with a functional h*XRCC3* gene on a cosmid vector, CXR3 (17). The sensitivity to both MMS and MNNG in irs1SF cells is partially reverted by the human *XRCC3* gene in Chinese hamster cells (Figure 1). The incomplete complementation by the human *XRCC3* gene in hamster cells has previously been reported (17,22). We found that the NHEJ-deficient cell line V3-3 is insensitive to MNNG and only slightly sensitive to MMS (Figure 1), which is interesting given that NHEJ is recognized to repair the majority of DNA DSBs in mammalian cells (23–25), and yKu80 mutant cells are MMS sensitive (26). The EM9 cell line was found sensitive to MMS and MNNG consistent with what was previously reported (13).

MMS and MNNG induce heat-labile sites that convert to DNA DSBs *in vitro*

We examined the DNA isolated from cells treated with alkylating agents for evidence of DNA DSBs, a candidate lesion leading to increased HR. To do this, DNA was displayed by PFGE and compared to positive control samples from cells

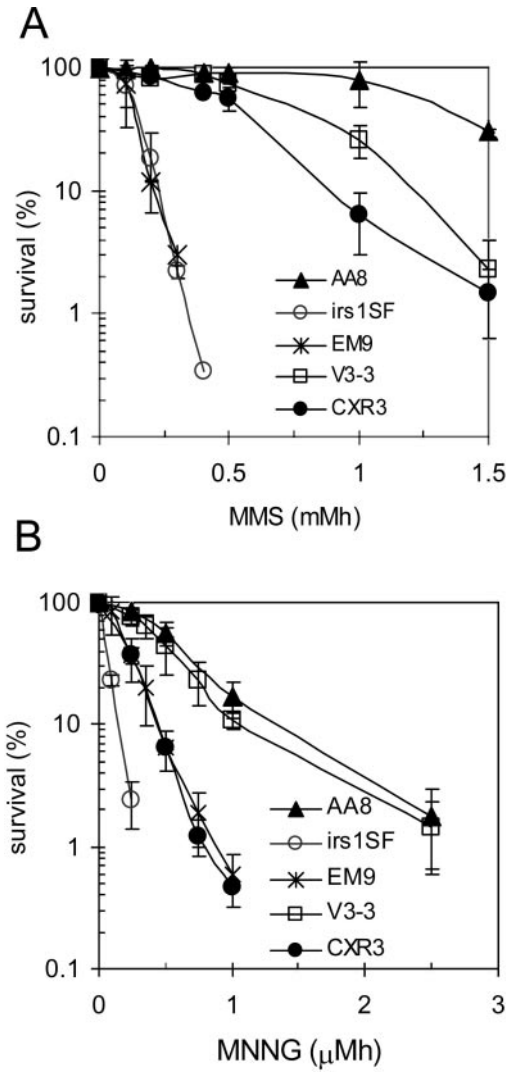

Figure 1. Homologous recombination and base excision repair are required for survival from toxic lesions induced by MMS and MNNG in mammalian cells. Colony forming ability in AA8, irs1SF, V3-3, CXR3 and EM9 cells after a 0.5 h treatment with MMS (**A**) or MNNG (**B**). The mean (symbols) and SD (bars) values of two to four experiments are shown.

treated with etoposide (VP-16), hydroxyurea (HU) or ionizing radiation, all known to induce DSBs (27). Under the conditions used, chromosomal DNA remains in the wells and shorter DNA molecules, arising from the presence of DSBs, enters the gel creating a smear. Initial results using standard preparation for PFGE gave the impression that both MMS and MNNG produce high levels of DSBs (Figure 2A). However, because sample preparation for PFGE commonly involves treatment with proteinase-K at 50°C, and alkylated bases on DNA are known to be heat-labile (28,29), we investigated this issue further. Our concern was that the DSBs could be formed by spontaneous hydrolysis of alkylated bases to apurinic/apyrimidinic (AP) sites (28). These AP sites are, in turn, heat-labile and can be transformed into DNA SSBs (29,30). Subsequent alkylation on the opposite strand (and SSB conversion), and sufficiently close to the first alkylation, would result in a DSB.

After incubation with proteinase K at 20°C for 48 h (Figure 2A), positive control AA8 cells treated with etoposide (VP-16), hydroxyurea (HU) or IR, revealed smearing due to the presence of DSBs, as expected from previous findings (27,31). In contrast, no smearing was detectable for MMS or MNNG treated in samples prepared in 20°C (Figure 2B). This indicates that MMS and MNNG form lesions, *in vivo*, that are heat-labile but, at this level of resolution no *in vivo* DSBs are apparent.

To confirm that the sensitivity to MMS and MNNG in the EM9 and irs1SF cell lines are not due to the induction of DSBs, we compared induction of MMS- and MNNG-induced lesions in CHO cells deficient in different repair pathways. Also, chromosomal aberrations and sister chromatid exchanges (SCEs) formed after MMS and MNNG treatment have been suggested to arise from repair of DSBs produced by futile repair of O^6-MeG by mismatch repair (MMR) proteins (32,33). To evaluate if release of DNA fragments after MMS and MNNG treatment is dependent on MMR, we also treated the MLH1-deficient human fibroblast cell line, HCT116, to the agents. All cell lines showed a similar induction of DNA fragments after both MMS and MNNG treatment as seen in Figure 2C (lanes 1–5; 11–15). DNA fragments were released in HCT116 cells as well, indicating that MMR is not sufficient for formation of these lesions. In samples incubated at 20°C (Figure 2C, lanes 6–10 and 16–20), no induction of DSBs were seen in either cell line.

To confirm that these results were not specific to mammalian cells, similar experiments were undertaken in yeast. Consistent with the results from mammalian cells, we found profound breakage of the DNA following preparation for PFGE at 55°C (Figure 3A), with significant repair of heat-labile sites being evident 16 h after MMS exposure. No smearing was detected for DNA from MMS-treated *S.cerevisiae* when preparing PFGE plugs at 30°C (Figure 3B). To enhance the sensitivity of the assay, the DNA was transferred onto a membrane for Southern hybridization using a probe specific to chromosome *VIII*. Still, we found no evidence of breakage of chromosome *VIII* following MMS treatment, supporting the view that DSBs are not formed *in vivo* following MMS treatment at 30°C.

One possible explanation for the dearth of DSBs after treatment at 30°C was because insufficient numbers of DSBs had been created by the level of MMS used (0.05%). To maximize our chances of capturing MMS-induced DSBs, *rad52* mutant cells (which are unable to repair DSBs by HR) were exposed for up to 90 min to either 0.05% MMS or 0.1% MMS (Figure 4). The chromosomes of cells treated with 0.05% MMS for only 30 min were severely degraded when prepared for PFGE using a 55°C incubation step (Figure 4A). In contrast, no low molecular weight smearing was obtained for cells treated with either 0.05% MMS or 0.1% MMS for up to 90 min with sample preparation at 30°C (Figure 4B). To assess the impact of MMS on the genome, we measured HR as the appearance of Arg4+ prototrophs in diploids containing inserts of *arg4–nsp* and *arg–bgl* alleles (18,19). Exposure to 0.05% MMS for 30 min increased gene conversion to *ARG4* about 6.5-fold compared to untreated cells (Figure 5A). Lastly, we checked that, as expected, *rad52* cells were sensitive to MMS in our hands (Figure 5B). With this confirmed, we are confident that the

