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Multiple recombination pathways for sister chromatid exchange in *Saccharomyces cerevisiae*: role of *RAD1* and the *RAD52* epistasis group genes

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ABSTRACT

Sister chromatid exchange (SCE) can occur by several recombination mechanisms, including those directly initiated by double-strand breaks (DSBs), such as gap repair and break-induced replication (BIR), and those initiated when DNA polymerases stall, such as template switching. To elucidate SCE recombination mechanisms, we determined whether spontaneous and DNA damage-associated SCE requires specific genes within the *RAD52* and *RAD3* epistasis groups in *Saccharomyces cerevisiae* strains containing two *his3* fragments, *his3-Δ5′* and *his3-Δ3′::HOcs*. SCE frequencies were measured after cells were exposed to UV, X-rays, 4-nitroquinoline 1-oxide (4-NQO) and methyl methanesulfonate (MMS), or when an HO endonuclease-induced DSB was introduced at *his3-Δ3′::HOcs*. Our data indicate that genes involved in gap repair, such as *RAD55*, *RAD57* and *RAD54*, are required for DNA damage-associated SCE but not for spontaneous SCE. *RAD50* and *RAD59*, genes required for BIR, are required for X-ray-associated SCE but not for SCE stimulated by HO-induced DSBs. In comparison with wild type, rates of spontaneous SCE are 10-fold lower in *rad51 rad1* but not in either *rad51 rad50* or *rad51 rad59* double mutants. We propose that gap repair mechanisms are important in DNA damage-associated recombination, whereas alternative pathways, including a template switch pathway, play a role in spontaneous SCE.

INTRODUCTION

Sister chromatid exchange (SCE) results from recombination between two essentially identical DNA duplexes that are generated when a chromosome undergoes DNA replication. Sister chromatids are thus ideal templates for accurate gap repair of double-strand breaks (DSBs). The frequency of SCE is increased in both yeast (1) and mammalian cells (2) after exposure to DNA-damaging agents that generate DSBs.

Specific types of DNA damage impede replication fork progression and initiate SCE through complex pathways (3). DSBs resulting from replication fork collapse can initiate SCE and re-establish a replication fork; such a mechanism is referred to as break-induced replication (BIR) (4). Other proposed SCE mechanisms include replication fork reversal, which leads to template switching and repair that is initiated by single-strand gaps (5). Identifying eukaryotic DNA repair (*RAD*) genes required for SCE is one approach by which to elucidate these complex recombinational pathways.

We studied SCE in *Saccharomyces cerevisiae*, an organism in which double and triple mutants can be constructed easily to define epistastic relationships. *RAD* genes involved in recombinational repair of DSBs belong to the *RAD52* epistasis group (6,7). *RAD52* is required for most homologous recombination pathways involved in gene conversion or reciprocal exchange (8), as well as those involved in BIR (4,9). Genes required for gap repair mechanisms but which are not necessary for BIR include the *recA* homologs *RAD51*, *RAD55*, *RAD57* (10), and *RAD54*, which encodes a Swi4/Snf1-related kinase (11); these genes are referred to as the *RAD51* subgroup. Genes involved in BIR include *RAD50* and *RAD59* (4,9). Mammalian *RAD50*, *RAD51*, *RAD52* and *RAD54* orthologs, and *RAD51* paralogs also function in DSB repair (12,13); however, there is no known mammalian *RAD59* ortholog (13). Thus, understanding the genetic interactions of the yeast *RAD* genes in SCE may elucidate the genetics of SCE in mammals.

Genes in the *RAD3* epistasis group (excision repair pathway), such as *RAD1* and *RAD10*, also participate in recombinational repair of direct (14–16) and inverted repeats (10). Rad1/Rad10 nuclease cleaves 3′ single-stranded ends adjacent to double-stranded DNA (17); this activity functions in either the resolution or the stabilization of recombination intermediates that result from single-strand annealing (SSA) (18) or occur in the initiation of recombination (19).

