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Characterization of mammalian *RAD51* double strand break repair using non-lethal dominant-negative forms

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In contrast to yeast *RAD51*, mammalian *mRAD51* is an essential gene. Its role in double strand break (DSB) repair and its consequences on cell viability remain to be characterized precisely. Here, we used a hamster cell line carrying tandem repeat sequences with an I-*Sce*I cleavage site. We characterized conservative recombination after I-*Sce*I cleavage as gene conversion or intrachromatid crossing over associated with random reintegration of the excised reciprocal product. We identified two dominant-negative *RAD51* forms that specifically inhibit conservative recombination: the yeast *ScRAD51* or the yeast–mouse chimera *SMRAD51*. In contrast, the mouse *MmRAD51* stimulates conservative recombination. None of these *RAD51* forms affects non-conservative recombination or global DSB healing. Consistently, although resistance to γ-rays remains unaffected, *MmRAD51* stimulates whereas *ScRAD51* or *SMRAD51* prevents radiation-induced recombination. This suggests that *mRAD51* does not significantly affect the global DSB repair efficiency but controls the classes of recombination events. Finally, both *ScRAD51* and *SMRAD51* drastically inhibit spontaneous recombination but not cell proliferation, showing that *RAD51*-dependent spontaneous and DSB-induced conservative recombination can be impaired significantly without affecting cell viability.

Keywords: double strand break repair/homologous recombination/mammalian cells/*RAD51*/trans-species dominant-negative allele

Introduction

A DNA double strand break (DSB) is a lesion that can be produced by genotoxic agents such as ionizing radiation or that can occur during physiological processes such as yeast meiosis (Sun *et al*., 1989). Repair of DSBs is essential to ensure genome integrity and cell viability, but may also result in genome rearrangements. Two major processes can repair DSBs: non-homologous end-joining (NHEJ) or a homology-directed (HD) process that takes advantage of a homologous sequence to repair the DSB (Liang *et al*., 1998; for reviews see Baumann and West, 1998; Kanaar and Hoeijmakers, 1998). Actually an HD process refers to two independent pathways: the non-conservative single strand annealing (SSA) process that occurs between direct repeat sequences (Lin *et al*., 1984), and the conservative homologous recombination, initiated by a DNA strand invasion step. In yeast, conservative recombination is thus dependent on *ScRAD51*. In contrast, SSA is *RAD51* independent (Ivanov *et al*., 1996). Thus, in mammalian cells, the comprehension of DSB repair regulation presupposes the precise determination of the different pathways involved.

The search for *RAD51* orthologues has led to the identification of a growing number of mammalian *RAD51* homologues (for a review see Thacker, 1999). This suggests either redundancy of functions or the splitting up of *ScRAD51*'s role on several mammalian genes, or an evolutionary divergence of function for some of the homologues. In this context, the mammalian *mRAD51* presents both similarities to and differences from *ScRAD51*, and its exact role in DSB repair remains to be established. Human Rad51 is able to promote DNA strand exchange *in vitro*, but less efficiently and with some biochemical differences compared with the yeast protein (Baumann *et al*., 1996; Gupta *et al*., 1997; Benson *et al*., 1998). *In vivo*, chromosome breakages and a decrease in sister chromatid exchanges have also been reported prior to cell death in *rad51*$^{-/-}$ depleted DT40 cells, a chicken immortalized B-cell line harbouring a particularly high frequency of spontaneous gene conversion (Sonoda *et al*., 1998, 1999). Moreover, mouse Rad51 protein was shown to associate with mouse meiotic chromosomes and to relocalize to nuclear foci in cells treated with genotoxic agents (Haaf *et al*., 1995; Barlow *et al*., 1997). However, contrasting with the situation in yeast and bacteria, *mRAD51* has been described to be involved in cell proliferation and is an essential gene in vertebrates since null mutants are not viable (Tsuzuki *et al*., 1996; Sonoda *et al*., 1998). Moreover, *mRAD51* participates, via *BRCA2*, in the regulation of p53 transactivation activity (Marmorstein *et al*., 1998). Since the transactivation activity of p53 is involved in cell cycle control but is not correlated with the role of p53 in recombination (Dudenhoffer *et al*., 1999; Saintigny *et al*., 1999), this result may suggest the participation of *mRAD51* in the cell cycle checkpoint controlled by p53 transactivation activity. Thus, chromosomal aberrations observed in *rad51*$^{-/-}$ depleted cells could also be explained by cell cycle defects.

Several hypotheses could account for these apparent paradoxes. The first hypothesis is that *mRAD51* controls one DSB repair pathway essential for cell viability, as proposed for the chicken DT40 lines (Sonoda *et al*., 1998, 1999; Morrison *et al*., 1999). The second hypothesis is that *mRAD51* possesses two distinct roles: a non-essential role controlling DSB repair and another essential for cell viability. A third hypothesis is that *mRAD51* is involved in an essential function but not in homologous

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recombination, while other homologues of *RAD51* would be required for homologous recombination. This latter hypothesis is based on the facts that a growing number of *RAD51* homologues are being reported and that some of them, such as *XRCC2* and *XRCC3*, are involved in HD DSB repair (Johnson *et al*., 1999; Pierce *et al*., 1999).

To determine whether *mRAD51* actually acts on DSB repair in mammalian cells and to characterize its putative role precisely, we used the CHO cell line DRA10 carrying a recombinant substrate with a unique site for the rare cutting endonuclease I-*Sce*I (Liang *et al*., 1998). We show that overexpression of mouse *MmRAD51* does not substantially stimulate the overall DSB repair but specifically stimulates the conservative recombination DSB repair pathway. More importantly, we also identified forms of *RAD51* (the yeast *ScRAD51* or the yeast–mouse chimera *SMRAD51*) that specifically inhibit DSB-induced conservative recombination as well as spontaneous recombination. None of the *RAD51* forms used here affect SSA. These results indicate that *mRAD51* participates in DSB repair, does not modify the global efficiency of DSB repair but acts on the balance controlling the different classes of events: conservative versus non-conservative recombination. Finally, our results show that it is possible to decrease the efficiency of *RAD51* DSB repair and *RAD51* spontaneous recombination substantially without affecting cell viability and proliferation.

Results

Strategy and cell lines used

In order to define the DSB repair pathway controlled by *mRAD51* precisely, we measured the impact of the overexpression of different forms of *RAD51* on recombination induced either by an acute DSB targeted to the recombination substrate or by ionizing radiation. It has been shown that the overexpression of *mRAD51* in mammalian cells results in an increase in spontaneous recombination between intrachromosomal repeat sequences (Vispé *et al*., 1998; Arnaudeau *et al*., 1999; Huang *et al*., 1999). However, it has been argued that overexpression of *RAD51* interferes with DNA metabolism and results in DNA lesions that could induce recombination, as has been pointed out (Huang *et al*., 1999). Importantly, the role of *mRAD51* in DSB repair and the putative pathways involved have not been addressed in mammalian cells.

