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An *xrcc4* defect or Wortmannin stimulates homologous recombination specifically induced by double-strand breaks in mammalian cells

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ABSTRACT

Non-homologous end joining (NHEJ) and homologous recombination (HR) are two alternative/competitor pathways for the repair of DNA double-strand breaks (DSBs). To gain further insights into the regulation of DSB repair, we detail here the different HR pathways affected by (i) the inactivation of DNA-PK activity, by treatment with Wortmannin, and (ii) a mutation in the *xrcc4* gene, involved in a late NHEJ step, using the XR-1 cell line. Here we have analyzed not only the impact of NHEJ inactivation on recombination induced by a single DSB targeted to the recombination substrate (using I-*Sce*I endonuclease) but also on γ-ray- and UV-C-induced and spontaneous recombination and finally on Rad51 foci formation, i.e. on the assembly of the homologous recombination complex, at the molecular level. The results presented here show that in contrast to embryonic stem cells, the *xrcc4* mutation strongly stimulates I-*Sce*I-induced HR in adult hamster cells. More precisely, we show here that both single strand annealing and gene conversion are stimulated. In contrast, Wortmannin does not affect I-*Sce*I-induced HR. In addition, γ-ray-induced recombination is stimulated by both *xrcc4* mutation and Wortmannin treatment in an epistatic-like manner. In contrast, neither spontaneous nor UV-C-induced recombination was affected by *xrcc4* mutation, showing that the channeling from NHEJ to HR is specific to DSBs. Finally, we show here that *xrcc4* mutation or Wortmannin treatment results in a stimulation of Rad51 foci assembly, thus that a late NHEJ step is able to affect Rad51 recombination complex assembly. The present data suggest a model according to which NHEJ and HR do not simply compete for DSB repair but can act sequentially: a defect in a late NHEJ step is not a dead end and can make DSB available for subsequent Rad51 recombination complex assembly.

INTRODUCTION

Faithful genome transmission requires the cooperation of a network of pathways, including the cell cycle checkpoint, DNA replication, repair and recombination. The different DNA repair pathways must also be coordinated as a function of the type of damage, the cell cycle and differentiation. DNA double-strand breaks (DSBs) can be generated by physiological cell processes such as meiosis and V(D)J recombination. Accidental highly toxic DSBs can also be produced by genotoxic stresses such as ionizing radiation and replication inhibition (1–3). Two major classes of processes can repair DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ involves two protein complexes: (i) the heterodimer Ku80–Ku70 associated with DNA-PKcs in mammalian cells and (ii) ligase IV with its co-factor Xrcc4. Ku80–Ku70 binds the DNA ends and recruits the other NHEJ components, then, in a second step, Xrcc4/ligaseIV reseals the DNA ends. HR takes advantage of a homologous sequence to repair the DSB and includes non-conservative single-strand annealing (SSA) and conservative gene conversion associated or not with crossing-over. The Rad52 protein can be involved in SSA as well as in gene conversion (4–6), but the two processes are different since SSA is *RAD51*-independent whereas gene conversion is *RAD51*-dependent, in yeast as well as in mammalian cells (7,8).

In yeast, HR is prominent in repair of DSBs and radiation sensitivity of mutants for the Ku homologs can be revealed if the HR pathway is inactivated (9–11). The NHEJ pathway is often considered as the main pathway for DSB repair in mammalian cells. Mouse embryonic stem (ES) cells defective for NHEJ show increased HR induced by a single DSB targeted to the recombination substrate by the rare-cutting endonuclease I-*Sce*I. This effect is essentially observed in null mutants for Ku proteins involved in early NHEJ steps, whereas an *xrcc4* defect, i.e. in a late NHEJ step, shows

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very little effect on HR (12). It has been proposed that competition for binding to the DNA ends between the Ku80–Ku70 heterodimer (involved in NHEJ) and Rad52 protein (involved in HR) would allow channeling of DSB repair to one or the other pathway (6). However, the effect of *xrcc4* inactivation on the different HR pathways (SSA and gene conversion) and the effects on spontaneous and radiation-induced recombination have not been addressed. Moreover, this model should be carefully examined since different cell situations can modify the relative implication of NHEJ and HR in DSB repair. First, the stage of embryonic mouse development is important since HR is mainly involved in ES cells whereas NHEJ is prominent in adult mice (13). Second, cell cycle can affect the choice of the DSB repair mechanism. In chicken cells NHEJ is prominent in G_1/early S phase and HR in late S/G_2 phase (14). In mammalian cells, the sister chromatid is a preferred substrate for HR (15). In line with this, the Rad51 and Rad52 proteins, involved in homologous recombination, are mainly expressed in late S/G_2 phase (16–18). Third, depending on the persistence and/or accumulation of DSBs, cells may sequentially use NHEJ then HR (3). In addition, HR and NHEJ can also cooperate in DSB repair (19), indicating that the two pathways can coexist, despite cell cycle or developmental regulation.

In order to detail the regulation of DSB repair, we have here studied the impact of alteration of late NHEJ steps on HR, i.e. in adult differentiated hamster CHO cells. We used two means of NHEJ inactivation. One is treatment with Wortmannin, which inhibits phosphatidylinositol-3 (PI-3) kinases, including DNA-PK, involved in NHEJ (20,21). Treatment with Wortmannin increases radiation sensitivity of the cells, but does not specifically act on DNA-PK and can affect other pathways. Thus, we also used an *xrcc4*-deleted cell line (XR-1), which exhibits enhanced radiation sensitivity. Xrcc4 protein acts as a co-factor of ligase IV, in a late phase of NHEJ (22,23). This strategy also allows easy combination of both inactivations (*xrcc4* and DNA-PKcs) by treatment of the XR-1 cells with Wortmannin. Importantly, the inactivation of NHEJ studied here acts on late steps of the process, which are not supposed to directly reflect the competition between Ku proteins and Rad52 protein. However, Xrcc4 facilitates the binding of Ku to DNA, thus indirectly affecting the competition between NHEJ and HR (24). We show here that *xrcc4* mutation results in a strong increase in I-*Sce*I-induced HR in adult CHO hamster lines, compared to ES cells. We also analyzed the impact of Wortmannin treatment on I-*Sce*I-induced HR. In the present paper, we have more precisely analyzed the impact not only on the balance between NHEJ and HR but also on the balance between SSA and gene conversion. Indeed, the strategy depicted in Figure 1 allows us to analyze these two balances. In addition, we have here analyzed the impact of *xrcc4* inactivation and Wortmannin treatment on homologous recombination induced by γ-rays, an efficient DSB inducer, in comparison with the effect on spontaneous HR and UV-induced HR, both of which induce DSBs poorly. Finally, since it has been proposed that Ku proteins may impair access of other enzymes, such as nucleases and recombination proteins, to DNA (6,25,26), the present paper shows the impact of *xrcc4* deletion and Wortmannin treatment on intranuclear Rad51 foci assembly after ionizing radiation. The present data suggest a model according to which NHEJ and HR do not simply compete but can sequentially act for DSB repair.