Previous studies found that SCE requires genes belonging to both the *RAD52* (1) and the *RAD3* epistasis groups (5). In *rad52* mutants, spontaneous SCE was reduced at least 10-fold (1,20), and DNA damage-associated SCE was abolished (5). However, *rad51* mutants did not exhibit lower rates of spontaneous unequal SCE but were defective in DNA damage-associated SCE (1). Although rates of spontaneous SCE are similar in *rad1* and wild-type strains, *rad1 rad52*

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Table 1. Yeast strains

Lab name	Genotype	Source (synonym)
YA165	*MAT*α *ura3-52 his3-Δ200 trp1-Δ1 leu2-Δ1*	F. Winston (FY250)
YA166	*MAT***a** *ura3-52 his3-Δ200 ade2-101 trp1-Δ1 leu2-Δ1*	F. Winston (FY251)
YA187	*MAT***a** *ura3-Δ0 his3-Δ1 lys2-Δ0 leu2-Δ0 met15-Δ0 rad59::KanMX*	Resgen company (3756)
YA188	*MAT*α *ura3-Δ0 his3-Δ1 lys2-Δ0 leu2-Δ0 rad59::KanMX*	Resgen company (13756)
YA189	*MAT*α *ura3-Δ0 his3-Δ1 lys2-Δ0 leu2-Δ0 rad4::KanMX*	Resgen company (16158)
Strains to measure SCE[a]		
YB163	*MAT***a**-inc *ura3-52 his3-Δ200 ade2-101 lys2-801 trp1-Δ1 gal3⁻ trp1::[his3-Δ3′::HOcs, his3-Δ5′]*	This lab
YB213	*MAT***a**-inc *rad50::URA3*	*rad50::URA3* disruption in YB163
YB177	*MAT***a**-inc *rad51::URA3*	This lab
YB203	*MAT***a**-inc *ADE2 leu2-Δ1*	From cross of YB163 with YA165
YB204	*MAT*α *leu2-Δ1*	From cross of YB177 with YA165
YB205	*MAT*α *leu2-Δ1 rad51::URA3*	From cross of YB177 with YB204
YB206	*MAT***a**-inc *ADE2 rad55::LEU2*	*rad55::LEU2* disruption in YB203
YB207	*MAT*α *rad55::LEU2*	From cross of YB206 with YB204
YB208	*MAT***a**-inc *ADE2 rad57::LEU2*	*rad57::LEU2* disruption in YB203
YB209	*MAT*α *ADE2 rad57::LEU2*	From cross of YB208 with YB204
YB211	*MAT***a**-inc *ADE2 rad55::LEU2 rad57::LEU2*	From cross of YB206 with YB209
YB212	*MAT***a**-inc *ADE2 rad51::URA3 rad55::LEU2 rad57::LEU2*	From cross of YB211 with YB205
YB214	*MAT***a**-inc *ADE2 rad51::URA3 rad50::URA3*	From cross of YB213 with YB205
YB215	*MAT*α *rad50::URA3*	From cross of YB213 with YB205
YB216	*MAT***a**-inc *ADE2 rad54::KanMX*	*rad54::KanMX* disruption in YB203
YB217	*MAT***a**-inc *rad50::URA3 rad54::KanMX*	From cross of YB216 with YB215
YB218	*MAT***a**-inc *ADE2 ura3 rad59::KanMX*	From cross of YB163 with YA188
YB219	*MAT***a**-inc *ADE2 ura3 rad51::URA3 rad59::KanMX*	From cross of YB205 with YA187
YB220	*MAT***a**-inc *ura3 ADE2 rad50::URA3 rad59::KanMX*	From cross of YB218 with YB215
YB221	*MAT***a**-inc *ADE2 rad1::KanMX*	*rad1::KanMX* disruption inYB203
YB222	*MAT***a**-inc *rad50::URA3 rad1::KanMX*	From cross of YB221 with YB215
YB223	*MAT*α *ADE2 rad51::URA3 rad1::KanMX*	From cross of YB221 with YB205
YB224	*MAT***a**-inc *rad50::URA3 rad51 rad1::KanMX*	From cross of YB222 with YB223
YB225	*MAT***a**-inc *ura3 rad4::KanMX*	From cross of YB163 with YA189
YB226	*MAT***a**-inc *ura3 rad4::KanMX rad51::URA3*	From cross of YB225 with YB205

[a]If not indicated, the genotype is the same as YB163; mating-type added for clarity.

mutants exhibited a synergistic decrease in the rate of spontaneous SCE (5).

In this study, we determined the *RAD* dependence of unequal SCE in yeast. We found that the genetic requirements for DNA damage-associated SCE depend on the type of DNA lesion and that there are distinct *RAD1*-dependent and *RAD51*-dependent pathways for spontaneous SCE.