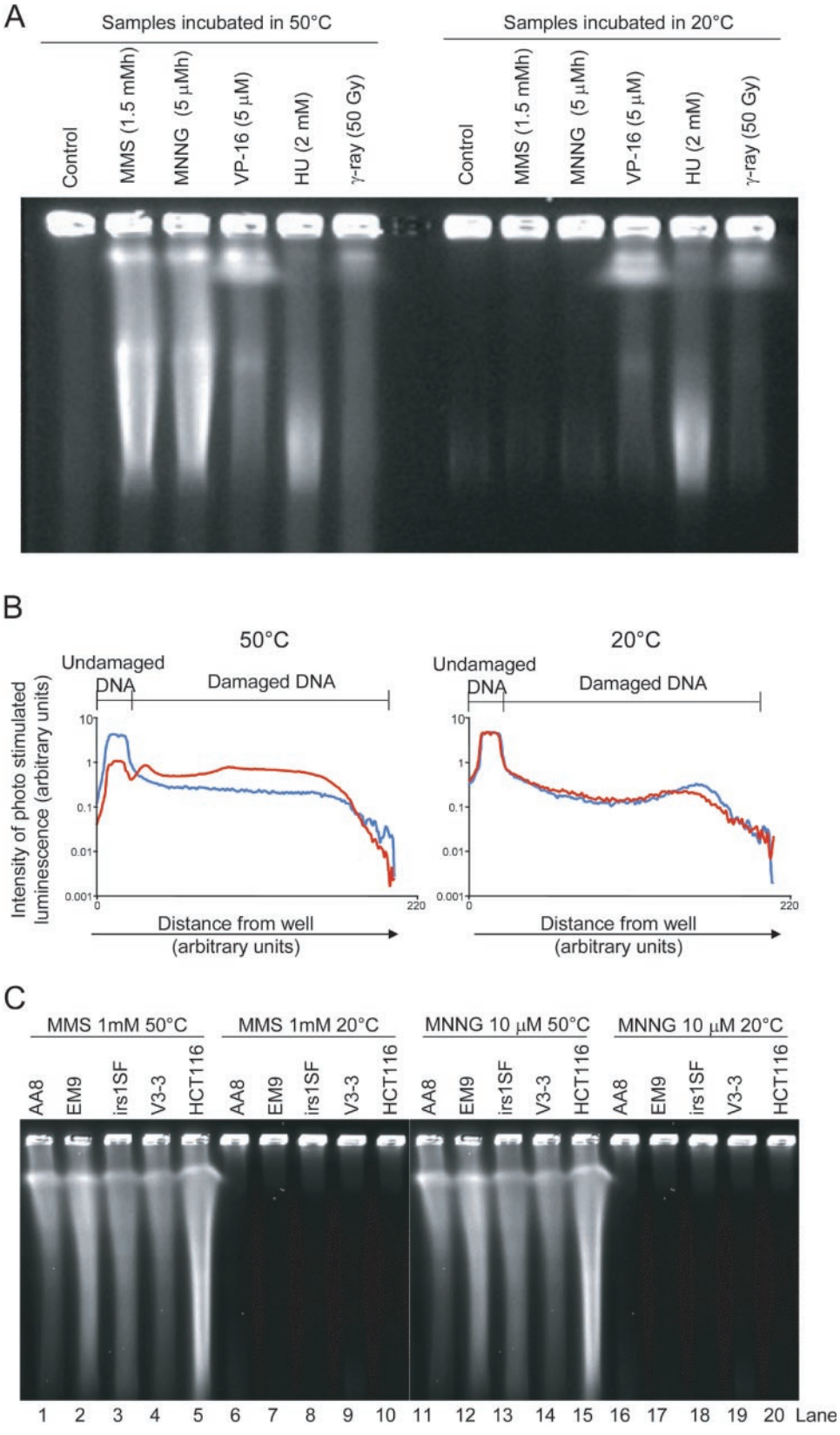

Figure 2. Methylating agents induce heat-labile DNA damage. (**A**) DSBs visualized on PFGE in AA8 cells after treatment of 3 mM MMS, 10 μM MNNG, 2 mM HU, 5 μM VP-16 or 50 Gy. Treatments were performed for 0.5 h for MMS and MNNG and 24 h for HU and VP-16. Samples were incubated in either 50°C or 20°C during preparation for PFGE. (**B**) Quantification of the intensity of DNA fragments released in control (blue line) and following MMS-treatment (red line) and incubation in either 50°C or 20°C during preparation for PFGE. (**C**) Induction of heat-labile sites in cells deficient in different repair pathways after treatment to 0.5 mM h MMS or 5 μM h MNNG and after sample preparation in either 50°C (lanes 1–5 and 11–15) or 20°C (lanes 6–10 and 16–20) before separation on PFGE.

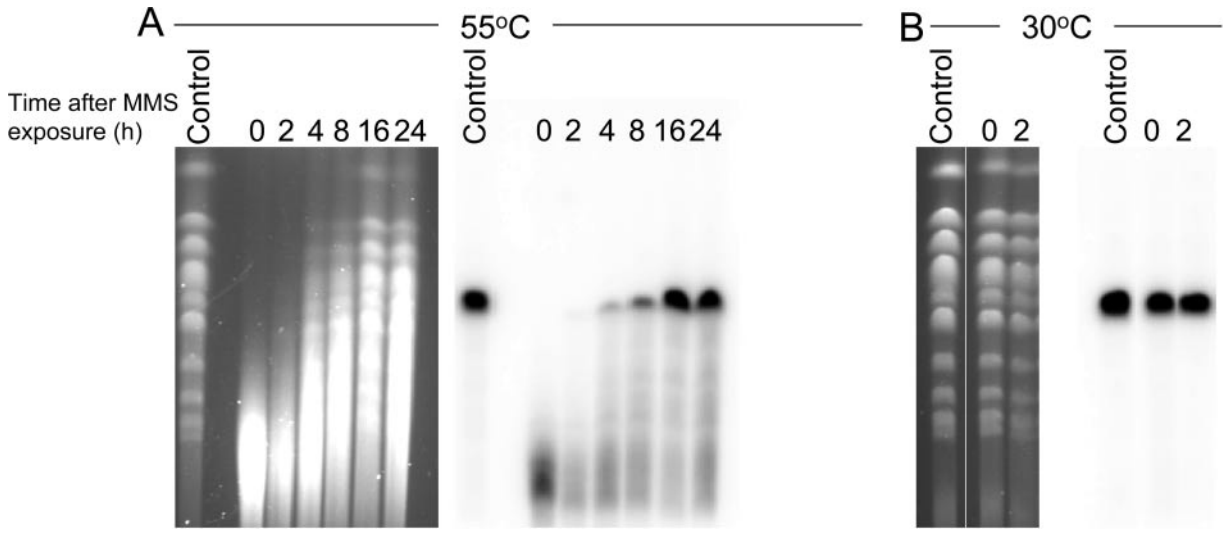

Figure 3. MMS produces heat-labile DNA damage in *S.cerevisiae*. PFGE of yeast chromosomes after a 0.5 h MMS treatment (0.05%) and treatment with proteinase K at 50°C (**A**) or at 30°C (**B**) for 24 h. Chromosomes were visualized by ethidium bromide or Southern hybridization to highlight chromosome *VIII* directly after MMS treatments or following repair as indicated.