MATERIALS AND METHODS

DNA manipulations

All DNA manipulations were performed as described previously (27,28).

Cells

Cell lines were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) for CHO-DRA10 cells and DMEM without sodium pyruvate for XR-1 (*xrcc4* mutant cell line), 4364 (the corresponding wild-type) and complemented cell lines (X4C and X4V). All media were supplemented with 10% fetal calf serum, 2 mM glutamine and 200 IU/ml penicillin.

The X4C and X4V cell lines correspond to XR-1 complemented with *HsXRCC4* and V5-tagged *HsXRCC4* cDNA cloned in pcDNA6 (InVitrogen), respectively.

Measurement of recombination

Survival and recombination frequency after ionizing or UV-C radiation. Cells were irradiated in phosphate-buffered saline (PBS), using either a ^{137}Cs source (0.7 Gy/min) or 254 nm UV-C light at the dose indicated. After irradiation, cells were incubated in their respective medium at 37°C for 24 h. Cells were then trypsinized, counted and divided into two fractions. The first fraction was used to calculate the viability by cloning efficiency. The second fraction was plated under 1 mg/ml G418 selection to measure recombination frequency.

Recombination after induction of a single DSB by I-SceI. A total of 3×10^5 cells were plated and transfected with 2 μg I-*Sce*I expression vector (pCMV I-SceI). Between 36 and 40 h post-transfection, G418 (1 mg/ml) or G418 (1 mg/ml) + hygromycin (500 μg/ml) selection was applied.

PCR recombination assay. The PCR recombination assays were performed as previously described (29,30). Cells were electroporated with the I-*Sce*I expression vector and grown in non-selective medium for 0, 4 or 48 h after electroporation. Genomic DNA was then isolated and subjected to PCR with primers that flank the I-*Sce*I cleavage site. The 5′ primer is located 410 bp upstream of the cleavage site and the 3′ primer 302 bp downstream. With this set of primers, imprecise NHEJ products with small deletions and insertions in the locus are amplified, as are the gene conversion products.

Wortmannin treatment. Cells were preincubated with 20 μM Wortmannin 1 h before γ-irradiation or I-*Sce*I transfection. Wortmannin treatment was maintained for 24 h (irradiation) or 36–40 h (I-*Sce*I) after treatment.

Spontaneous recombination. Spontaneous recombination was measured by fluctuation analysis as previously described (8,31). For each cell line analyzed, several independent cultures were plated and cultured to confluence. Cells were then trypsinized, counted and one fraction was used to estimate plating efficiency. The remaining cells were plated

under G418 selection. The resulting number of Neo^R clones allowed us to calculate the recombination frequency. The rate of recombination per cell per generation was calculated using the Luria and Delbrück fluctuation test (32).

Western blot analysis and Rad51 foci kinetics

All extract preparation steps were performed at 4°C. After washing with PBS, cells were suspended in lysis buffer (25 mM Tris, pH 7.5, 1 mM EDTA, 600 mM NaCl, 0.5% NP-40, 5 µg/ml leupeptin, 2 µM pepstatin, 1 mM phenylmethylsulfonyl fluoride) and incubated for 45 min on ice. Extracts were centrifuged at 15 000 *g* for 30 min, the supernatant was retrieved and the protein concentration was determined using the Bio-Rad Protein Assay. Boiled protein extract (25–50 µg) was loaded on a 10% polyacrylamide–SDS gel for electrophoresis. After migration, the proteins were electrotransferred to nitrocellulose membrane and probed with specific antibodies: anti-Rad51 (Oncogene Research) and anti-actin (Sigma) antibodies. Antibodies were visualized using the ECL detection kit (Amersham). The Rad51 foci were analyzed as described (33) using the anti-Rad51 antibody.

DNA-PK 'pull-down' assay

All extract preparation steps were performed at 4°C. After washing with Tris-buffered saline, 10^7 cells were suspended in lysis buffer [50 mM NaF, 20 mM HEPES pH 7.8, 450 mM NaCl, 0.2 mM EDTA, 25% v/v glycerol, 0.5 µM dithiothreitol and protease inhibitors cocktail (Roche)] and then frozen in liquid nitrogen and thawed at 37°C three times. After centrifugation for 15 min at 15 000 *g*, the supernatant was stored at –70°C. An aliquot of 20 µl of protein extract was incubated for 30 min at 4°C with 5 mg of double-stranded (ds)DNA–cellulose (Sigma) in a 100 µl total volume of Z′0.05 buffer (34). The extract plus dsDNA–cellulose was washed three times with 1 ml of Z′0.05 buffer and resuspended in Z′0.05 with 0.2 mM p53 peptide, 0.2 µM cold ATP and 0.5 µCi [γ-^{32}P]ATP (20 µl total volume). The kinase assay was performed for 10 min at 30°C. The reaction was stopped by adding 5 µl of tricine sample buffer and boiling for 4 min. Half of the volume (12.5 µl) was loaded on an 18% tricine gel. After migration, the gel was fixed (10% acetic acid, 40% methanol) and dried (85°C for 1–2 h) and then analyzed by autoradiography or quantified with a Storm phosphorimager (Molecular Dynamics).