In addition, in an attempt to identify a dominant-negative *RAD51* form, we expressed *ScRAD51* from *Saccharomyces cerevisiae*. Trans-species dominant-negative effects have been described between *RAD52* from *Kluyveromyces lactis* and *S.cerevisiae* (Milne and Weaver, 1993). More specifically, the active species for recombination is the RecA/Rad51 nucleoprotein filament (Radding, 1991). Alignment of mouse MmRad51 and ScRad51 shows that the yeast protein is longer than that of the mouse, with a block of 55 additional amino acids at the N-terminal extremity (Figure 1A). Furthermore, *ScRAD51* interacts with the human *RAD51* and *XRCC3* (Schild *et al*., 2000). Our hypothesis was that *ScRAD51* may act as a dominant-negative allele in mammalian cells by interacting with endogenous *mRAD51*, leading to the poisoning of the *mRAD51* nucleoprotein filament via the extra N-terminal part of the yeast protein. Another possibility could be that *ScRAD51* would lead to the titration of the endogenous recombination complex. To check these hypotheses, we also expressed the chimera SMRad51 composed of the 55 N-terminal amino acids from ScRad51 fused to the entire MmRad51, and ΔNtScRad51 corresponding to ScRad51 with its 55 N-terminal amino acids deleted (see Figure 1A).

These different *RAD51* forms are expressed in the parental CHO-DRA10 line (Liang *et al*., 1998), and the names of the derivative cell lines are listed in Figure 1A. Figure 1B shows the expression of the endogenous and exogenous *RAD51*s, verified by western blotting. Expression of ΔNt*ScRAD5*1 has been verified by RT–PCR (data not shown).

Characterization of the DSB-induced recombination events

The CHO-DRA10 cell line and its derivatives carry a unique recombination substrate with the cleavage site for the rare-cutting endonuclease I-*Sce*I (see Figure 2). Transient expression of I-*Sce*I efficiently produces a DSB in the recombination substrate (Liang *et al*., 1998).

Single-drug resistance to G418 (Neo^R) monitors recombinant clones arising by an HD recombination process, both conservative and non-conservative (SSA) events (Figure 2). Double resistance to both G418 (Neo^R) and hygromycin (Hyg^R) monitors only conservative events due to gene conversion not associated with crossing over or associated with crossing over, but with a faithful reintegration of the excised Hyg^R sequence (Figure 2B). Neo^R–Hyg^s monitors SSA events plus crossing over with loss of the pop out circle (POC) (Figure 2A).

To demonstrate the occurrence of the recombination products predicted in Figure 2, we analysed the molecular structure of the recombination locus from Neo^R–Hyg^R clones by Southern blotting (Figure 3A). We analysed 11 Neo^R–Hyg^R recombinant clones, seven from the control line Cm3 and four from the *MmRAD51*-overexpressing line Rm2 (Figure 3). With the S2neo sequence probe (probe A), nine clones show the gene conversion 4 kb band and two recombinant clones show the 1.1 kb deletion event band (Figure 3B), despite the fact that these two clones exhibit a hygromycin-resistant phenotype. We thus probed the same filters with the hygromycin resistance gene sequence (probe B). The structure of the nine gene conversion events previously identified is confirmed, since they show the 4 kb restriction band with this new probe (Figure 3C). In the two deleted clones, however, the presence of the hygromycin resistance gene sequence is revealed but at a different location (Figure 3C), suggesting a random integration after a crossing over event. These results show that Neo^R–Hyg^R clones actually score gene conversion and some crossing over events, both initiated by strand invasion (Szostak *et al*., 1983). In addition, these results demonstrate the existence of the predicted DNA fragment excised by an intrachromatid crossing over (see Figure 2B), followed by its random integration into the genome.