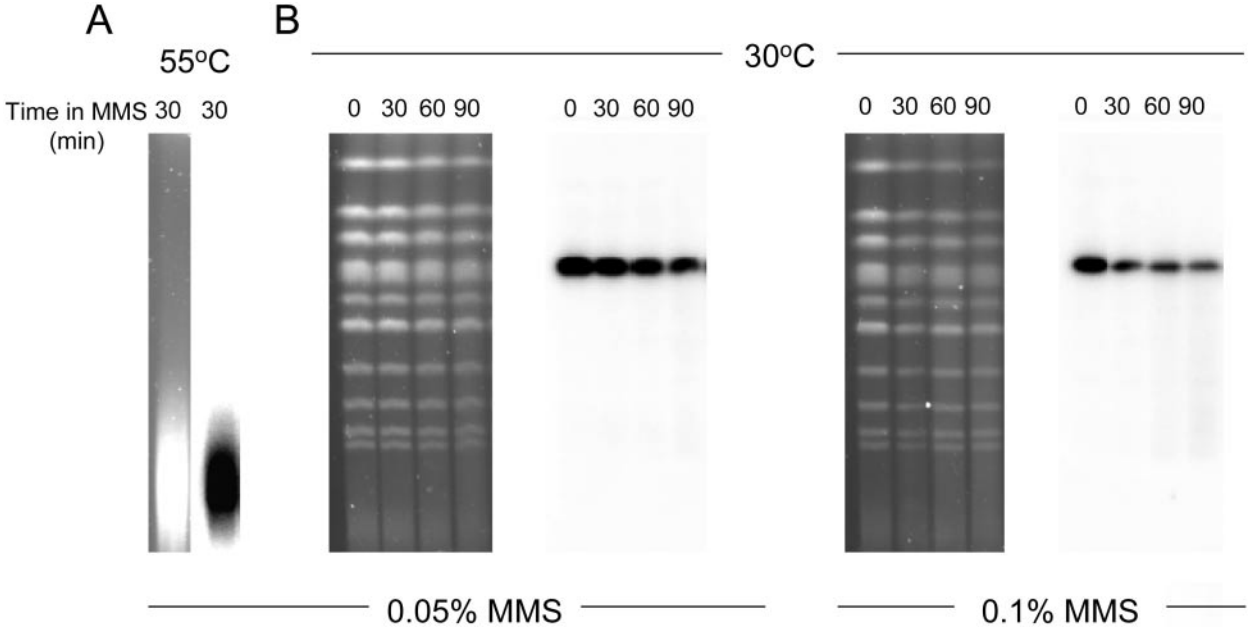

Figure 4. Double-strands breaks do not accumulate in *rad52* cells treated with MMS. Chromosomes were visualized by ethidium bromide or Southern hybridization. (**A**) After exposing *rad52* cells to 0.05% MMS for 30 min heating to 55°C, which causes massive chromosome fragmentation. (**B**) No chromosome fragmentation was observed in *rad52* cells treated with either 0.05 or 0.1% MMS for 30–90 min.

MMS treatment is causing DNA damage, and the failure to detect severely damaged chromosomes by PFGE for samples treated at 30°C is because *in vivo* DSBs do not arise from exposure to MMS.

MMS and MNNG do not induce DSBs close to replication forks

We reasoned that the lack of DSBs found in samples treated at 20°C could reflect the fact that they are specifically associated *in vivo* with replicating DNA, which forms a small proportion of the total DNA examined. To enhance our ability to detect replication-associated DSBs, we labelled nascent DNA *in vivo* by incorporation of ^{14}C-thymidine for 0.5 h or genome-wide DNA for 24 h labelling in wild-type Chinese hamster AA8 cells. Following labelling, cells were treated with MMS, MNNG, HU or ionizing radiation. A high level of labelled DNA fragments entered the gel following incubation of the plugs at 50°C (Figure 6A). For the cells treated with a 0.5 h pulse of ^{14}C-thymidine, labelling is constrained to recently replicated, nascent DNA. Nascent DNA associated with replication forks is expected to form bubble structures preventing

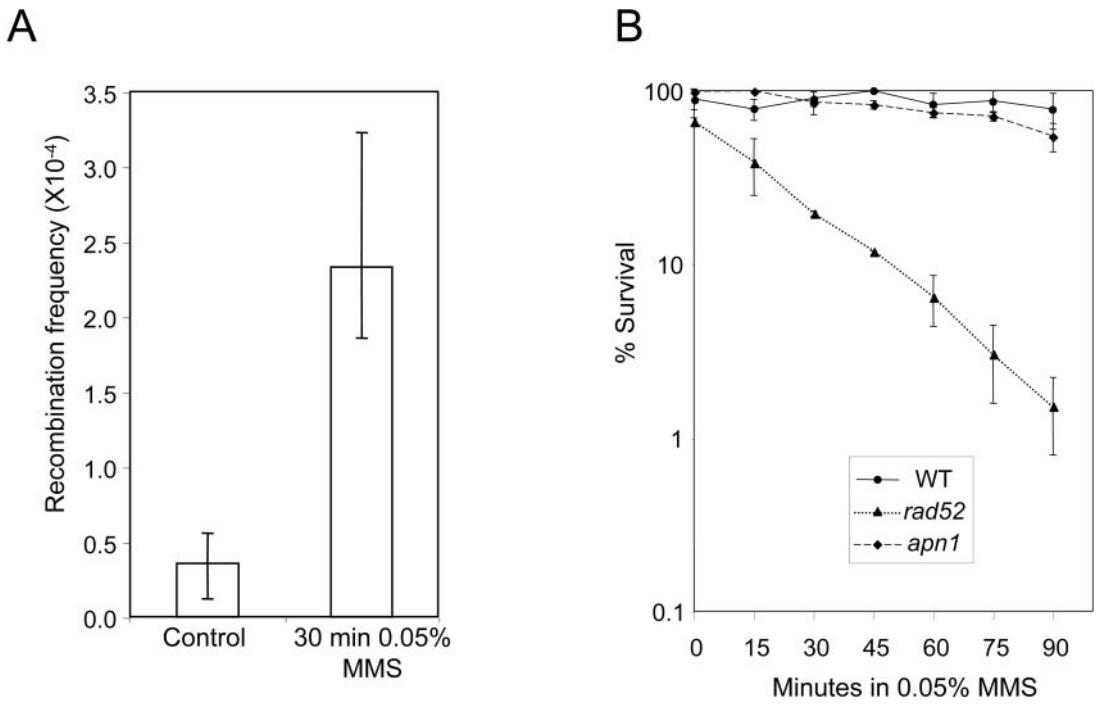

Figure 5. HR induced by MMS in *S.cerevisiae*. (**A**) Recombination was measured between *arg4-nsp* and *arg4-bgl* alleles following 30 min MMS (0.05%) treatment, by measuring the frequency of *ARG4* cells in the population. (**B**) The viability of *rad52* cells was assessed after exposure to MMS to confirm that the MMS was causing DNA damage in the cells, which did not display DSBs. As expected, viability of *rad52* cells is reduced significantly compared to wild type on exposure to MMS. The *APN1* gene alone has little impact on viability after exposure to MMS.

it from entering the gel (34). DSBs forming within these replicons, or close to replication forks, releases them into the gel (27,31). Only a small amount of nascent DNA fragments (0.5 h ^{14}C-thymidine labelling) were released following MMS or MNNG treatments. Thus, most of these DSBs do not release the labelled DNA within replication bubbles, presumably because the heat-labile damage is spread throughout the genome and not restricted to replication forks. The same small release of nascent DNA fragments are also found following γ-ray treatments, consistent with random distribution of IR-induced DSBs. In contrast, HU releases large amounts of nascent DNA fragments into the gel, consistent with suggestions that HU produces DSBs close to replication forks (31).

For samples treated at 20°C, HU and IR treatments lead to labelled DNA being released into the gel (Figure 6B). It is particularly important to note that no nascent DNA was released into the gels by MMS or MNNG when cells were pulse labelled and treated at 20°C. This result suggests that alkylating agents do not induce replication fork associated DSBs *in vivo*.

Taken together, the data indicate that DSBs produced following MMS treatment are an *in vitro* artefact following a 50°C or 55°C treatment of the heat-labile alkylated DNA. To confirm that appearance of DSBs is a purely chemical process without biological relevance at physiological temperatures, we embedded control cells in agarose and stripped these with Proteinase K for 48 h. The naked DNA was treated with 1 mM MMS for 30 min (i.e. 0.5 mM h) and samples were incubated at 50°C or 20°C for 48 h, prior to analysis with PFGE. DSBs were induced by MMS in DNA exposed to 50°C, while no DSBs could be detected in DNA exposed to 20°C (Figure 7).