RESULTS

Cell lines used

The cell lines used contained the recombination substrate depicted in Figure 1A integrated in their genome. This substrate is made of direct repeats of two inactive cassettes of the neomycin resistance gene (Neo) separated by the hygromycin resistance gene (Hyg). Recombination reconstitutes a functional Neo gene leading to resistance to G418 (29).

One recipient is the hamster CHO-DRA10 cell line, already described (29). The other recipients are the X-ray-sensitive XR-1, deleted for the *xrcc4* gene (35), and the corresponding wild-type 4364 cell lines. The lines carrying the marker are XD17 (XR-1 with the recombination marker) and 4D22 (4364 with the recombination marker).

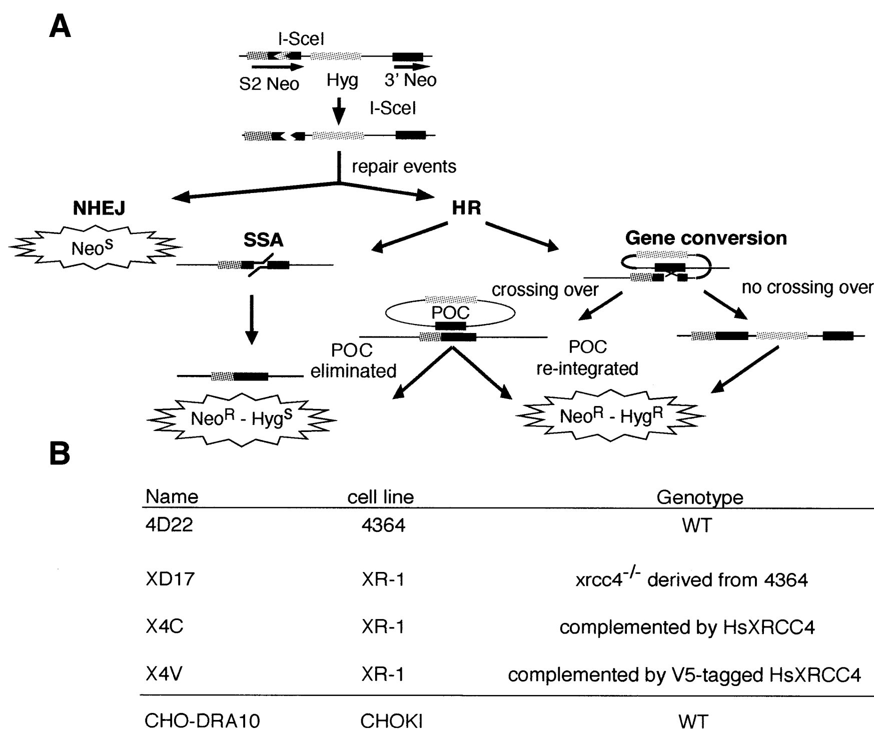
We also devised two other control cell lines, corresponding to XD17 complemented with the human *HsXRCC4* cDNA (named X4C) or with V5-tagged *HsXRCC4* cDNA (cell line X4V). The different cell lines are summarized in Figure 1B.

A defect in *xrcc4* or treatment with Wortmannin stimulates radiation sensitivity and radiation-induced HR by a common pathway

We first measured the radiation sensitivity of the XR-1 (*xrcc4* deletion) and X4C and X4V cell lines (XR-1 cell lines expressing *HsXRCC4* cDNA) to verify the efficiency of complementation by the *XRCC4* cDNA. As expected, the *xrcc4*-deleted cells showed a higher sensitivity to ionizing radiation than the control cell lines. Interestingly, expression of the *XRCC4* cDNA restored radiation resistance in both the X4C and X4V cell lines, indicating that it is functional (Fig. 2A). Radiation-induced recombination was measured in parallel. Recombination was induced by γ-rays at a higher level in the *xrcc4*-deleted cell line than in the control (Fig. 2B). At a dose of 6 Gy, recombination was 26-fold higher in the $xrcc4^{-/-}$ cell than in the control lines. Interestingly, expression of *XRCC4* cDNA lowered the level of radiation-induced recombination to the level of the control cell line. Remarkably, radiation resistance complementation was slightly less efficient in the X4C line than in the X4V line, despite the presence of the V5 tag (Fig. 2A). This may be due to potential differences in the expression level of the exogenous *XRCC4* cDNA. Consistently, radiation-induced recombination was more efficiently repressed in the X4V cell line than in the X4C line. Taken together, these results show that the stimulation of radiation-induced recombination in the XD17 line actually results from the *xrcc4* defect and that recombination repression is linked to the level of *XRCC4* activity for radiation resistance. In addition, since the level of Rad51 protein has been shown to modify the efficiency of radiation-induced recombination (8), we measured by western blotting the amount of Rad51 protein in the different cell lines (Fig. 2C) and found it to be similar in the different lines, i.e. *xrcc4* deletion was not compensated by an increase in the amount of Rad51 protein.

Wortmannin inhibits PI-3 kinases, among them ATM and DNA-PK. Since DNA-PK and Xrcc4 act in the same pathway, we addressed the question of whether treatment with Wortmannin can affect radiation-induced recombination, in a similar way to *xrcc4* inactivation. As expected, treatment of the CHO-DRA10 cell line with Wortmannin increased radiation toxicity (Fig. 3A). In parallel, Wortmannin strongly stimulated radiation-induced recombination (Fig. 3B). At a dose of 5 Gy, recombination was 9-fold higher in Wortmannin-treated cells than in untreated cells. At a 10% equivalent cell viability (i.e. 2.5 Gy for treated cells and 5 Gy for untreated cells), recombination was 4-fold higher in Wortmannin-treated than in untreated cells. These results show that recombination actually results from Wortmannin treatment rather than selection of a highly recombination-prone cell sub-population resistant to radiation. In addition, these results show that the recombination stimulation by NHEJ inactivation acts in a second type of CHO cell line.