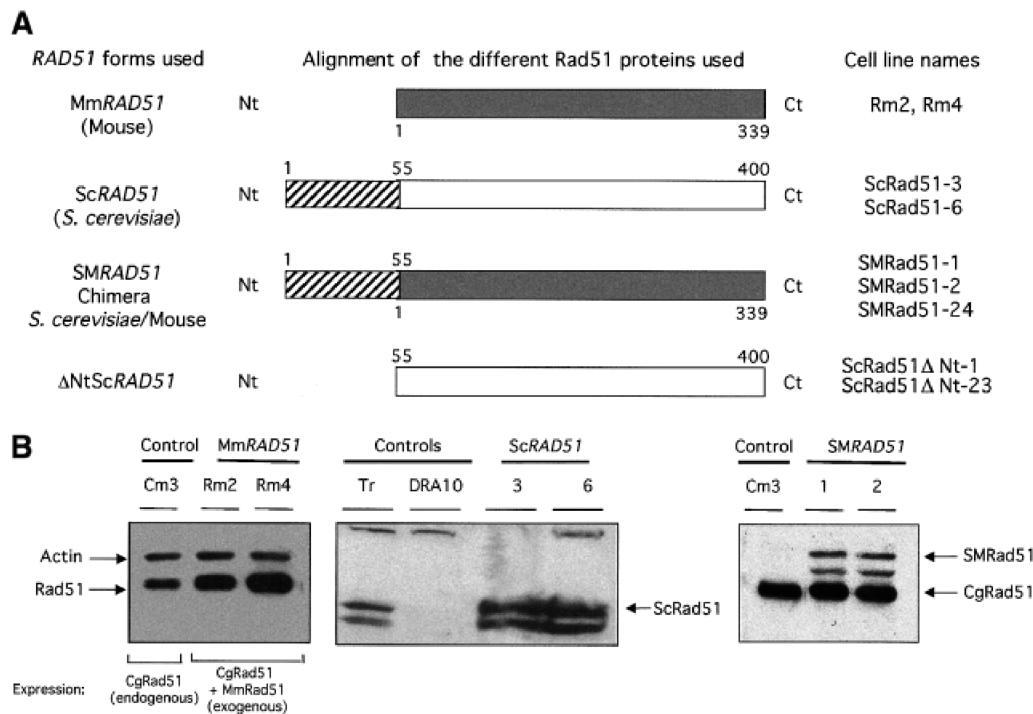


Fig. 1. Overexpression of the different *RAD51* genes in hamster cells. (**A**) Structure of the *RAD51* genes used. The boxes correspond to the homologous region between the different *RAD51* genes: grey, mouse *MmRAD51*; white, yeast *ScRAD51*. *ScRAD51* is longer at the N-terminal part. The white box corresponds to the region homologous to *MmRAD51* and the hatched box to the extra sequence. The numbers correspond to the positions of the amino acids. The numbers in black correspond to the yeast amino acids and the numbers in grey correspond to the mouse amino acids. The chimeric protein SMRad51 was constructed with the N-terminal part of ScRad51 (hatched box) fused to the entire MmRad51 (white box). ΔNtScRad51 corresponds to ScRad51 with 55 N-terminal amino acids deleted. These proteins are expressed in the CHO-DRA10 line, and the corresponding derivative lines are listed on the right. (**B**). Overexpression of exogenous *RAD51*. Protein extracts were obtained from stable transfectants. The expression of *MmRAD51* and *SMRAD51* was verified by western blotting using an antibody raised against the human Rad51 protein and normalized with an anti-actin antibody. Rm2 and 4 correspond to two independent stable transfectants overexpressing *MmRAD51*. SMRad51-1 and SMRad51-2 correspond to two independent stable transfectants overexpressing the fusion *SMRAD51*. Cm3 is a control clone corresponding to the CHO-DRA10 line transfected with the empty pCDNA3.1puro plasmid. Expression of *ScRAD51* is visualized by western blotting using an anti-ScRad51 antibody. The first lane corresponds to transient expression (Tr) of *ScRAD51* (used as control). The second lane corresponds to extracts from mock-transfected cells (CHO-DRA10). The third and fourth lanes correspond to two independent clones with stable expression of *ScRAD51*. MmRad51 is Rad51 from *Mus musculus*; ScRad51 is Rad51 from *S.cerevisiae*; CgRad51 is Rad51 from *Cricetellus griseus*; and SMrad51 is a chimeric fusion protein MmRad51–ScRad51.

RAD51 specifically affects conservative recombination events in mammalian cells

We measured here the impact of the overexpression of the different *RAD51* forms (Figure 1) on recombination induced by an I-*Sce*I DSB, targeted to the recombination substrate.

We first verified that the transfection efficiency of the I-*Sce*I expression vector was equivalent in the control lines and the overexpressing *RAD51* cell lines (data not shown). We also measured the same 100- to 1000-fold increase in the frequency of G418-resistant colonies (total recombinant clones) following transient transfection of the I-*Sce*I expression vector (Liang *et al.*, 1998). As compared with the control cell line (Cm3), increases in the frequency of G418-resistant clones by factors of 1.8–2.6 are found in lines expressing *MmRAD51* and decreases in the frequency of G418-resistant clones by factors of 1.7–2.4 in lines expressing *ScRAD51* or *SMRAD51* (Table I).

The impact of overexpression of the different *RAD51* forms is much stronger on the frequency of Neo^R–Hyg^R recombinants. Indeed, overexpression of *MmRAD51* stimulates the frequency of Neo^R–Hyg^R colonies by 4–5 times compared with the control line Cm3 (Table I). In contrast, the frequency of Neo^R–Hyg^R colonies is decreased 48 times by expression of *ScRAD51*. The chimera *SMRAD51* also exhibits a pronounced dominant-negative effect since inhibition of the occurrence of Neo^R–Hyg^R colonies is decreased 42- to 127-fold compared with the control line Cm3 (Table I).

The percentage of conservative events (double-resistant Neo^R–Hyg^R colonies) relative to all the recombinant colonies (Neo^R alone) was examined (Figure 4). Since this value is normalized to the frequency of Neo^R colonies (representing the whole recombinant population), the calculation is based on an internal standard and is independent of transfection and cleavage efficiencies. In the Cm3 control line, the frequency of conservative events (Neo^R–Hyg^R colonies) is 27%, and thus the non-conservative events represent 73% of the total recombinant colonies. Comparable ratios are obtained with the parental CHO-DRA10 line (Liang *et al.*, 1998). In the lines overexpressing the *MmRAD51* gene, the relative proportion of Neo^R–Hyg^R colonies rises to 65–72% (Figure 4). In contrast, the percentage of Neo^R–Hyg^R colonies falls to 2% in the line expressing *ScRAD51*, and to 1.6 or 0.9% in lines expressing *SMRAD51* (Figure 4). The expression of

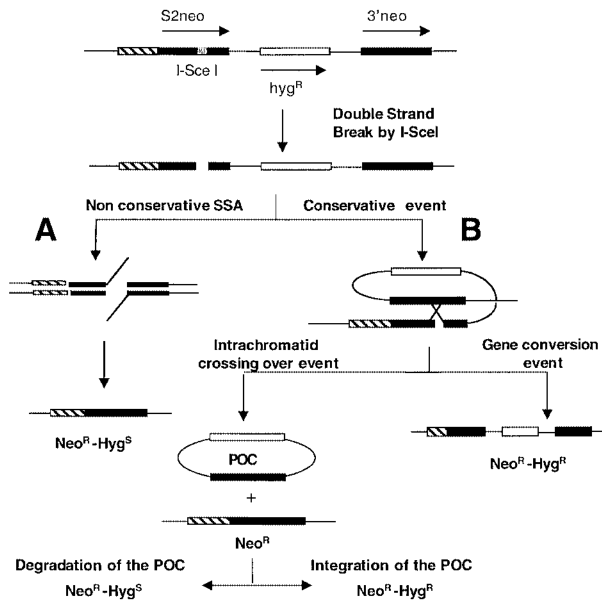


Fig. 2. Strategy used to measure DSB-induced recombination events. After the formation of a DSB induced by I-*Sce*I, two processes can compete. (**A**) The non-conservative SSA that produces Neo^R and hygromycin-sensitive recombinants. (**B**) Homologous recombination events leading to gene conversion either with or without crossing over. The products of gene conversion events are resistant to both G418 and hygromycin (Neo^R–Hyg^R). Intrachromatid crossing over leads to the formation of POCs. If the POCs are eliminated, the products are only Neo^R; if the POC is reintegrated into the genome, it could result in a Neo^R–Hyg^R colony. Thus, double resistance (Neo^R–Hyg^R) scores only conservative recombination events. Not shown are unequal sister chromatid exchange events in which the Hyg^R segregates from the Neo^R gene.

the deleted ΔNt*ScRAD51* has no effect on the distribution of the different events, compared with the control lines (Figure 4). These results demonstrate a dominant-negative effect of *ScRAD51* and of the chimera *SMRAD51* with respect to the homologous recombination initiated by strand invasion. In addition, the dominant-negative effect requires the 55 N-terminal amino acids of the *ScRAD51* product.

The present data agree completely with the view that DSBs can be repaired by NHEJ, by a conservative strand invasion mechanism or by SSA. Indeed, expression of *MmRAD51, ScRAD51* or *SMRAD51* only very moderately affects the total frequency of recombinants, indicating that the ratio of NHEJ to HD repair is not modified significantly. In addition, overexpression of *MmRAD51* specifically increases the occurrence of conservative events, whereas overexpression of *ScRAD51* or of the chimera *SMRAD51* prevents conservative events. Thus, the different *RAD51* forms do not affect the SSA process itself, but modify the ratio of conservative events to SSA.