MNNG-induced heat-labile sites appear to be clustered

MMS or MNNG-induced heat-labile methylated bases may convert to a SSB following incubation at 50°C (29,30). One possibility is that DNA fragments visualized by PFGE are only released when two heat-labile methylated bases are in close proximity, and on opposite strands (so that two SSBs, are converted into a single DSB). By this argument, the amount of DSBs induced by methylations will depend on how the damage is distributed. Clustered alkylations would convert to high levels of DSBs, whereas randomly distributed damage would convert lower levels of DSBs.

We calculated the DNA alkylation grade following MMS or MNNG treatments using DNA reactivity data for MMS and MNNG (35–38). We found that 0.044% and 0.014% bases will be alkylated with MMS and MNNG, respectively, under the conditions used here for mammalian cells (Table 1).

Based on a random distribution of methylations, we calculated the expected number of DSBs that would be produced by MMS or MNNG. In the simulation, we hypothesized that 100 or 10% of the alkylations would give rise to a heat-labile site (Table 1). We also assumed that there is equal methylation on opposite strands and that heat-labile sites must be within 14 bases of each other on opposite strands to be converted into a DSB. According to the simulations, after *in vitro* heat treatment, MMS (1.5 mM h) would lead to 17 364 DSBs per cell if all alkylated sites are converted by heat into SSBs or 173 DSBs if only 10% of alkylated sites are converted by heat into SSBs. Similarly, MNNG (5 μM h) would lead to, respectively, 1653 DSBs or 17.3 DSBs at 100% and 10% efficiency of damage conversion to SSBs (Table 1). The DNA fragments released by PFGE following MMS and MNNG treatments

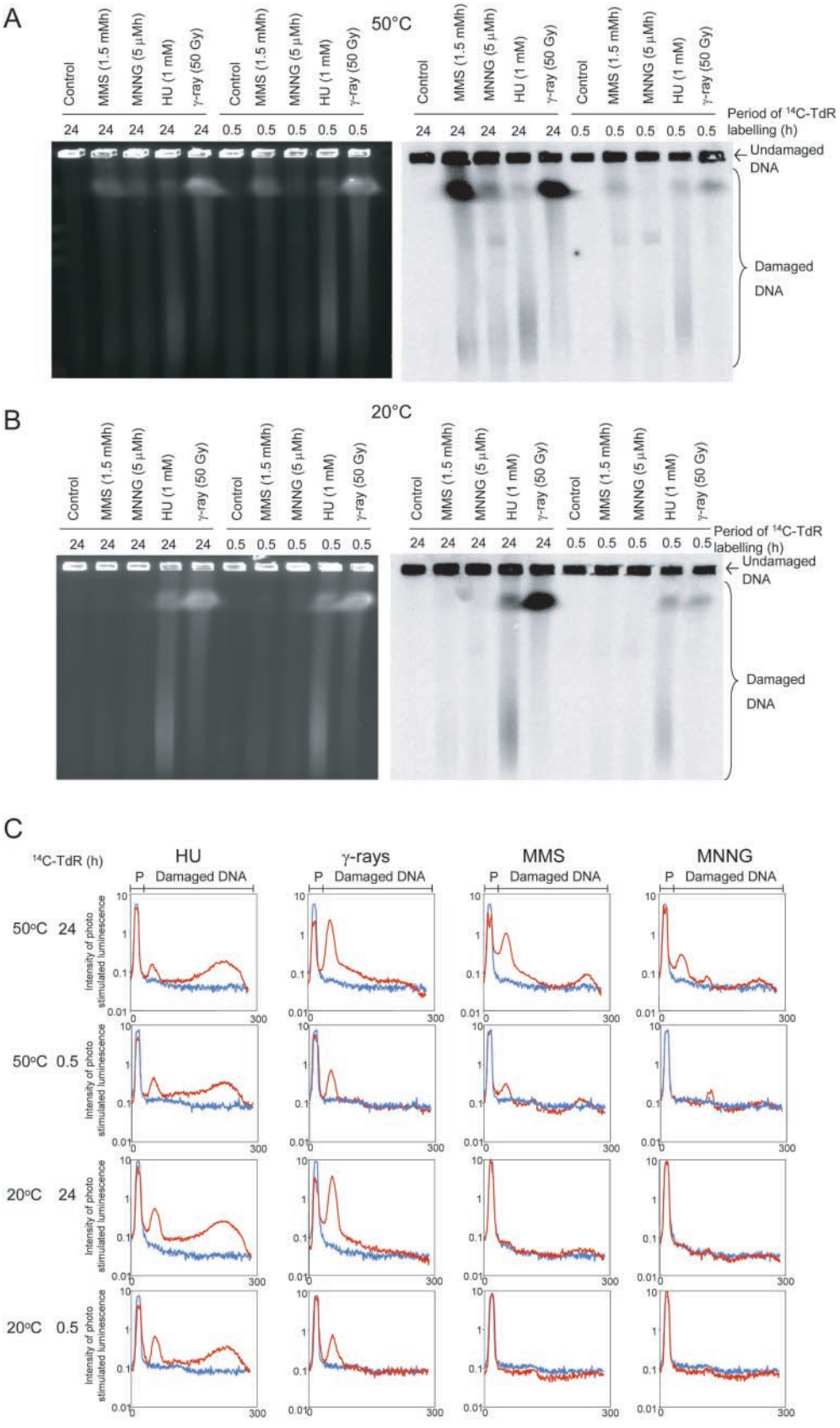

Figure 6. Alkylating agents induce DSBs randomly in DNA. The DNA of AA8 cells was labelled with ^{14}C-thymidine (^{14}C-TdR), either homogeneously for 24 h or specifically at sites of replication for 30 min, prior to exposure to 3 mM MMS, 10 μM MNNG, 2 mM HU or γ-rays (50 Gy). DNA was separated utilizing PFGE and visualized by ethidium bromide staining (**A**) and autoradiography (**B**).

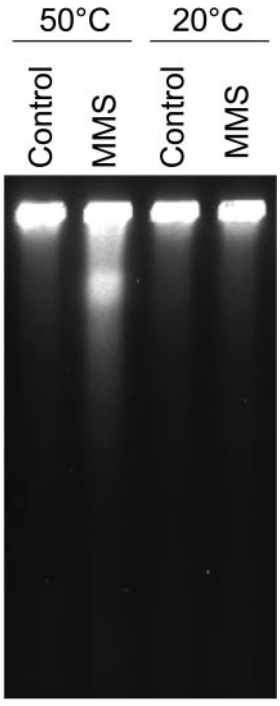

Figure 7. Genomic DNA treated with 1 mM MMS for 0.5 h and incubated in either 50 or 20°C before separation on PFGE.

(and 50°C incubation) are equivalent to a treatment with ~200 Gy, producing ~8000 DSBs (Figure 2) (39,40). The predicted number of DSBs from conversion of heat-labile sites close on opposite strands is in 2-fold excess for MMS at 100% efficiency, but 5-fold lower for MNNG. From this different implications for the distribution of damage induced by MMS and MNNG can be considered. The inference is that damage caused by MMS could be randomly distributed to generate the quantity of heat-labile sites, demonstrated from the amount of low molecular weight DNA released. In contrast, MNNG damage sites appear to be clustered, otherwise based on our calculations MNNG heat-labile sites would not be sufficiently close to each other to liberate the amount of DNA fragments detected after heat treatment.