Wortmannin acts on both ATM and DNA-PK and inactivation of ATM leads to high level homologous recombination (36). Thus Wortmannin may stimulate



Name	cell line	Genotype
4D22	4364	WT
XD17	XR-1	xrcc4$^{-/-}$ derived from 4364
X4C	XR-1	complemented by HsXRCC4
X4V	XR-1	complemented by V5-tagged HsXRCC4
CHO-DRA10	CHOKI	WT

Figure 1. Substrates to measure homologous recombination and cell lines. (**A**) Cells carry in their genome a single copy of the recombination substrate depicted in the figure as already described. This substrate contains direct repeats of two inactive copies of the neomycin resistance gene (S2neo and 3′neo) separated by the hygromycin resistance gene. The cells are thus sensitive to G418 and resistant to hygromycin (Neo^S-Hyg^R). 3′ neo is not expressed because of the absence of a promoter and of the first amino acids. A promoter drives S2neo but an I-*Sce*I restriction site interrupts the coding sequence. Expression of the I-*Sce*I rare-cutting endonuclease cleaves the S2neo cassettes. The cut can be repaired by either NHEJ or HR (29). NHEJ does not restore a functional Neo gene and the cells are thus G418-sensitive (Neo^S). HR restores a functional Neo gene and all such HR events are thus G418-resistant (Neo^R). Gene conversion without associated crossing-over keeps the hygromycin sequence and the recombinant cell becomes resistant to both G418 and hygromycin (Neo^R-Hyg^R). When associated with a crossing-over, if the reciprocal product (POC) is eliminated, cells are Neo^R-Hyg^S, but if the POC is re-integrated, cells are Neo^R-Hyg^R (8). Thus, single G418 resistance (Neo^R) monitors all HR events and double G418 + hygromycin (Neo^R-Hyg^R) resistance gives an estimation specific to gene conversion. This substrate also allows measurement of spontaneous and radiation-induced recombination resulting in G418 resistance. (**B**) The cell line used.

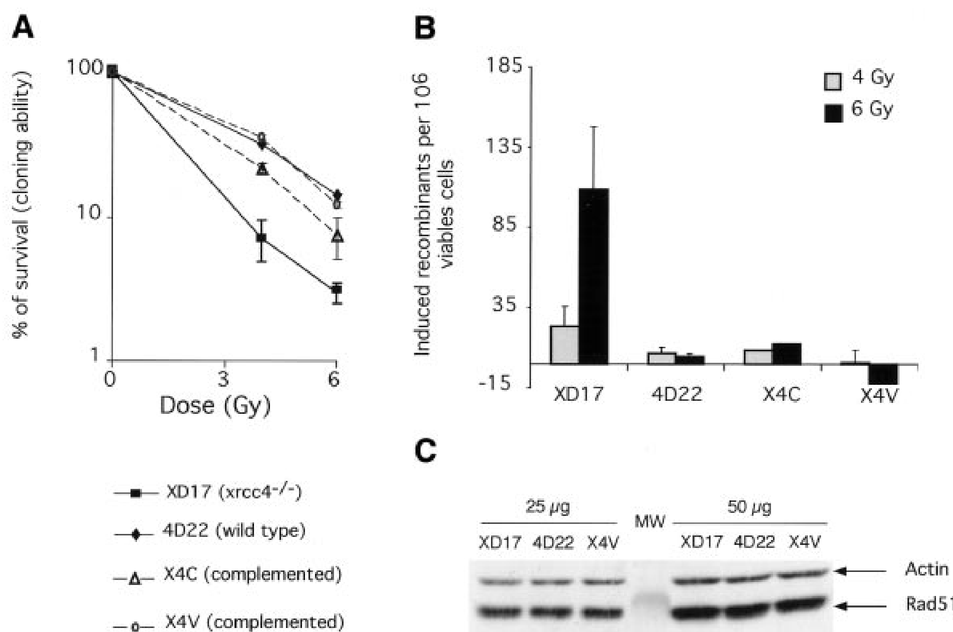


Figure 2. Effects of *xrcc4* deletions on (**A**) radiation sensitivity, (**B**) radiation-induced recombination and (**C**) expression of Rad51 protein. The names and phenotypes of the cell lines are indicated in the figure. The values correspond to the mean of three independent experiments.

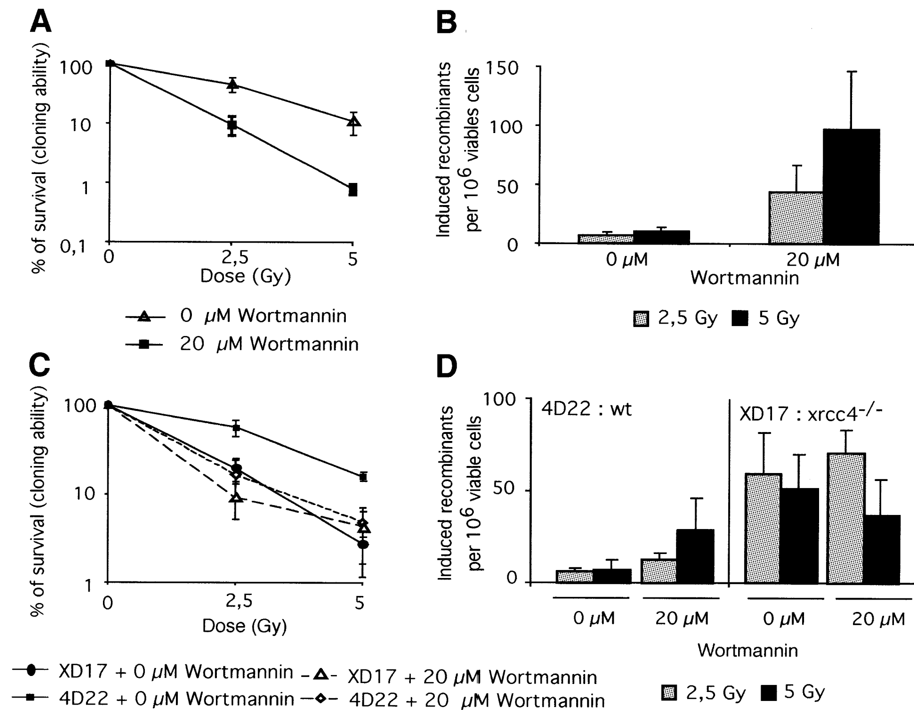


Figure 3. Effects of Wortmannin treatment on radiation survival of the CHO-DRA10 cell line (**A**) and radiation-induced recombination of CHO-DRA10 (**B**). Effects of both Wortmannin treatment and *xrcc4* deletion on radiation survival (**C**) and radiation-induced recombination (**D**). The names and phenotypes of the cell lines are indicated in the figure. The values correspond to the means of three independent experiments.