RAD51 controls radiation-induced recombination but not radiation resistance

In the above experiments, *RAD51* does not substantially affect the total efficiency of DSB healing but rather the ratio of the different recombination classes. Since DSB is one of the main lethal lesions induced by ionizing radiation, we measured the impact of expression of

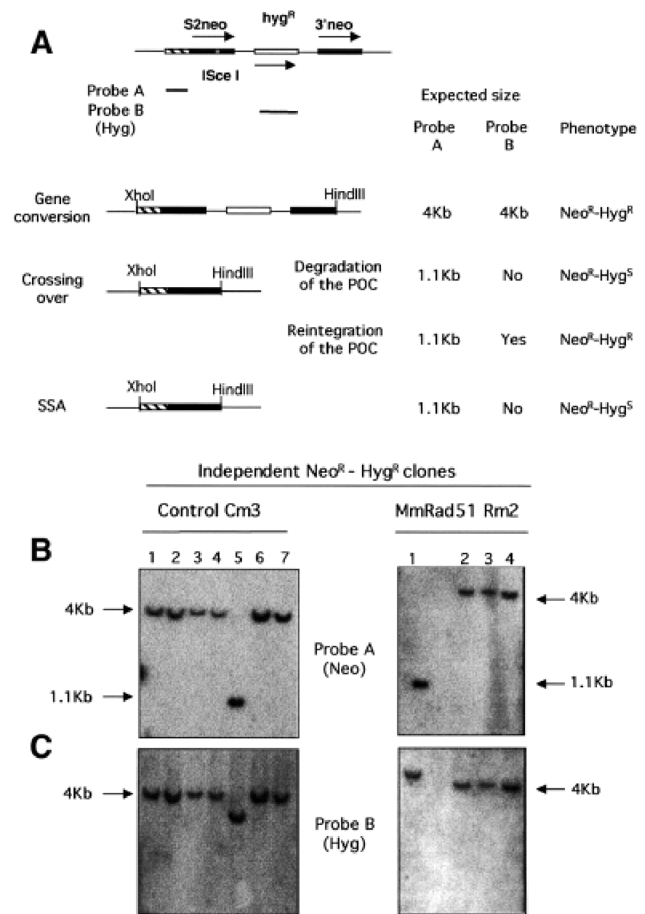


Fig. 3. (**A**) Restriction pattern of recombinant clones induced by an I-*Sce*I-generated DSB. Probe A corresponds to the S2neo promoter sequence and probe B to part of the hygromycin-resistant gene, located between the two *neo* cassettes. The corresponding expected *Xho*I–*Hin*dIII sizes (with both probes) and the corresponding resistance phenotype are indicated. POC: pop out circle (see Figure 2). (**B**) Southern blot analysis with probe A. The sizes of the bands are indicated on the sides of the panels. Left panel: seven independent Neo^R–Hyg^R clones (numbered on the top of the panel) from the control cell line Cm3. Right panel: four independent Neo^R–Hyg^R clones (numbered on the top of the panel) from the Rm2 cell line, overexpressing *MmRAD51*. (**C**) The same filters as in (B) but hybridized to probe B, characteristic of the intervening sequence.

different *RAD51* forms on radiation resistance and on radiation-induced recombination.

None of the different *RAD51* forms modify the radiation resistance of the recipient cell lines in the dose range tested (Figure 5A). These results are consistent with our previous I-*Sce*I experiments showing that *mRAD51* does not affect the efficiency of global DSB healing but controls the classes of recombination events. Indeed, radiation-induced recombination is strongly dependent on the type of exogenous *RAD51* expressed: *MmRAD51* expression stimulates recombination frequency 10- to 20-fold after a dose of 6 Gy, whilst *SMRAD51* as well as *ScRAD51* completely abolish the induction of radiation-induced recombination (Figure 5B). These results show that in an asynchronous cell population of mammalian cells, radiation resistance is a *RAD51*-independent process whereas radiation-induced recombination is a *RAD51*-dependent process.

Table I. Effect of different Rad51 proteins on DSB-induced recombination

Cell line	Mean Neo^R frequency ($\times 10^{-3}$)[a]	Stimulation factor[b]	Mean Neo^R–Hyg^R frequency ($\times 10^{-3}$)[a]	Stimulation factor[b]
Cm3	1.19 ± 0.2		0.38 ± 0.1	
Rm2	3.09 ± 0.7	2.6	2 ± 0.4	5.3
Rm4	2.1 ± 0.9	1.8	1.59 ± 0.9	4.2
		Inhibition factor[b]		Inhibition factor[b]
ScRad51-3	0.64 ± 0.4	1.8	0.008 ± 0.005	47.5
SMRad51-1	0.71 ± 0.1	1.7	0.009 ± 0.007	42.2
SMRad51-2	0.5 ± 0.2	2.4	0.003 ± 0.002	126.7

[a]Mean values from three independent experiments.
[b]Compared with the Cm3 cell line control.

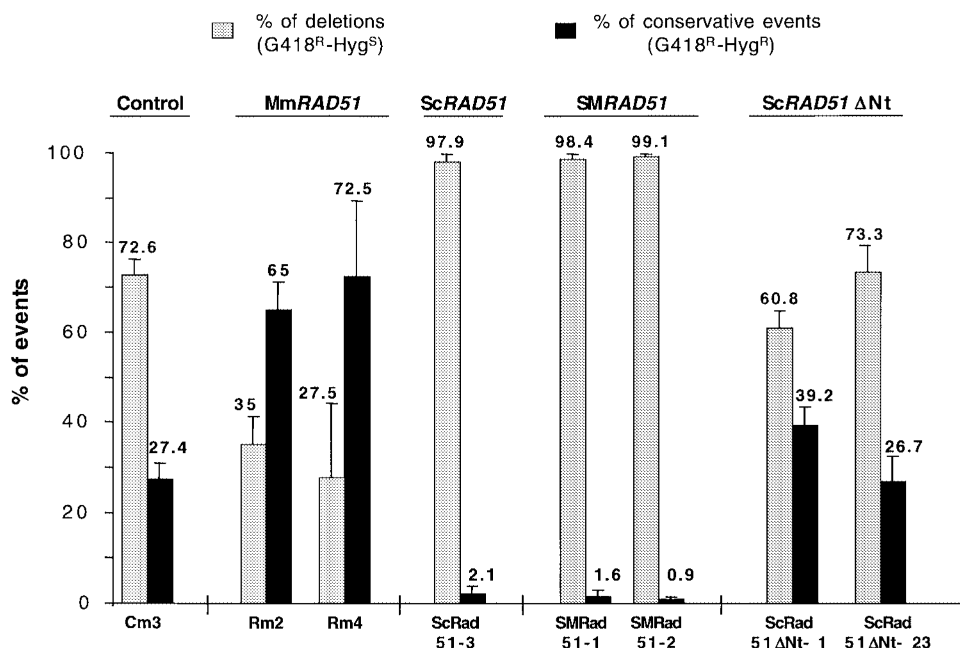


Fig. 4. Effect of the overexpression of *MmRAD51*, *ScRAD51* or *SMRAD51* on the frequency of conservative recombination events. Control refers to the parental line transfected with empty expression vector (Cm3). *MmRAD51* refers to two independent transfectants overexpressing *MmRAD51*; *ScRAD51* refers to one transfectant expressing yeast *ScRAD51*; SMRad51-1 and SMRad51-2 refer to two independent clones expressing the chimera *SMRAD51*; ScRad51ΔNt-1 and ScRad51ΔNt-23 refer to two independent clones expressing the deleted ΔNt*ScRAD51*. Values correspond to the percentage of double-resistant (Neo^R–Hyg^R) colonies (black boxes) and single-resistant (Neo^R) colonies (grey boxes). The percentage of conservative recombination events is calculated from the ratio of the number of Neo^R–Hyg^R clones to the Neo^R clone frequency. The percentages are indicated on the top of the corresponding histograms.