Repair of MMS-induced heat-labile sites requires XRCC1 and is independent of recombination

Next, we set out to test whether or not the heat-labile damage caused by MMS is responsible for HR sensitivity to alkylating agents. To test this, we examined the DNA of wild-type and HR compromised cells treated with MMS. For wild-type mammalian cells, repair of the heat-labile sites was apparent by the gradual disappearance of DSB DNA band (>2 Mbp) from cells incubated at 50°C (Figure 8A). For stationary phase wild-type yeast cells, similar repair was detected by the gradual increasing molecular weight of the smear and reappearance of chromosome-specific bands (Figure 3A).

Although the XRCC3 mutant cell line is severely compromised for HR (15,41) and sensitive to MMS (Figure 1A), this mutation had very little impact on the loss of heat-labile moieties, such that the DSB band from these cells disappeared with similar kinetics as wild-type cells (Figure 8). Similarly,

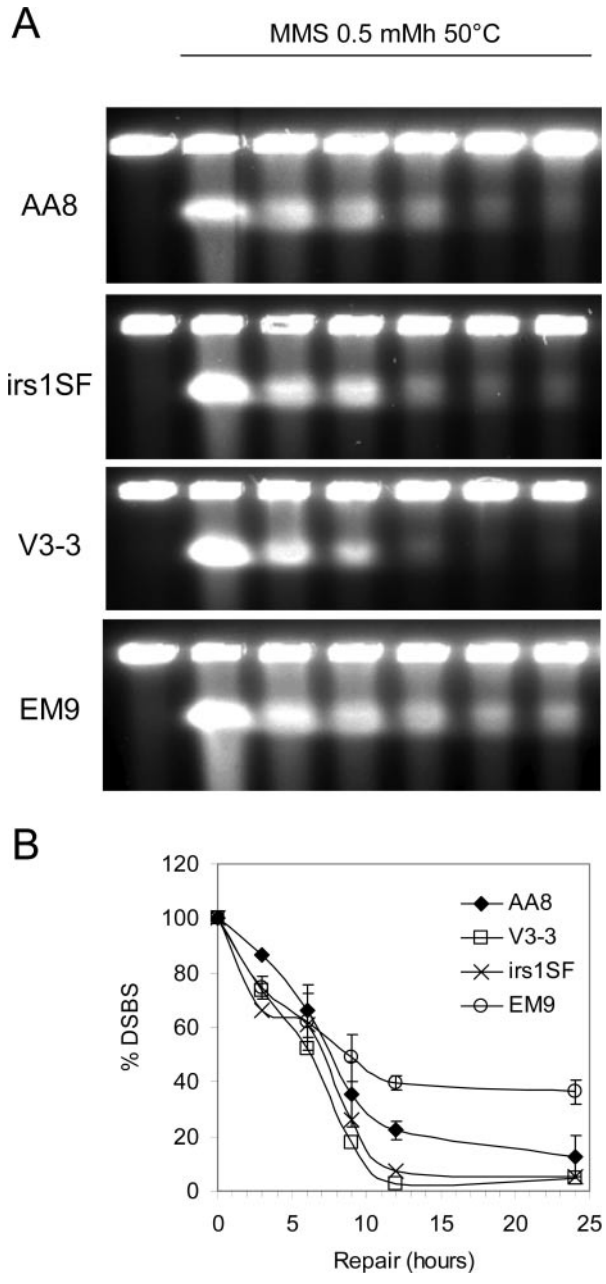

Figure 8. Delayed repair of MMS-induced heat-labile DNA damage in XRCC1-deficient cells. (**A**) The repair of MMS-induced heat-labile DNA damage was determined in wild-type hamster AA8 cells and compared with HR-deficient irs1SF, NHEJ-deficient V3-3 and BER-deficient EM9 cells. (**B**) Quantification of repair kinetics in AA8, irs1SF, V3-3 and EM9 cells. The average (symbol) and SD (error bars) from three experiments are depicted.

Δ*rad52* yeast cells can regain whole chromosomes at virtually the same rate as wild-type cells (Figure 9). These data indicate that the hypersensitivity of HR-deficient cells is unlikely to be due to the presence of either DNA DSBs or loss of repair of heat-labile damage caused by MMS.

NHEJ is the quickest and most commonly used DSBs repair pathway in mammalian cells (23,24). If MMS produced an excessive amount of DSBs, one would imagine that the repair of these DSBs would be slow in an NHEJ-deficient cell line. To test this, we determined the repair of MMS-induced

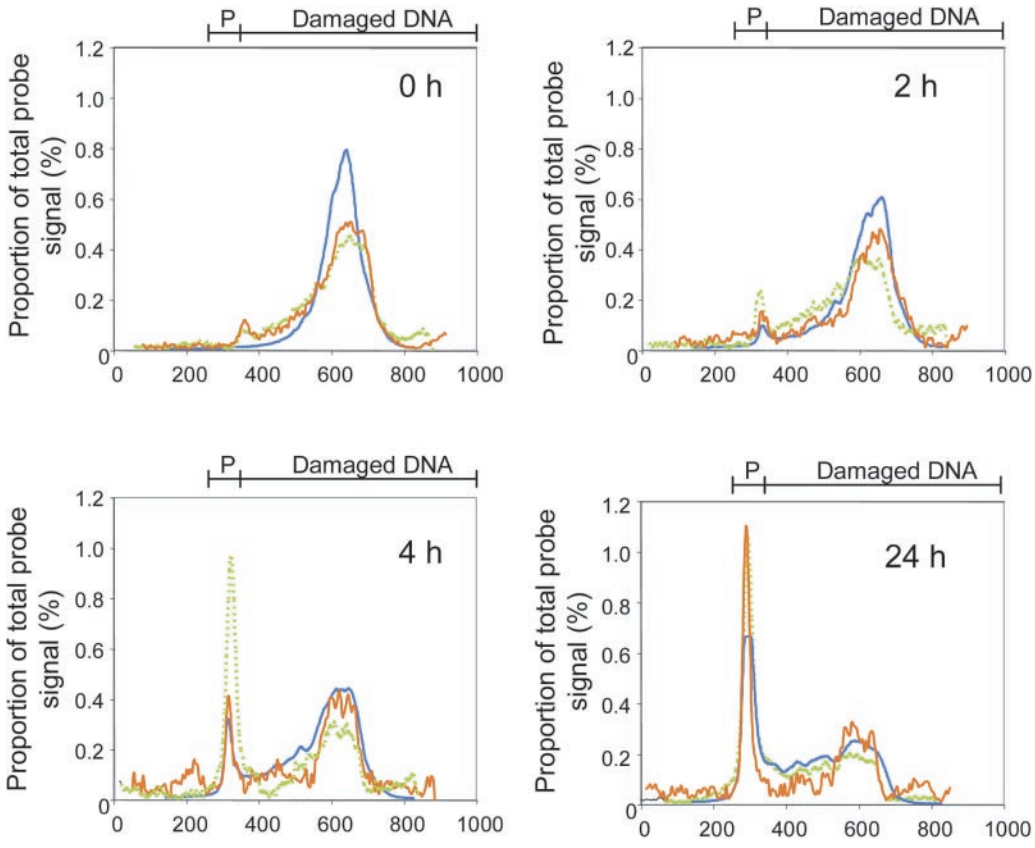

Figure 9. No defect in repair of MMS-induced heat-labile DNA damage in *rad52* or *apn1* deficient *S.cerevisiae*. Profiles of hybridization signals for chromosome *VIII* following Southern analysis of wild-type (blue), *rad52* (red) and *apn1* (green) cells in pulsed-field gels at different repair time points of MMS-treated cells. 'P' indicates the position of whole chromosome band.

heat-labile sites in the DNA-PKcs deficient V3-3 cell line. We found that V3-3 cells repair MMS-induced heat-labile sites as efficiently as the wild-type HR deficient cells (Figure 8).