Table 1. Effect of *xrcc4* deletion on spontaneous homologous recombination

Cell line	Genotype	No. of independent cultures	No. of cells ($\times 10^7$)	No. of Neo^R clones	Luria and Delbruck test (rate/locus/generation $\times 10^7$)
XD17	*xrcc4*$^{-/-}$	14	12.7	560	11.8 ± 7.5
X4C	Complemented	8	9.8	512	11.8 ± 6.0

recombination by inhibiting either ATM and/or DNA-PK. In order to check whether Wortmannin and Xrcc4 act on recombination via the same pathway, we treated the *xrcc4*-deleted cell line with Wortmannin. Treatment of the wild-type 4D22 line with Wortmannin significantly increased radiation sensitivity (Fig. 3C) and radiation-induced recombination, by 2-fold at 2.5 Gy and 4-fold at 5 Gy (Fig. 3D). Although the effect is less pronounced than in CHODRA10 cells, this shows that Wortmannin also acts in this cell line. Treatment of the *xrcc4*$^{-/-}$ cell line with Wortmannin increased neither radiation toxicity (Fig. 3C) nor radiation-induced recombination (Fig. 3D). These results suggest that Wortmannin and Xrcc4 act on recombination via the same pathway: this pathway must be NHEJ inactivation.

A defect in *xrcc4* does not stimulate spontaneous and UV-induced recombination

One may ask whether the NHEJ defect stimulates all homologous recombination events or whether it is specific to some genotoxic stresses, as would be predicted by the challenge for DSB repair between the two pathways. It is thus essential to verify the impact of NHEJ inhibition on recombination in the absence of strong DSB induction.

We first measured the impact of *xrcc4* inactivation on spontaneous recombination measured by fluctuation analysis (Table 1). We compared the *xrcc4*-deleted XR-1 cell line to an XR-1 line complemented with *XRCC4* cDNA, because this allows measurement of recombination at the same locus. The rate of spontaneous recombination is similar in the *xrcc4*-defective line and the complemented lines. This result shows that the defect in *xrcc4* requires a genotoxic stress to stimulate recombination. We then used UV-C, a genotoxic stress different from γ-rays. UV-C induces DSBs poorly but strongly stimulates recombination in mammalian cells (37,38). We thus measured whether *xrcc4* alteration affects UV sensitivity and UV-induced recombination. Both UV-C resistance and UV-C-induced recombination were at similar levels in *xrcc4*-defective lines and in a complemented line (Fig. 4), showing that a defect in NHEJ does not stimulate UV-induced recombination.

Effect of an *xrcc4* defect and of Wortmannin on a single DSB targeted to the recombination substrate

Transfection of an expression vector encoding the rare-cutting endonuclease I-*Sce*I produced a DSB targeted to the corresponding site in the recombination substrate (see Fig. 1A). DSB

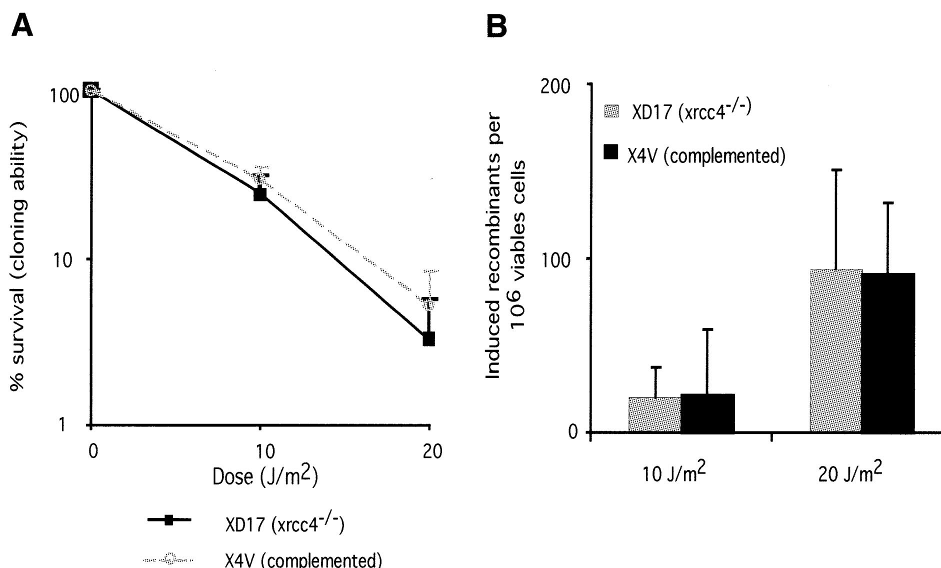


Figure 4. (**A**) UV-C sensitivity. (**B**) UV-C induced recombination. The UV-C doses and names and phenotypes of the different lines are indicated in the figure. The values correspond to the means of three independent experiments.

repair can be promoted by NHEJ or HR, comprising gene conversion associated or not with crossing-over and SSA. Resistance to G418 (Neo^R) monitors all HR DSB repair events (SSA plus gene conversion) and reflects the balance between NHEJ and HR. Double resistance to G418 and hygromycin (Neo^R-Hyg^R) monitors only gene conversion (29). The Neo^R-Hyg^R/Neo^R ratio gives an estimation of the balance between SSA and gene conversion (see Fig. 1A).

We measured the impact of *xrcc4* on these two balances. We first verified that the transfection efficiency was similar in the different cell lines (data not shown). The frequencies of both HR (Neo^R) and gene conversion (Neo^R-Hyg^R) were, respectively, 4.5- and 7-fold higher in the *xrcc4* deletion line than in the control and complemented lines (Fig. 5A). Consequently, the Neo^R-Hyg^R/Neo^R ratio, corresponding to the SSA versus gene conversion balance, is not or is only slightly (to the limit of significance) increased in *xrcc4*$^{-/-}$ cells. These results indicate that *xrcc4* deletion affects the NHEJ versus HR balance but not, or very moderately, the SSA versus gene conversion balance.