Spontaneous recombination is affected by ScRAD51 and SMRAD51

RAD51 is an essential gene in non-irradiated mammalian cells. It has been proposed that spontaneous recombination controlled by *mRAD51* would be essential for cell viability by repairing spontaneous damage occurring during replication; this would also be different from yeast. However, the above results show that *RAD51* recombinational DSB repair can be decreased substantially without modifying the cell viability after radiation. Taking into account our results, the proposed hypothesis should thus consider that spontaneous and DSB-induced recombination are two separable mechanisms involving *mRAD51*. We thus tested whether spontaneous recombination can also be inhibited by *ScRAD51* and *SMRAD51*, as is the case for DSB-induced recombination.

Overexpression of *mRAD51* has previously been reported to increase spontaneous recombination (Vispé *et al*., 1998; Arnaudeau *et al*., 1999; Huang *et al*., 1999). However, none of these studies used a fluctuation analysis to measure the process. In order to measure spontaneous recombination in an exponentially growing population, we performed fluctuation analysis using the Luria and Delbruck assay (Luria and Delbruck, 1943), adapted by Capizzi and Jameson (1973). As expected, we confirmed that *MmRAD51* stimulates spontaneous recombination 3- to 4-fold per cell per generation (Figure 6A).

In contrast to *MmRAD51*, *ScRAD51* and the chimera *SMRAD51* strongly decrease the spontaneous recombination frequency in mammalian cell lines, in an interspecies dominant-negative manner (Figure 6B). The recombination inhibition is so pronounced that the recombination

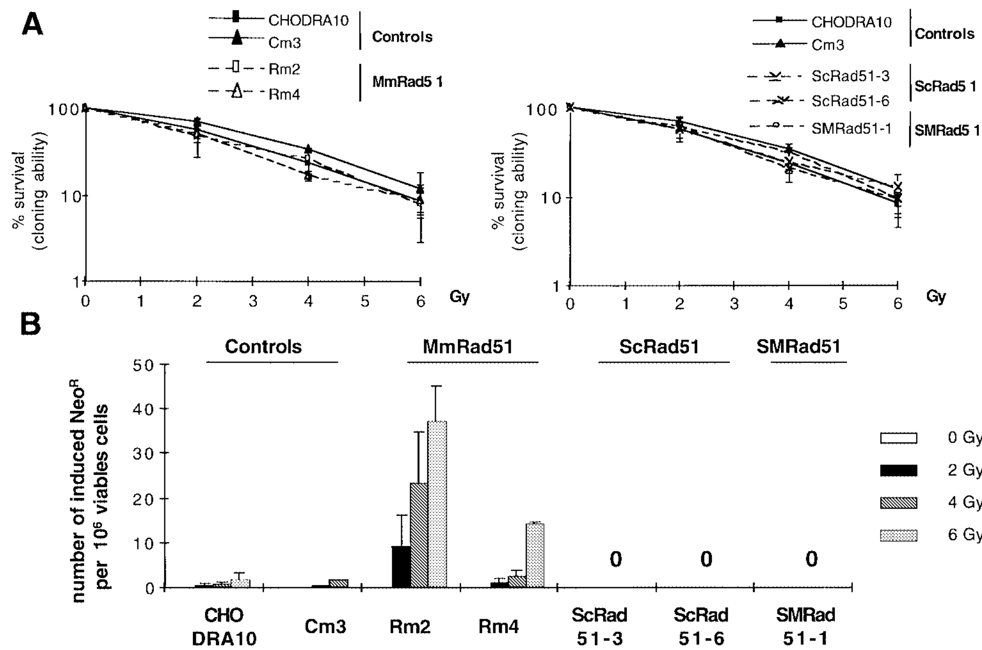


Fig. 5. Effect of *RAD51* on radiation resistance (**A**) and on radiation-induced recombination (**B**). Cells were irradiated at the doses indicated. Controls correspond to the parental CHO-DRA10 line and to Cm3, which is CHO-DRA10 transfected with the empty expression vector. Rm2 and Rm4 are two independent clones overexpressing *MmRAD51*. ScRad51-3 and ScRad51-6 are two independent clones expressing *ScRAD51*. Radiation-induced recombination (B): the values correspond to the number of Neo^R in 10^6 surviving irradiated cells, following subtraction of the number of Neo^R in 10^6 non-irradiated cells.

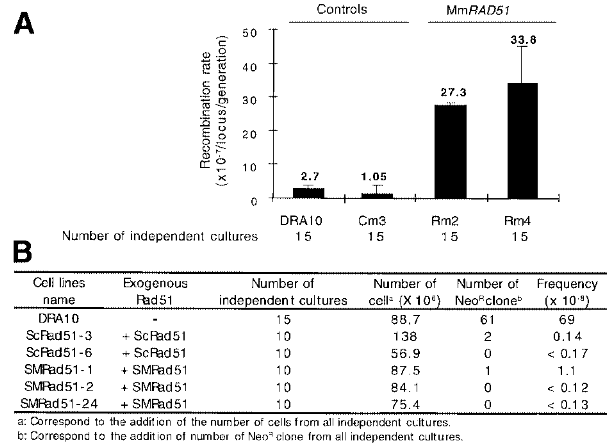
rate cannot be calculated by the Luria and Delbruck test. Indeed, most of the cultures contain no recombinant colonies. Consequently, *ScRAD51* as well as *SMRAD51* also inhibit spontaneous recombination in growing cells, in a dominant-negative manner.

Finally, despite the fact that spontaneous recombination is almost totally defective, neither the generation time, the plating efficiency nor the cell cycle measured by flow cytometry (data not shown) are modified in the different lines expressing *ScRAD51* or *SMRAD51*, showing that cell viability and proliferation are unaffected in these lines.