Proteins in the BER pathway play an important role in processing alkylated bases as well as clustered damage sites *in vitro* (42). We used the Chinese hamster cell line EM9, deficient in XRCC1 (12,20) to test if the repair of MMS-induced heat-labile sites, possibly multiple damage sites, is dependent on this enzyme. We found that ~40% of MMS-induced heat-labile sites remained in EM9 cells following 24 h, while most were repaired in wild-type cells (Figure 7B). This result implicates the XRCC1 protein in repair of MMS-induced heat-labile, possibly multiple damage sites. We also determined the repair of MMS-induced heat-labile sites in the *S.cerevisiae apn1*Δ mutant, deficient in the major yeast apurinic endonuclease (43). In contrast to EM9 cells, we found no defect in the repair of MMS-induced heat-labile sites in *apn1*Δ mutants (Figures 6B and 9).

DISCUSSION

Methylating agents methylate DNA mainly on oxygen or nitrogen residues present in either the DNA backbone or in the DNA bases. Methylated bases are efficiently repaired by BER or DNA methyltransferases (2). Further to this, alkylating agents induce both SCE and HR (6,44–47). The significance of this response to DNA alkylation is revealed by the well-established fact that budding yeast HR mutants are hypersensitive to alkylating damage, which we confirm to be the case also with mammalian cells deficient in HR. The HR-deficient irs1SF cell line is more sensitive to MNNG than the BER-deficient EM9 cell line, whereas the EM9 and irs1SF cell lines show similar sensitivity to MMS. One explanation for this difference could be that MNNG methylates the O^6-guanine position much more efficiently than MMS, and O^6-methyl guanine is linked with induction of HR (35,48,49).

Since it is well established that HR is involved in repair of alkylation-induced damage in yeast, such agents are regularly used in experimental systems to induce DNA damage responses for studying HR. Early reports using sucrose density sedimentation suggested that high MMS doses were required to induce DSBs in yeast, thought due to the proximity of multiple SSBs on opposite strands (50,51). More recently, low doses of MMS have been shown by PFGE to induce DSBs (5) and various authors continue to refer to MMS as a radiomimetic or DSB-inducing agent. A possible explanation for published PFGE data (5) was that DSBs arise during replication, when a replication fork encounters an MMS-induced SSB, causing the replication fork to collapse into a DSB (10,11). Replication fork collapse is known to trigger break-induced replication by HR in both yeast and mammalian cells (52,53). Our experiments were designed to search for such DSBs following exposure of mammalian and yeast

cells to alkylating agents. Here, we present evidence that no such DSBs form *in vivo* following treatment with alkylating agents. First, chromosomes were not fragmented when analysed by PFGE after preparation at low temperatures in either wild-type or *rad52* cells. Second, a higher resolution analysis of nascent mammalian DNA revealed that replicons remain trapped in the wells of agarose gels as long as heat-labile moieties remained stable, implying that they are not associated with DSBs. On the other hand, converting heat-labile MMS damage to DSBs by treating samples at 50°C permitted nascent DNA to travel into the gel. Similarly, DSBs induced *in vivo* by IR or HU also released replicons into the gel.

We did find extensive DNA fragmentation following preparation of the samples for PFGE at 50°C. Since isolated DNA can also be fragmented by exposure to MMS *in vitro* and heating to 50°C, we suggest that no enzymatic activity is required for MMS-associated DSB formation, but such DSBs are an experimental artefact. These DSBs may form *in vitro* following spontaneous hydrolysis of alkylated bases to AP sites (28), and heat-induced transformation into SSBs (29). Such SSBs could then be converted to DSBs through two routes. Either the SSBs are at sufficient density to be very close to each other on both strands (30) or SSBs can be formed on ssDNA of the lagging strand of replication forks. Significantly fewer nascent DNA fragments (after treatment at 50°C) are released by MMS and MNNG compared to HU. Therefore, the data support the view that the distribution of MMS and MNNG-induced heat-labile lesions is not concentrated in single-stranded DNA regions of replication forks. Computer modelling lead to the conclusion that, assuming 100% efficiency conversion of heat-labile sites to SSBs, SSBs separated by 6 bp (for MMS) or 70 bp (for MNNG) would be required to yield DSBs if the alkylated damage is randomly distributed. These calculations support the view that damage caused by MMS may be randomly distributed. For MNNG, the distance between randomized sites is almost certainly too great to explain the amount of DSB DNA generated by heat treatment, thus it is more likely that damage caused by MNNG is clustered.

While our data does not support that MMS or MNNG directly induce *in vivo* DSBs, HR repair may still be involved in the repair of heat-labile sites. To test this, we investigated the repair of MMS-induced heat-labile sites in wild-type and repair-deficient cell lines. For both mammalian and yeast cells, these sites could be repaired as quickly in HR-deficient cells as wild-type cells. The data suggest that, in mammalian cells, BER is involved in repair of MMS-induced heat-labile damage sites, since these are persisting for longer time in the XRCC1-deficient EM9 cell line. This is consistent with data showing that proteins involved in short-patch repair are important in repair of multi-damaged sites *in vitro* (42). It is not clear why we found no heat-labile repair defect in yeast *apn1* mutants, though it could reflect the known partial redundancy between Apn1 and Apn2 (54). Another possibility is that heat-labile alkylated DNA bases are primarily repaired by bifunctional DNA glycolsylases in *S.cerevisiae*. Interestingly, expression of a human DNA glycolsylase in *S.cerevisiae* increase resistance to MMS (55).

Why then are HR-deficient cells hypersensitive to alkylating agents? One possibility is that at physiological temperatures, the number of alkylated sites converted to SSBs and then DSBs are too few to detect, but are sufficient to elicit a requirement for HR. If this is true, it is still important to note that studies based on analysis of chromosome fragmentation using PFGE are examining repair of heat-labile sites and not *in vivo* DSBs. Others have previously suggested that the HR may be required to correct various forms of DNA damage other than DSBs (56,57). One possibility is that stalling replication forks at sites of alkylated bases reverse to form a chicken foot including a Holiday junction (HJ), as has been suggested to occur at stalled replication forks (58–61). Alternatively, other structures resembling HJs may form at stalled replication forks that could mediate HR repair of MMS-induced damage (62,63). This notion is supported by the known sensitivity of *mus81* mutant yeast to MMS and the need for a helicase, such as Srs2 or Sgs1, for both *RAD52*-mediated MMS resistance and removal of MMS-induced S-phase cruciform structures (62,64,65).

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Conflict of interest statement. None declared.