We also repeated similar experiments in CHO-DRA10 cells after treatment with Wortmannin (Fig. 5B). Whereas treatment with Wortmannin enhanced radiation sensitivity and radiation-induced recombination, it did not modify the NHEJ versus HR balance after cleavage by I-*Sce*I. To check whether Wortmannin was actually efficient, we measured the DNA-PK activity with an *in vitro* assay (Fig. 5C). Wortmannin significantly decreased the DNA-PK kinase activity, but traces of kinase activity were still detectable (Fig. 5C).

In order to verify the effect of the *xrcc4* defect on the NHEJ versus HR balance and to gain further insight into recombination mechanisms, we used other cell lines containing a slightly different substrate, SCneo (Fig. 6A), and we measured NHEJ and gene conversion at the molecular level. This new strategy does not monitor SSA. Since the status of *xrcc4* does not substantially affect the SSA versus gene conversion balance, we could compare NHEJ and HR without the complication of SSA using this new substrate. We examined NHEJ, in conjunction with homologous repair, with the SCneo substrate using a PCR assay (Fig. 6B; 29). Results from one experiment are shown in Figure 6B. The homologous repair products were *Nco*I$^+$ and the NHEJ products were I-*Sce*I$^-$ and also *Nco*I$^-$. In the control wild-type cell line, both homologous and NHEJ products were readily detected. I-*Sce*I cleavage of the amplified product indicated that a portion of the cells had retained the cleavage site, either because the genome was never cleaved or because it was cleaved but then precisely repaired to retain the site. The *xrcc4*$^{-/-}$ cell line also showed both homologous and NHEJ repair products, although there was a shift in the proportion of the two products. The homologous repair product was substantially more prominent than the NHEJ product, in contrast to the wild-type cells in which the two products were more similar in intensity. There was also a visibly lower amount of the I-*Sce*I$^+$ band.

Several such experiments were performed using two different SCneo-containing cell lines and the bands were quantified (Fig. 6C and D). In the parental wild-type cell lines the relative proportion of the homologous and NHEJ repair products was similar, as has been reported previously (29,30). In the XR-1 cell lines (*xrcc4*$^{-/-}$ cell lines) there was an ~3-fold reduction in the NHEJ product and an increase in the homologous product (Fig. 6C). There was also a reproducible decrease (27%) in the amount of amplified product with an intact I-*Sce*I site (Fig. 6D). Since the imprecise NHEJ product is reduced, the reduction in this I-*Sce*I$^+$ band in the mutant suggests that precise rejoining of a cleaved I-*Sce*I site is also reduced.

These results confirm at a molecular level that the defect in *xrcc4* results in increased homologous recombination repair of a single DSB.

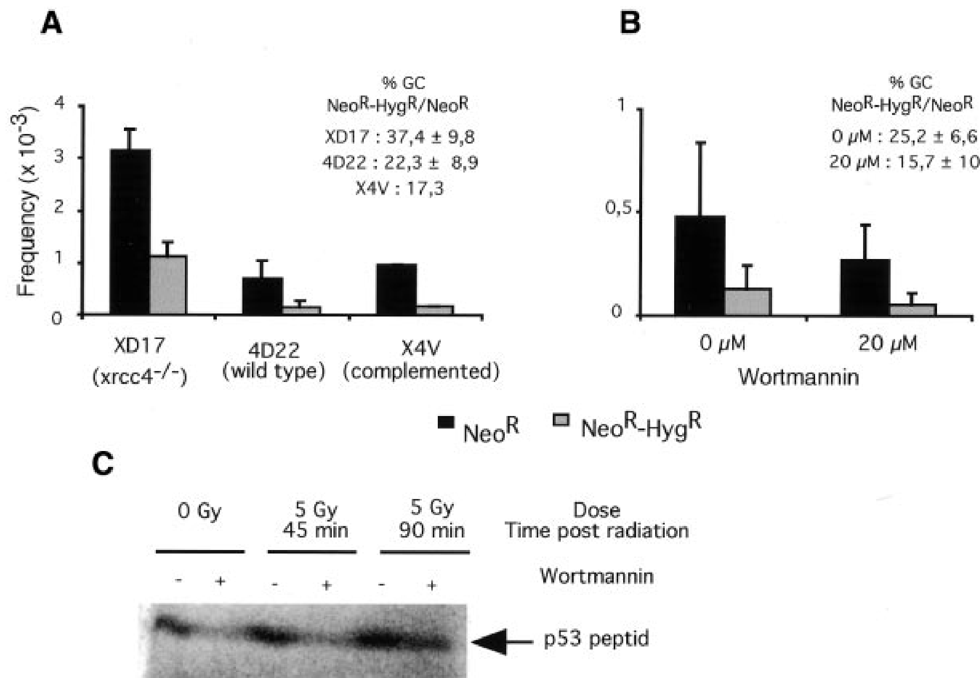


Figure 5. Recombination induced by a single DSB in the recombination substrate produced by I-*Sce*I. Effect of *xrcc4* deletion (**A**) and Wortmannin treatment (**B**) on single and double recombination resistances. The names and phenotypes of the cell lines are indicated in the figure. The values correspond to the means of three independent experiments. (**C**) *In vitro* DNA-PK activity in cell extracts treated or not with Wortmannin.