Discussion

The different lines devised here derive from the same parental line. Thus, recombination frequencies are calculated for one copy of substrate located at the same locus in each cell line. Using these lines, we have determined the precise pathway involving *mRAD51* for DSB repair in mammalian cells: it acts specifically on conservative recombination and does not affect non-conservative SSA. This phenotype is similar to that of *ScRAD51* in yeast, despite the numerous differences between the yeast and mammalian *RAD51* protein products. However, an important difference from the yeast model is that in mammalian cells, *mRAD51* DSB repair can be decreased substantially without significant effects on the global DSB repair efficiency. Indeed, in yeast, alteration of the *RAD51* pathway leads to a drastic decrease in the global DSB repair efficiency. In mammalian cells, a balanced regulation maintains the total level of DSB repair efficiency, but modifies the class of recombination events.

Southern blot analysis of DSB-induced Neo^R–Hyg^R clones shows the existence of both gene conversion and



Cell lines name	Exogenous Rad51	Number of independent cultures	Number of cell[a] (X 10⁶)	Number of NeoR clone[b]	Frequency (x 10⁻⁸)
DRA10	-	15	88,7	61	69
ScRad51-3	+ ScRad51	10	138	2	0.14
ScRad51-6	+ ScRad51	10	56.9	0	< 0.17
SMRad51-1	+ SMRad51	10	87.5	1	1.1
SMRad51-2	+ SMRad51	10	84.1	0	< 0.12
SMRad51-24	+ SMRad51	10	75.4	0	< 0.13

a: Correspond to the addition of the number of cells from all independent cultures.
b: Correspond to the addition of number of NeoR clone from all independent cultures.

Fig. 6. Effect of the overexpression of the different *RAD51* forms on spontaneous recombination. (**A**) *MmRAD51* stimulates the spontaneous recombination rate measured by fluctuation analysis using the Luria and Delbruck test (Luria and Delbruck, 1943). The names of the different lines are indicated under the histograms. DRA10, parental line; Cm3 (control line), DRA10 transfected with empty expression vector; Rm2 and Rm4 correspond to two independent clones overexpressing *MmRAD51*. The number of independent cultures for each line is reported under the histograms. (**B**) *ScRAD51* and *SMRAD51* inhibit the frequency of spontaneous recombination. A recombination rate cannot be calculated because most of the cultures do not contain any recombinant. The frequency of recombination reported here corresponds to the sum of the recombinants from all the independent cultures in relation to the total number of cells summed from all the independent cultures. DRA10 is the parental line; ScRad51-3 and ScRad51-6 correspond to two independent clones expressing *ScRAD51*; SMRad51-1, SMRad51-2 and SMRad51-24 refer to three independent clones expressing the chimera *SMRAD51*.

crossing over events, the former being detected more frequently in the system used here. The restriction patterns of some clones are compatible with an intrachromatid crossing over followed by a random reintegration of the excised DNA. Although the putative circular nature of the intermediate excised DNA remains to be established, the present data demonstrate the genome rearrangement predicted by the intrachromatid crossing over model (see Figure 2). Such processes have important biological consequences with regard to genome stability/variability. For example, POCs created by intrachromatid crossing over in rDNA repeats are involved in cell ageing in yeast (Sinclair and Guarente, 1997). In addition, random integration of an excised fragment generated by a DSB-induced intrachromatid crossing over can inactivate a recipient integration locus. Taking into account the high number of homologous repeat sequences dispersed throughout the genome, the process described here could correspond to an actual mutagenic mechanism, particularly after treatments generating DSBs, such as ionizing radiation.

When inducing one DSB, targeted to one repeat of the duplication, the overexpression of *MmRAD51* only slightly stimulates the frequency of total recombinant clones (Neo^R). However, the ratio of Neo^R–Hyg^R to Neo^R clones is increased several-fold. These results demonstrate that *MmRAD51* specifically increases conservative recombination events and not SSA events (most of the Neo^R clones). In yeast, *ScRAD51* acts in a complex comprising the components of the *RAD52* epistasis group (Milne and Weaver, 1993; Hays *et al*., 1995; Johnson and Symington, 1995; Rattray and Symington, 1995). In mammalian cells, homologues of most of these genes have been described. In addition, *mRAD51* interacts with another set of proteins without homologues in yeast, such as the tumour suppressor and cell cycle control proteins p53, BRCA1 and BRCA2 (Sturzbecher *et al*., 1996; Buchhop *et al*., 1997; Mizuta *et al*., 1997; Scully *et al*., 1997; Marmorstein *et al*., 1998). In line with this, p53 is involved in homologous recombination independently of its role in G_1/S transition and of its transactivation activity (Dudenhoffer *et al*., 1999; Saintigny *et al*., 1999), a result compatible with a role for p53 in the homologous recombination process. In addition, *BRCA1* has been shown to be involved in DSB repair in mammalian cells (Moynahan *et al*., 1999). Taken together, these data suggest that *mRAD51* acts in one (or several) highly elaborated complex(es). This raises the question as to how overexpression of only one component of such (a) complex(es) can stimulate the whole recombination process. The present results suggest that *RAD51* is a limiting factor for homologous recombination in mammalian cells. Recombination stimulation by overexpression of *RAD52*, another component of the complex, has also been described in mammalian cells (Park, 1995). However, it is not known whether both conservative recombination and SSA events are stimulated since *RAD52* also promotes SSA (Fishman-Lobell *et al*., 1992; Mortensen *et al*., 1996; Van Dyck *et al*., 1999). Thus, it cannot be excluded that this observation in mammalian cells refers to stimulation of SSA events. Consistent with this hypothesis, it has been suggested that Rad52 and Ku proteins compete for binding to DSBs, leading to the channelling of the DSB repair to an HD DSB repair or to an NHEJ process (Van Dyck *et al*., 1999). Our results suggest that *RAD51* does not substantially affect the channelling of the NHEJ versus the HD repair process, but that inside the HD pathway (the *RAD52* pathway), *RAD51* may favour the strand invasion over the SSA recombination pathway. This observation is consistent with the fact that *mRAD51* does not modify radiation sensitivity since the global efficiency of DSB healing remains essentially unaffected. Moreover, the fact that the status of *RAD51* affects the extent of radiation-induced recombination is compatible with the hypothesis of a channelling role for *mRAD51* towards the conservative recombination pathway. The simplest explanation would be that *RAD51* constitutes a limiting factor for the homologous recombination pathway. *RAD52* would bind the DSB and would predominantly channel repair to SSA, the most efficient HD process in these experiments (Liang *et al*., 1998; this study). Alternatively, *RAD52* could load *mRAD51* on the broken DNA, resulting in the channelling of DSB repair towards a conservative homologous recombination process (Figure 7). With such a hypothesis, increasing the intracellular amount of *mRAD51* should favour the channelling of the repair to strand invasion conservative events, as is actually the case in our experiments.