REFERENCES

1. Beranek,D.T. (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutat. Res.*, **231**, 11–30.
2. Lindahl,T. and Wood,R.D. (1999) Quality control by DNA repair. *Science*, **286**, 1897–1905.
3. Krogh,B.O. and Symington,L.S. (2004) Recombination proteins in yeast. *Annu. Rev. Genet.*, **38**, 233–271.
4. Ui,A., Seki,M., Ogiwara,H., Onodera,R., Fukushige,S., Onoda,F. and Enomoto,T. (2005) The ability of Sgs1 to interact with DNA topoisomerase III is essential for damage-induced recombination. *DNA Repair* (*Amst.*), **4**, 191–201.
5. Choy,J.S. and Kron,S.J. (2002) NuA4 subunit Yng2 function in intra-S-phase DNA damage response. *Mol. Cell. Biol.*, **22**, 8215–8225.
6. Myung,K. and Kolodner,R.D. (2003) Induction of genome instability by DNA damage in *Saccharomyces cerevisiae*. *DNA Repair*, **2**, 243–258.
7. Prakash,L. and Prakash,S. (1977) Isolation and characterization of MMS-sensitive mutants of *Saccharomyces cerevisiae*. *Genetics*, **86**, 33–55.
8. Hryciw,T., Tang,M., Fontanie,T. and Xiao,W. (2002) MMS1 protects against replication-dependent DNA damage in *Saccharomyces cerevisiae*. *Mol. Genet. Genomics*, **266**, 848–857.
9. Odagiri,N., Seki,M., Onoda,F., Yoshimura,A., Watanabe,S. and Enomoto,T. (2003) Budding yeast mms4 is epistatic with rad52 and the function of Mms4 can be replaced by a bacterial Holliday junction resolvase. *DNA Repair* (*Amst.*), **2**, 347–358.
10. Sobol,R.W., Kartalou,M., Almeida,K.H., Joyce,D.F., Engelward,B.P., Horton,J.K., Prasad,R., Samson,L.D. and Wilson,S.H. (2003) Base excision repair intermediates induce p53-independent cytotoxic and genotoxic responses. *J. Biol. Chem.*, **278**, 39951–39959.
11. Pascucci,B., Russo,M.T., Crescenzi,M., Bignami,M. and Dogliotti,E. (2005) The accumulation of MMS-induced single strand breaks in G_1 phase is recombinogenic in DNA polymerase beta defective mammalian cells. *Nucleic Acids Res.*, **33**, 280–288.

12. Thompson,L.H., Brookman,K.W., Jones,N.J., Allen,S.A. and Carrano,A.V. (1990) Molecular cloning of the human XRCC1 gene, which corrects defective DNA strand break repair and sister chromatid exchange. *Mol. Cell. Biol.*, **10**, 6160–6171.
13. Thompson,L.H., Brookman,K.W., Dillehay,L.E., Carrano,A.V., Mazrimas,J.A., Mooney,C.L. and Minkler,J.L. (1982) A CHO-cell strain having hypersensitivity to mutagens, a defect in DNA strand-break repair, and an extraordinary baseline frequency of sister-chromatid exchange. *Mutat. Res.*, **95**, 427–440.
14. Blunt,T., Finnie,N.J., Taccioli,G.E., Smith,G.C., Demengeot,J., Gottlieb,T.M., Mizuta,R., Varghese,A.J., Alt,F.W., Jeggo,P.A. *et al.* (1995) Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. *Cell*, **80**, 813–823.
15. Liu,N., Lamerdin,J.E., Tebbs,R.S., Schild,D., Tucker,J.D., Shen,M.R., Brookman,K.W., Siciliano,M.J., Walter,C.A., Fan,W. *et al.* (1998) XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages. *Mol. Cell*, **1**, 783–793.
16. Fuller,L.F. and Painter,R.B. (1988) A Chinese hamster ovary cell line hypersensitive to ionizing radiation and deficient in repair replication. *Mutat. Res.*, **193**, 109–121.
17. Tebbs,R.S., Zhao,Y., Tucker,J.D., Scheerer,J.B., Siciliano,M.J., Hwang,M., Liu,N., Legerski,R.J. and Thompson,L.H. (1995) Correction of chromosomal instability and sensitivity to diverse mutagens by a cloned cDNA of the XRCC3 DNA repair gene. *Proc. Natl Acad. Sci. USA*, **92**, 6354–6358.
18. Goldman,A.S. and Lichten,M. (1996) The efficiency of meiotic recombination between dispersed sequences in *Saccharomyces cerevisiae* depends upon their chromosomal location. *Genetics*, **144**, 43–55.
19. Wu,T.C. and Lichten,M. (1995) Factors that affect the location and frequency of meiosis-induced double-strand breaks in *Saccharomyces cerevisiae*. *Genetics*, **140**, 55–66.
20. Caldecott,K.W., Tucker,J.D. and Thompson,L.H. (1992) Construction of human XRCC1 minigenes that fully correct the CHO DNA repair mutant EM9. *Nucleic Acids Res.*, **20**, 4575–4579.
21. Whitehouse,C.J., Taylor,R.M., Thistlethwaite,A., Zhang,H., Karimi-Busheri,F., Lasko,D.D., Weinfeld,M. and Caldecott,K.W. (2001) XRCC1 stimulates human polynucleotide kinase activity at damaged DNA termini and accelerates DNA single-strand break repair. *Cell*, **104**, 107–117.
22. Hinz,J.M., Helleday,T. and Meuth,M. (2003) Reduced apoptotic response to camptothecin in CHO cells deficient in XRCC3. *Carcinogenesis*, **24**, 249–253.
23. Sargent,R.G., Brenneman,M.A. and Wilson,J.H. (1997) Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. *Mol. Cell. Biol.*, **17**, 267–277.
24. Wang,H., Zeng,Z.C., Bui,T.A., Sonoda,E., Takata,M., Takeda,S. and Iliakis,G. (2001) Efficient rejoining of radiation-induced DNA double-strand breaks in vertebrate cells deficient in genes of the RAD52 epistasis group. *Oncogene*, **20**, 2212–2224.
25. Rothkamm,K., Kruger,I., Thompson,L.H. and Lobrich,M. (2003) Pathways of DNA double-strand break repair during the mammalian cell cycle. *Mol. Cell. Biol.*, **23**, 5706–5715.
26. de la Torre-Ruiz,M. and Lowndes,N.F. (2000) The *Saccharomyces cerevisiae* DNA damage checkpoint is required for efficient repair of double strand breaks by non-homologous end joining. *FEBS Lett.*, **467**, 311–315.
27. Lundin,C., Schultz,N., Arnaudeau,C., Mohindra,A., Hansen,L.T. and Helleday,T. (2003) RAD51 is involved in repair of damage associated with DNA replication in mammalian cells. *J. Mol. Biol.*, **328**, 521–535.
28. Brookes,P. and Lawley,P.D. (1963) Evidence for the action of alkylating agents on deoxyribonucleic acid. *Exp. Cell Res.*, **24**, 521–524.
29. Lindahl,T. and Andersson,A. (1972) Rate of chain breakage at apurinic sites in double-stranded deoxyribonucleic acid. *Biochemistry*, **11**, 3618–3623.
30. Rydberg,B. (2000) Radiation-induced heat-labile sites that convert into DNA double-strand breaks. *Radiat. Res.*, **153**, 805–812.
31. Lundin,C., Erixon,K., Arnaudeau,C., Schultz,N., Jenssen,D., Meuth,M. and Helleday,T. (2002) Different roles for nonhomologous end joining and homologous recombination following replication arrest in mammalian cells. *Mol. Cell. Biol.*, **22**, 5869–5878.
32. Kaina,B., Ziouta,A., Ochs,K. and Coquerelle,T. (1997) Chromosomal instability, reproductive cell death and apoptosis induced by O6-methylguanine in Mex−, Mex+ and methylation-tolerant mismatch repair compromised cells: facts and models. *Mutat. Res.*, **381**, 227–241.
33. Ochs,K. and Kaina,B. (2000) Apoptosis induced by DNA damage O6-methylguanine is Bcl-2 and caspase-9/3 regulated and Fas/caspase-8 independent. *Cancer Res.*, **62**, 1524–1530.
34. Iliakis,G.E., Cicilioni,O. and Metzger,L. (1991) Measurement of DNA double-strand breaks in CHO cells at various stages of the cell cycle using pulsed field gel electrophoresis: calibration by means of, 125I decay. *Int. J. Radiat. Biol.*, **59**, 343–357.
35. Roberts,J.J., Pascoe,J.M., Plant,J.E., Sturrock,J.E. and Crathorn,A.R. (1971) Quantitative aspects of the repair of alkylated DNA in cultured mammalian cells. I. The effect on HeLa and Chinese hamster cell survival of alkylation of cellular macromolecules. *Chem. Biol. Interact.*, **3**, 29–47.
36. Lawley,P.D. and Thatcher,C.J. (1970) Methylation of deoxyribonucleic acid in cultured mammalian cells by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. The influence of cellular thiol concentrations on the extent of methylation and the, 6-oxygen atom of guanine as a site of methylation. *Biochem. J.*, **116**, 693–707.
37. Lawley,P.D. and Shah,S.A. (1972) Reaction of alkylating mutagens and carcinogens with nucleic acids: detection and estimation of a small extent of methylation at O-6 of guanine in DNA by methyl methanesulphonate *in vitro*. *Chem. Biol. Interact.*, **5**, 286–288.
38. Lawley,P.D. and Shah,S.A. (1973) Methylation of DNA by, 3H-14C-methyl-labelled *N*-methyl-*N*-nitrosourea—evidence for transfer of the intact methyl group. *Chem. Biol. Interact.*, **7**, 115–120.
39. Erixon,K. and Cedervall,B. (1995) Linear induction of DNA double-strand breakage with X-ray dose, as determined from DNA fragment size distribution. *Radiat. Res.*, **142**, 153–162.
40. Prise,K.M., Ahnstrom,G., Belli,M., Carlsson,J., Frankenberg,D., Kiefer,J., Lobrich,M., Michael,B.D., Nygren,J., Simone,G. and Stenerlow,B. (1998) A review of dsb induction data for varying quality radiations. *Int. J. Radiat. Biol.*, **74**, 173–184.
41. Pierce,A.J., Johnson,R.D., Thompson,L.H. and Jasin,M. (1999) XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. *Genes Dev.*, **13**, 2633–2638.
42. Eot-Houllier,G., Eon-Marchais,S., Gasparutto,D. and Sage,E. (2005) Processing of a complex multiply damaged DNA site by human cell extracts and purified repair proteins. *Nucleic Acids Res.*, **33**, 260–271.
43. Popoff,S.C., Spira,A.I., Johnson,A.W. and Demple,B. (1990) Yeast structural gene (APN1) for the major apurinic endonuclease: homology to *Escherichia coli* endonuclease IV. *Proc. Natl Acad. Sci. USA*, **87**, 4193–4197.
44. Hendricks,C.A., Razlog,M., Matsuguchi,T., Goyal,A., Brock,A.L. and Engelward,B.P. (2002) The *S. cerevisiae* Mag1 3-methyladenine DNA glycosylase modulates susceptibility to homologous recombination. *DNA Repair*, **1**, 645–659.
45. Kaina,B., Fritz,G., Mitra,S. and Coquerelle,T. (1991) Transfection and expression of human O6-methylguanine-DNA methyltransferase (MGMT) cDNA in Chinese hamster cells: the role of MGMT in protection against the genotoxic effects of alkylating agents. *Carcinogenesis*, **12**, 1857–1867.
46. Rasouli-Nia,A., Sibghat,U., Mirzayans,R., Paterson,M.C. and Day,R.S.,III (1994) On the quantitative relationship between O6-methylguanine residues in genomic DNA and production of sister-chromatid exchanges, mutations and lethal events in a Mer-human tumor cell line. *Mutat. Res.*, **314**, 99–113.
47. Helleday,T., Arnaudeau,C. and Jenssen,D. (1998) Effects of carcinogenic agents upon different mechanisms for intragenic recombination in mammalian cells. *Carcinogenesis*, **19**, 973–978.
48. Zhang,H., Tsujimura,T., Bhattacharyya,N.P., Maher,V.M. and McCormick,J.J. (1996) O6-methylguanine induces intrachromosomal homologous recombination in human cells. *Carcinogenesis*, **17**, 2229–2235.
49. Zhang,H., Marra,G., Jiricny,J., Maher,V.M. and McCormick,J.J. (2000) Mismatch repair is required for O(6)-methylguanine-induced homologous recombination in human fibroblasts. *Carcinogenesis*, **21**, 1639–1646.
50. Chlebowicz,E. and Jachymczyk,W.J. (1979) Repair of MMS-induced DNA double-strand breaks in haploid cells of *Saccharomyces cerevisiae*,