A defect in *xrcc4* or treatment with Wortmannin stimulates radiation-induced Rad51 foci

It has been suggested that the binding of Ku proteins to DNA ends impairs the access of other enzymes such as exonucleases or Rad51 to form the nucleoprotein filament. We here address the question of whether *xrcc4* deletion or Wortmannin treatment may also affect intranuclear Rad51 foci assembly. Rad51 protein is involved in radiation-induced recombination and in I-*Sce*I-induced gene conversion in mammalian cells (8). In addition, Rad51 protein has been shown to relocalize in nuclear foci after genotoxic stress (33). Since NHEJ alteration stimulates both radiation-induced recombination and I-*Sce*I gene conversion, two Rad51-dependent processes, we measured whether the frequency of Rad51 foci formation was correlated with the recombination stimulation observed in NHEJ-defective situations. Examples of Rad51 foci in *xrcc4*-defective cells or in Wortmannin-treated cells are shown (Fig. 7A and B). The number of Rad51 foci per cell appears higher in NHEJ-inactivated cells (see Fig. 7A and B). In addition, the frequency of Rad51 foci is significantly higher in *xrcc4*-defective cell lines than in control and complemented cell lines (Fig. 7C). Similarly, treatment with Wortmannin enhanced Rad51 foci formation after treatment with ionizing radiation (Fig. 7D). Thus, in NHEJ-defective situations, Rad51 foci formation is correlated with recombination stimulation. This suggests that a functional NHEJ process reduces the frequency of Rad51 foci assembly and homologous recombination and that even late NHEJ steps are involved in such regulation.

DISCUSSION

The results presented here show that deletion of the *XRCC4* gene or inhibition of the PI3 kinases (Wortmannin) results in an increase in γ-ray-induced recombination. Wortmannin has been shown to affect the DNA-PK-dependent NHEJ pathway, which includes Xrcc4, both for radiation resistance and for rejoining of DSBs (39–43). However, the effect of Wortmanninn on HR has never been addressed. Importantly, Wortmannin also inhibits ATM. Since ATM inactivation increases homologous recombination (36), it is thus questionable whether Wortmannin stimulates recombination by inhibiting DNA-PK and/or ATM. Since Wortmannin treatment of the *xrcc4*-deleted line does not further increase radiation-induced cell sensitivity and HR, this indicates that the inhibition of a common pathway is responsible for the recombination stimulation. This pathway must be NHEJ. In contrast, *xrcc4* deletion does not stimulate spontaneous recombination and UV-C-induced recombination, showing that the stimulatory effect of NHEJ inactivation is specific to DSBs. On a single DSB targeted to the recombination substrate, it has been shown that deletion of the *xrcc4* gene results in a low stimulation of HR in ES cells (12), whereas we here show a strong HR stimulation in adult hamster cells. In addition we show here that *xrcc4* deletion stimulates the frequency of HR but has no or very little effect on the gene conversion/SSA ratio. This consistently indicates that, unlike Rad51 (8), Xrcc4 mainly participates in the NHEJ versus HR balance and not significantly in the SSA versus gene conversion balance. In contrast, although DNA-PK activity was substantially decreased, Wortmannin had no stimulatory

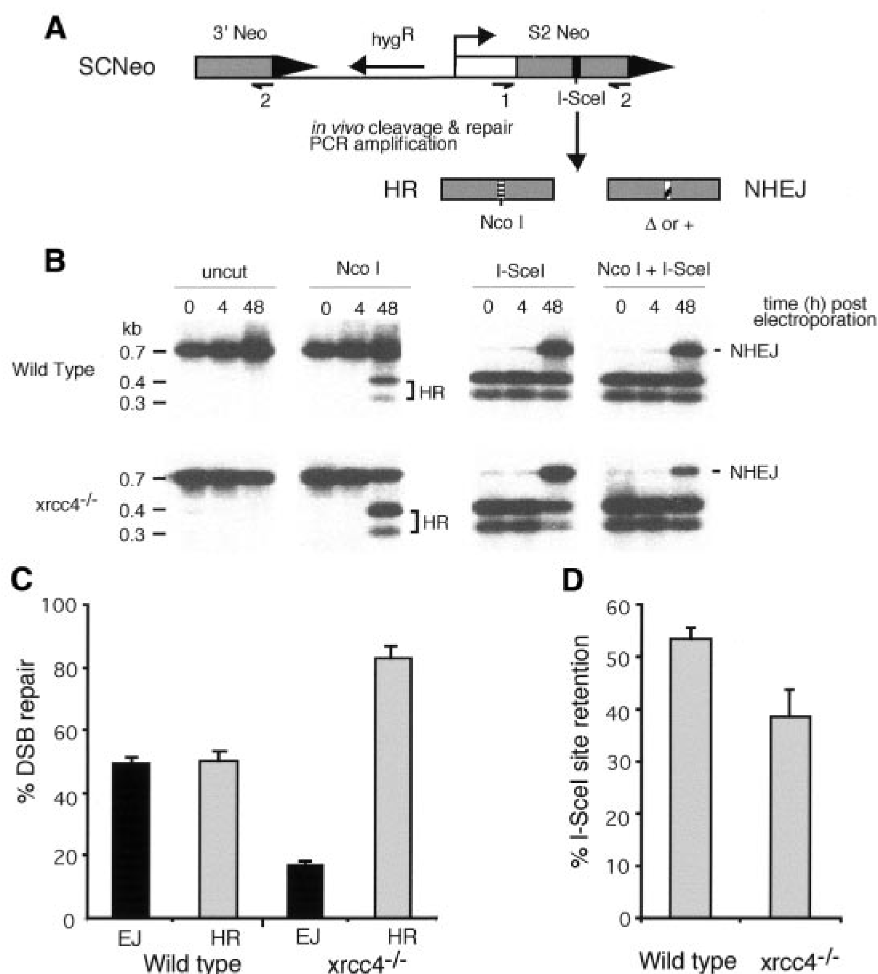


Figure 6. Molecular detection of repair events at a single DSB induced by a single I-*Sce*I cut. (**A**) Strategy of the PCR assay to detect DSB repair (29,30). (**B**) PCR products (digested or non-digested) were resolved by agarose gel electrophoresis. The digestion and phenotypes of the cell lines are indicated in the figure. (**C**) Quantification of the DSB repair products from several experiments. (**D**) Fraction of I-*Sce*I site retention indicating the absence of cleavage or the accuracy of DSB repair.

effect on HR after I-*Sce*I cleavage. However, DNA-PK activity was not completely abolished and the residual traces of activity may have been enough to ensure repair of a single DSB. In this context, a fully active DNA-PK would be required to repair multiple DSBs produced by ionizing radiation. Another hypothesis is that DNA-PKcs would not be required to repair a single DSB and/or that alternative pathways are active in the absence of this kinase activity. During the reviewing process of the present paper, it has been shown that spontaneous recombination and I-*Sce*I-induced recombination were both increased in DNA-PKcs mutant cell lines (44).