MATERIALS AND METHODS

Standard media for yeast culture

SC (synthetic complete, dextrose), SC-TRP (synthetic complete lacking tryptophan), SC-HIS (synthetic complete lacking histidine), SD (synthetic dextrose), YP (yeast extract, peptone), YPD (yeast extract, peptone, dextrose) and sporulation media are described by Sherman *et al*. (21). YPL medium contains YP medium with 2% lactate (pH 5.5); YPGlu medium contains YP medium with 2% ultra-pure glucose; YPGal medium contains YP medium with 2% ultra-pure galactose (Sigma, St Louis, MO). YPD (Kan) is YPD supplemented with 200 μg/ml of kanamycin sulfate. YPD (HU) is supplemented with 50 mM hydroxyurea (HU).

Yeast strains

A list of yeast strains and their relevant sources is shown in Table 1. All strains are derivatives of S288c. Strain YB203 is a Leu⁻ derivative of the YB163 strain and contains tandem *his3* fragments, *his3*-Δ5′ and *his3*-Δ3′*::HOcs*, at the *trp1* locus. Plasmids used for the gene disruptions included pSTL11 (Δ*rad55::LEU2*), pSM51 (Δ*rad57::LEU2*) (22) and pNKY83 (Δ*rad50::hisG-URA3-hisG*) (23). To make *rad55*, *rad57* and *rad50* null mutants, a *Hin*dIII fragment of pSTL11, a *Sac*I fragment of pSM51 and an *Eco*RI–*Bgl*II fragment of pNKY83, respectively, were introduced into YB203 by one-step gene disruption (24), and the appropriate prototrophs were selected. We confirmed that the *rad55*, *rad57* and *rad50* mutants were sensitive to both X-rays and HU.

To delete *RAD1* and *RAD54* in YB203, genomic DNA from the corresponding ResGen yeast deletion strains was used as a template for PCR amplification; the appropriate DNA fragments were introduced into YB203 by one-step gene disruption (24); and kanamycin-resistant transformants were selected. The primers used to amplify the *rad1::KanMX* fragment were 5′-CTTTATTTTGCGACTTTTCTTCATC-3′ and 5′-TAATGAATATGATTGTGCGCTTCTA-3′. The primers used to amplify the *rad54::KanMX* fragment were 5′-CTTTATTTTGCGACTTTTCTTCATC-3′ and 5′-TAATGAATATGATTGTGCGCTTCTA-3′. We confirmed that the *rad1* and *rad54* mutants were sensitive to UV and X-rays, respectively.

Strains containing a *rad59::KanMX* gene disruption were derived by a diploid cross of the ResGene strain 13756

(YA188) with the *MAT***a**-inc strain YB203, and the appropriate kanamycin-resistant meiotic segregant was obtained by tetrad dissection. We confirmed that the *rad59* mutant was sensitive to X-rays and methyl methanesulfonate (MMS). The presence of the *his3-Δ200* allele was confirmed by PCR analysis using the primers 5′-CACGGCAGAGACCAATCAGTA-3′ and 5′-GCACTCCTGATTCCGCTAATA-3′.

Other single, double and triple mutants were constructed by crossing the appropriate haploid strains and screening the meiotic segregants for prototrophic phenotypes, antibiotic resistance and radiation sensitivities.

Determining rates of spontaneous recombination and frequencies of DNA damage-associated recombinants

The rates (events per cell division) of spontaneous SCE were determined by the method of median (25), using 11 independent colonies for each rate calculation as described previously (26). At least three independent rate calculations were done for each strain, and the significance of the differences was determined by the Mann–Whitney U-test (27).

Protocols used to measure the recombinogenicity of MMS, 4-nitroquinoline 1-oxide (4-NQO), UV and X-rays have been described previously (26). At least three independent experiments were performed at 30°C for each DNA-damaging agent. Because radiation resistance in *rad55* and *rad57* mutants is enhanced at 30°C, we also measured the MMS and 4-NQO stimulation of SCE in *rad55* and *rad57* at room temperature. After exposure to the DNA-damaging agent at room temperature, cells were plated on the appropriate medium and incubated at 30°C.

Induction of HO endonuclease

pGHOT-*GAL3* (28), containing the *HO* gene under *GAL* control, was introduced into *rad51*, *rad50*, *rad54*, *rad55*, *rad57* and *rad59* strains by selecting for Trp$^+$ transformants. After growth in SC-TRP medium, cells were diluted 1:10 in YPL and incubated for a minimum of 12 h. At a density of 10^7 cells/ml, glucose or galactose was added to a final concentration of 2%, and cells were incubated for 2 h, as previously described (1).