which requires the presence of a duplicate genome. *Mol. Gen. Genet.*, **167**, 279–286.
51. Jachymczyk,W.J., Chlebowicz,E., Swietlinska,Z. and Zuk,J. (1977) Alkaline sucrose sedimentation studies of MMS-induced DNA single-strand breakage and rejoining in the wild type and in UV-sensitive mutants of *Saccharomyces cerevisiae*. *Mutat. Res.*, **43**, 1–10.
52. Haber,J.E. (2000) Lucky breaks: analysis of recombination in *Saccharomyces*. *Mutat. Res.*, **451**, 53–69.
53. Arnaudeau,C., Lundin,C. and Helleday,T. (2001) DNA double-strand breaks associated with replication forks are predominantly repaired by homologous recombination involving an exchange mechanism in mammalian cells. *J. Mol. Biol.*, **307**, 1235–1245.
54. Vance,J.R. and Wilson,T.E. (2001) Repair of DNA strand breaks by the overlapping functions of lesion-specific and non-lesion-specific DNA 3′ phosphatases. *Mol. Cell. Biol.*, **21**, 7191–7198.
55. Connor,E.E., Wilson,J.J. and Wyatt,M.D. (2005) Effects of substrate specificity on initiating the base excision repair of *N*-methylpurines by variant human, 3-methyladenine DNA glycosylases. *Chem. Res. Toxicol.*, **18**, 87–94.
56. Game,J.C. (2000) The *Saccharomyces* repair genes at the end of the century. *Mutat. Res.*, **451**, 277–293.
57. Helleday,T. (2003) Pathways for mitotic homologous recombination in mammalian cells. *Mutat. Res.*, **532**, 103–115.
58. McGlynn,P. (2004) Links between DNA replication and recombination in prokaryotes. *Curr. Opin. Genet. Dev.*, **14**, 107–112.
59. Postow,L., Ullsperger,C., Keller,R.W., Bustamante,C., Vologodskii,A.V. and Cozzarelli,N.R. (2001) Positive torsional strain causes the formation of a four-way junction at replication forks. *J. Biol. Chem.*, **276**, 2790–2796.
60. McGlynn,P. and Lloyd,R.G. (2001) Rescue of stalled replication forks by RecG: simultaneous translocation on the leading and lagging strand templates supports an active DNA unwinding model of fork reversal and Holliday junction formation. *Proc. Natl Acad. Sci. USA*, **98**, 8227–8234.
61. Michel,B., Grompone,G., Flores,M.J. and Bidnenko,V. (2004) Multiple pathways process stalled replication forks. *Proc. Natl Acad. Sci. USA*, **101**, 12783–12788.
62. Liberi,G., Maffioletti,G., Lucca,C., Chiolo,I., Baryshnikova,A., Cotta-Ramusino,C., Lopes,M., Pellicioli,A., Haber,J.E. and Foiani,M. (2005) Rad51-dependent DNA structures accumulate at damaged replication forks in sgs1 mutants defective in the yeast ortholog of BLM RecQ helicase. *Genes Dev.*, **19**, 339–350.
63. Lopes,M., Cotta-Ramusino,C., Liberi,G. and Foiani,M. (2003) Branch migrating sister chromatid junctions form at replication origins through Rad51/Rad52-independent mechanisms. *Mol. Cell*, **12**, 1499–1510.
64. Interthal,H. and Heyer,W.D. (2000) MUS81 encodes a novel helix–hairpin–helix protein involved in the response to UV- and methylation-induced DNA damage in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **263**, 812–827.
65. Mankouri,H.W., Craig,T.J. and Morgan,A. (2002) SGS1 is a multicopy suppressor of srs2: functional overlap between DNA helicases. *Nucleic Acids Res.*, **30**, 1103–1113.