The present results show that a *xrcc4* defect stimulates all DSB-induced HR events, including gene conversion and radiation-induced recombination, two Rad51-dependent processes (8). The impact of a late NHEJ step such as Xrcc4 on Rad51 foci assembly, i.e. an early homologous recombination step, has never been addressed. We show here that radiation-induced Rad51 foci assembly is increased in *xrcc4*-deleted lines, indicating that the Rad51 pathway is stimulated in the NHEJ-defective cells. However, the Rad51-independent SSA pathway also appeared to be increased.

When bound to the DNA, Ku proteins can block the access of nucleases preparing the DNA for HR and/or blocks Rad51 recombination-nucleoprotein filament formation. It has been proposed that Ku proteins (NHEJ) compete with Rad52 protein (HR) in binding the DSB and activate their respective pathways (Fig. 8). Ku protein is very efficient in such competition (12). Ku acts upstream of the other components of NHEJ and the present results show that inactivation of a late step of NHEJ (*xrcc4* deletion) stimulates DSB-induced recombination. Either the absence of Xrcc4 protein destabilizes the whole NHEJ complex or some key components, as described (24), or the Ku heterodimer can bind the DSB but, in a second step, could be replaced by the alternative HR, when NHEJ fails. This model implies: (i) that the competition between NHEJ and HR does not simply involve Ku70–Ku80 versus Rad52 but that even a component of a late NHEJ step can affect the choice of DSB repair; (ii) that the two pathways can act sequentially. Then, depending on the structure of the recombining molecules (for example, direct repeats) and on the intracellular level of Rad51 protein (8), HR can be channeled to SSA or gene conversion (Fig. 8).

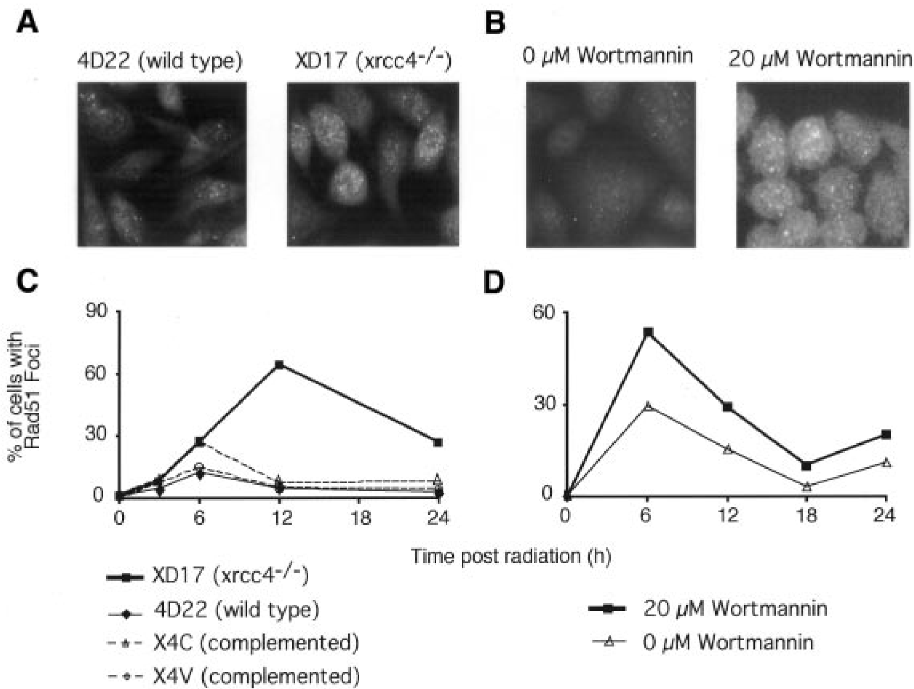


Figure 7. Rad51 foci formation induced by radiation. (**A** and **B**) Example of Rad51 foci. (**C**) Effects of *xrcc4* deletion on the frequency of radiation-induced Rad51 foci at different times (h) after radiation (4 Gy). The names and phenotypes of the different cell lines are indicated in the figure. (**D**) Effect of Wortmannin treatment of CHO-DRA10 cells on radiation-induced Rad51 foci at different times (h) after radiation (5 Gy).

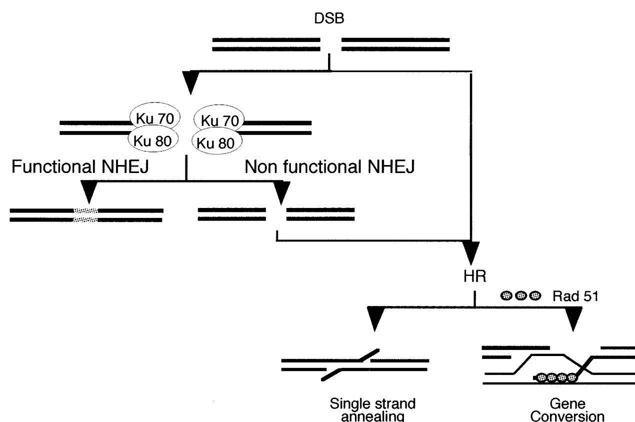


Figure 8. The sequential double competition model for DSB repair, NHEJ versus HR and SSA versus gene conversion. First, NHEJ and HR compete for DSB via the binding of heterodimer Ku70–Ku80 or Rad52, respectively (6). A defect in the binding of Ku70–Ku80 should favor HR (12). Our data indicate that even in the presence of Ku proteins a defect in the late ligation NHEJ step, i.e. a *xrcc4* defect, can favor DSB repair via HR, stimulating both SSA and gene conversion. Then, depending on the structure of the substrates and on the amount of Rad51 (8), HR can be channeled to SSA or gene conversion.

DSB is a highly toxic lesion and can generate genome rearrangement. DSB repair thus appears essential for cell viability, to maintain genome integrity and so to prevent neoplastic development. It is therefore important to unravel how the alternative DSB repair pathways cooperate and/or compete to elucidate whether and how they can protect against tumors.

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