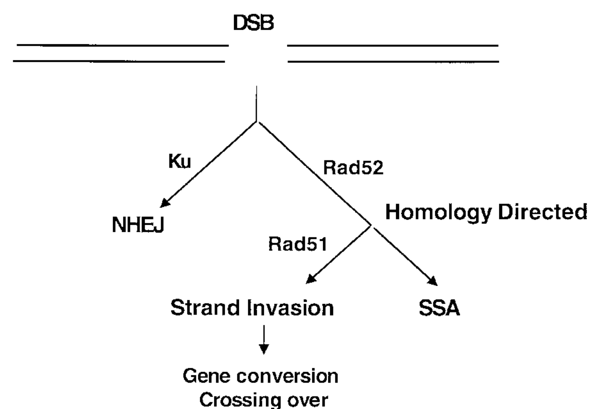


Fig. 7. Role of *RAD51* in DSB repair in mammalian cells. On a DSB, Ku and *RAD52* compete to process the DSB. Ku orientates DSB repair towards NHEJ. Conversely, *RAD52* orientates repair to an HD process that can be either SSA or homologous recombination. In the absence of *RAD51*, SSA takes place. This is the most frequent reaction in the parental lines. If *mRAD51* is present, it can bind to *RAD52* and channel the reaction to homologous recombination initiated by strand invasion.

Expression of *ScRAD51* specifically inhibits conservative events in mammalian cells. The 55 N-terminal amino acids of the protein appear to be essential for this specific dominant-negative effect. Indeed, ScRad51 lacking these N-terminal amino acids (ΔNtScRad51) loses its dominant-negative effect. In contrast, the fusion of these 55 amino acids to mouse MmRad51 (SMRad51) confers a specific dominant-negative effect to the chimera. Our hypothesis was that *ScRAD51* molecules would be incorporated into the *mRAD51* single-stranded DNA nucleoprotein filament, generally considered as the active intermediate in the homologous recombination process (Radding, 1989); indeed, *ScRAD51* has been shown to interact with human *RAD51* (Schild *et al*., 2000). However, our hypothesis was also that the divergences between the two kinds of *RAD51* molecules would then poison the

nucleoprotein filament and inhibit the *RAD51*-dependent recombination. Consistent with this hypothesis is the fact that the chimera *SMRAD51* specifically inhibits recombination initiated by a strand invasion, whereas the wild-type *MmRAD51* stimulates it. An alternative explanation would be that the remaining homologies between the protein products of *mRAD51* and *ScRAD51* are sufficient to promote interactions with some of *mRAD51*'s partners, but that the divergence would impair the formation of a functional recombination complex. *ScRAD51* could thus titrate the components of the recombination complex. Our results do not argue in favour of this second hypothesis. Indeed, the N-terminal part of ScRad51 is essential to poison mammalian homologous recombination, but this part of the protein is absent in its mammalian orthologue; in contrast, the rest of the yeast protein (amino acids 55–400) is unable to titrate the mammalian complex, despite its strong sequence homologies to mRad51 that should favour interactions with endogenous mRad51's partners. In addition, interactions at least with Rad52 have been reported to be species specific (Shen *et al*., 1996). Nevertheless, from the genetic point of view, *ScRAD51* as well as *SMRAD51* are dominant-negative alleles specific for homologous recombination in mammalian cells; they constitute useful and universal tools to devise cell lines specifically defective in conservative recombination without affecting cell proliferation and viability.

XRCC2 and XRCC3 proteins show homologies to Rad51 and they interact with each other and with Rad51 (Schild *et al*., 2000). Cell lines mutant for *XRCC2* or *XRCC3* are deficient in recombinational DSB repair (Johnson *et al*., 1999; Pierce *et al*., 1999). Moreover, these *XRCC2* or *XRCC3* mutant lines are sensitive to ionizing radiation, correlating the radiation resistance to the efficiency of recombination (Jones *et al*., 1987; Liu *et al*., 1998). In contrast, this correlation does not exist with our lines expressing the *SMRAD51* (or *ScRAD51*) dominant-negative form. Several hypotheses could account for these apparent differences. First, our lines contain an endogenous wild-type *RAD51*, and one can suggest that low or undetectable levels of homologous recombination would be sufficient to maintain radiation resistance; however, radiation-induced recombination is strongly decreased. Secondly, one of the other paralogues can substitute for *RAD51* for radiation resistance but not for radiation-induced recombination; they would be unable to substitute for *XRCC2* or *XRCC3*. These two hypotheses uncouple radiation resistance and homologous recombination efficiency. A third hypothesis reconciles all the different results. This hypothesis proposes that *RAD51* initiates the recombination process and that its partners, such as *XRCC2* or *XRCC3*, facilitate the completion of the process by maturing and resolving the recombination intermediates generated by *RAD51*. Non-processed intermediates would result in cell toxicity. We show here that radiation-induced recombination is a *RAD51*-dependent pathway; thus, radiation stimulates the initiation of recombination by *RAD51*. The absence of *XRCC2* or *XRCC3* would lead to the accumulation of non-processed intermediates and would result in radiation sensitivity of the *XRCC2* or *XRCC3* mutant cell lines. If the initiation of homologous recombination is impaired by the dominant-negative *SMRAD51*, on the one hand toxic recombination intermediates would not accumulate and on the other hand DSBs would be repaired by another pathway such as NHEJ. In this hypothesis, cells should not be radiation sensitive. We do no have direct evidence that *SMRAD51* acts at initiation or at a later step of strand invasion. However, our results are compatible with the channelling towards SSA or NHEJ for DSB repair. In addition, our results and the third hypothesis are fully consistent with the fact that no *RAD51* mutant alleles have been isolated in the screen for radiation-sensitive mutants in CHO cell lines.

RAD51 is an essential gene in non-irradiated mammalian cells. It has been proposed that spontaneous recombination controlled by *mRAD51* would be essential for cell viability by repairing spontaneous damage occurring during replication. Remarkably, the present results show that efficient *mRAD51* recombination repair of DSBs induced by I-*Sce*I or ionizing radiation is not required for cell viability. This could suggest that DSB-induced recombination and cell viability are two separable functions of *mRAD51*. It can be argued that the essential role of *mRAD51* is spontaneous recombination. This does not agree with our results showing that both DSB-induced recombination and spontaneous recombination are stimulated by *MmRAD51* and both are inhibited by *ScRAD51* or *SMRAD51*. However, since the endogenous wild-type *RAD51* is still present in our cell lines, we cannot exclude the possibility that low or undetectable levels of recombination would be sufficient to ensure its putative role in cell proliferation and viability. Nevertheless, the efficiency of the *mRAD51* recombination pathway, involved in spontaneous as well as DSB-induced conservative recombination, can be decreased substantially without affecting cell proliferation and viability. However, since deletion of the *RAD51* gene is lethal in mammalian cells, this could suggest that *mRAD51* has other role(s), essential to cell viability. Its interactions with p53, BRCA1 and BRCA2 (Sturzbecher *et al*., 1996; Buchhop *et al*., 1997; Mizuta *et al*., 1997; Scully *et al*., 1997; Marmorstein *et al*., 1998) and its participation in the regulation of p53 transactivation activity (Marmorstein *et al*., 1998) suggest a potential role in the cell cycle checkpoint (beside potential roles in DNA repair), which could be a good candidate for this new putative essential function of *RAD51*. Indeed, no good structural homologues of the *mRAD51* interactors p53, BRCA1 and BRCA2 have been identified in yeast; in addition, both null *BRCA*$^{-/-}$ mice and null *RAD51*$^{-/-}$ mice show embryonic lethality that can be partially rescued by inactivation of the *p53* gene (Lim and Hasty, 1996; Hakem *et al*., 1997). Another possible essential role could be the protective effect of *RAD51* against apoptosis (Huang *et al*., 1999). A connection between these two roles of *RAD51* cannot be excluded.

A growing amount of evidence connects *mRAD51* to tumorigenesis: *mRAD51* interacts with tumour suppressor gene products and, in addition, mutations or loss of heterozygosity have been reported in some cancers for chromosomal loci containing *BRCA1*, *BRCA2* and *RAD51B* or *mRAD51*'s partners *RAD52*, *RAD54* and *RAD54B* (Gonzalez *et al*., 1999; Hiramoto *et al*., 1999; Matsuda *et al*., 1999; Schoenmakers *et al*., 1999). Our results show that cell viability and proliferation remain

unaffected in the absence of an efficient *mRAD51*-dependent spontaneous and DSB-induced recombination process, but that *mRAD51* participates in DSB repair by controlling the types of recombination events, i.e. conservative recombination. This function should confer an important role on *mRAD51* in the maintenance of genetic stability of vegetative cells beside an essential function in cell proliferation.

Materials and methods

DNA manipulations

All DNA manipulations were performed as described (Sambrook *et al*., 1989).

Cells and plasmids

CHO-DRA10 cells (Liang *et al*., 1998) and their derivative lines were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Neo^R clones were selected in 500 µg/ml G418, and hygromycin-resistant clones were selected in 500 µg/ml hygromycin. Transfections were performed using Fugene 6 (Boehringer, Mannheim). The *SMRAD51* cDNA was constructed as follows: the N-terminal part of *ScRAD51* was amplified by PCR using a 5′ primer containing an *Eco*RI restriction site and the beginning of the yeast gene (ccggaattcATGtctcaagaacaacat), and a 3′ primer containing the sequence corresponding to the last amino acids of the N-terminal part of *ScRAD51* and the beginning of *MmRAD51* cDNA up to the *Ava*II site (and without the first ATG). The sequence of this 3′ oligonucleotide is: gctgtggaccaaaactttcttcttccactgaagtatctgcacttgcttcaa-gctgcatttgcatagcgccgttggt ggcctcaatatc. The rest of the *MmRAD51* sequence was amplified using a 5′ primer at the *Ava*II site (ttttgg-tccacagcctatttcaccgc) and a 3′ primer at the end of the *MmRAD51* cDNA and containing a *Xho*I site (cggccgctcgagggagtccagtctttggcatcgccc). Double digests of the PCR products with *Eco*RI–*Ava*II and *Ava*II–*Xho*I were co-cloned in pBluescript vector digested by *Eco*RI–*Xho*I. *MmRAD51*, *ScRAD51* and the fusion *SMRAD51* cDNAs were cloned in pcDNA3.1puro plasmid. This plasmid was constructed by replacing the 1.7 kb *Pvu*II fragment of pcDNA3.1Zeo (In Vitrogen) by the 1.4 kb *Pvu*II–*Bam*HI fragment from pPuro plasmid (Clontech). We used the I-*Sce*I (HA-tagged) expression vector described by Liang *et al*. (1998). The deleted ΔNt*ScRAD51* was constructed as follows: the cDNA coding for amino acids 55–400 was amplified by PCR using an intact *ScRAD51* cDNA as matrix, a 5′ primer (cggccgctcgagccaccatgtccggcgatggtggcgga) and a 3′ primer (atcaccaaatacctactcgtcttc). The amplimer was digested with *Xho*I and cloned in pBluescript vector. After verification of the sequence, an *Xho*I–*Eco*RV fragment was cloned in PcDNA3.1puro.

Western blot analysis

All extract preparation steps were performed at 4°C. After washing with phosphate-buffered saline (PBS), cells were suspended in lysis buffer A [25 mM Tris pH 7.5, 5 mM EDTA, 600 mM NaCl, 1 mM dithiothreitol (DTT), 0.1% NP-40, 5 µg/ml leupeptin, 2 µM pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol] and incubated for 40 min on ice. Extracts were centrifuged for 30 min at 15 000 *g*, the supernatant was retrieved and the protein concentration was determined using a Bio-Rad Protein Assay (Bio-Rad). Forty micrograms per well of the boiled samples were loaded on a 10% polyacrylamide gel in the presence of SDS. After migration, the proteins were electrotransferred onto a nitrocellulose membrane and probed with specific antibodies: anti-human Rad51 and anti-actin (Sigma). Standard procedures were used for the electrophoresis, transfer and western blotting. Antibodies were visualized using the ECL detection kit (Amersham).

Recombination measurements

Recombination after induction of a DSB. A total of 3×10^5 cells (for the Rm2, Rm4, DRA10 and Cm3 lines) and up to 1.8×10^6 cells (for the ScR and SM lines) were plated and transfected with 2 and 12 µg, respectively, of an expression vector for the I-*Sce*I endonuclease (pCMV I-*Sce*I). At 24 h post-transfection, G418 or G418/hygromycin selection was added. The Neo^R and Neo^R–Hyg^R colony frequencies are expressed in relation to the total number of cells plated. The relative percentage of conservative recombination events is calculated as the ratio of the frequency of double-resistant Neo^R–Hyg^R clones to the frequency of Neo clones.

Recombination frequency after γ-radiation. Cells were irradiated in PBS, using a ^{60}Co irradiator (2.5 Gy/min) at the dose indicated. After irradiation, the cells were incubated in DMEM at 37°C for 24 h. The cells were then trypsinized, counted and divided into two fractions. The first fraction was used to calculate the viability by measuring the plating efficiency. The second fraction was plated under G418 selection to measure the recombination frequency.

Fluctuation analysis for spontaneous recombination was performed as previously described (Liskay *et al*., 1984). For each line analysed, several independent cultures were plated and cultured to confluence. Cells were then trypsinized, counted and one fraction was used for plating efficiency estimation. The remaining cells were plated under G418 selection. The resulting number of TK^+ or Neo^+ clones allowed us to calculate the recombination frequency. The rate of recombination per cell per generation was calculated by using the fluctuation tests of Luria and Delbruck (Luria and Delbruck, 1943; Capizzi and Jameson, 1973).

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