Characterizing repair events after HO induction

Three different mechanisms may participate in the repair of the DSB generated by HO endonuclease digestion at the *trp1::his3-Δ3′::HOcs* locus. These include non-homologous end joining (NHEJ), SSA and homologous recombination. SSA events generate a chromosomal deletion and a single *his3* fragment lacking both 3′ and 5′ ends, rendering cells unable to generate His$^+$ recombinants (Fig. 1). To determine frequencies of chromosomal deletions (SSA), cells were plated on YPD plates after HO induction. The surviving colonies were replica plated onto SC-HIS to measure the number of His$^-$ colonies that cannot generate His$^+$ recombinants, and onto SC-TRP to measure the number of colonies that maintain the pGHOT-*GAL3* plasmid. The presence of the single *his3* fragment was confirmed by the presence of a 1.4 kb PCR product using primers 5′-CACGGCAGAGACCAATCAGTA-3′ and 5′-GCACTCCTGATTCCGCTAATA-3′ (Fig. 1).

RESULTS

Recombination assays

Our aim was to elucidate the genetic pathways for SCE by measuring spontaneous and DNA damage-associated SCE in particular *rad* mutants that are defective in recombinational repair. To measure unequal SCE, we selected His$^+$ recombinants that resulted from mitotic recombination between two tandem truncated *his3* fragments (29) (Fig. 1). To target DSBs at the site of recombinational substrate, the HO-cut site was inserted in *his3-Δ3′* at the *trp1* locus as previously described (28). For each *rad* mutant, we first measured rates of spontaneous SCE and then measured frequencies of DNA damage-associated SCE after exposure to UV, X-rays, MMS and 4-NQO, or after HO induction. The rate of spontaneous SCE in the wild-type strain (YB163) was 1.2×10^{-6}, which is consistent with previously reported data (1,29,30).

Spontaneous SCE does not require *RAD55*, *RAD57*, *RAD54* or *RAD59* and is modestly dependent on *RAD50*

Previous studies found that spontaneous SCE is not reduced in the *rad51* null mutant (1). We measured the rate of spontaneous recombination in other *rad* mutants that are defective in gap repair of DSBs (Table 2). The rates of spontaneous SCE in *rad51* (YB177), *rad55* (YB206), *rad57* (YB208), *rad54* (YB216) single mutants, the *rad55 rad57* double mutant (YB211), and the *rad51 rad55 rad57* (YB212) triple mutant were the same as in wild type (YB163) ($P > 0.05$). These results are consistent with the idea that *RAD51*, *RAD55*, *RAD57* and *RAD54* participate in a single pathway for gene conversion (13), which, however, is not necessary for spontaneous SCE.

One explanation for the *RAD51* independence of SCE is that the major pathway for spontaneous SCE is either *RAD59* or *RAD50* dependent, because both genes have been shown to function in several *RAD51*-independent pathways for DSB repair, including BIR (31) and SSA (32). We measured spontaneous rates of SCE in *rad50* (YB213) and *rad59* (YB218) single mutants and in *rad50 rad59* (YB220), *rad50 rad51* (YB214) and *rad59 rad51* (YB219) double mutants. The rate of spontaneous SCE in *rad59* was the same as in wild type ($P > 0.05$) (Table 2), whereas in the *rad51 rad59* double mutant, the rate of spontaneous SCE was reduced between 2- and 3-fold ($P < 0.05$) (Table 2). This indicates that *RAD51* and *RAD59* participate in independent SCE pathways. We found that the rates of spontaneous SCE in the *rad50* single mutant, as well as in *rad51 rad50*, *rad54 rad50* (YB217) and *rad59 rad50* double mutants were reduced 2-fold compared with wild type ($P < 0.05$) (Table 2). Thus, mutations in *rad50* conferred a modest reduction in rates of spontaneous SCE in combination with either *rad59* mutations or null mutations in the *RAD51* subgroup, implying that *RAD50* functions in all pathways for spontaneous SCE.

The rate of spontaneous SCE is reduced 10-fold in *rad1 rad51* mutants and 25-fold in *rad1 rad51 rad50* mutants

An alternative *RAD51*-independent pathway involves *RAD1*, a gene that participates in both intrachromosomal (15) and ectopic recombination events (33). There is a 2-fold higher rate of spontaneous SCE ($P < 0.05$) in the *rad1* mutant

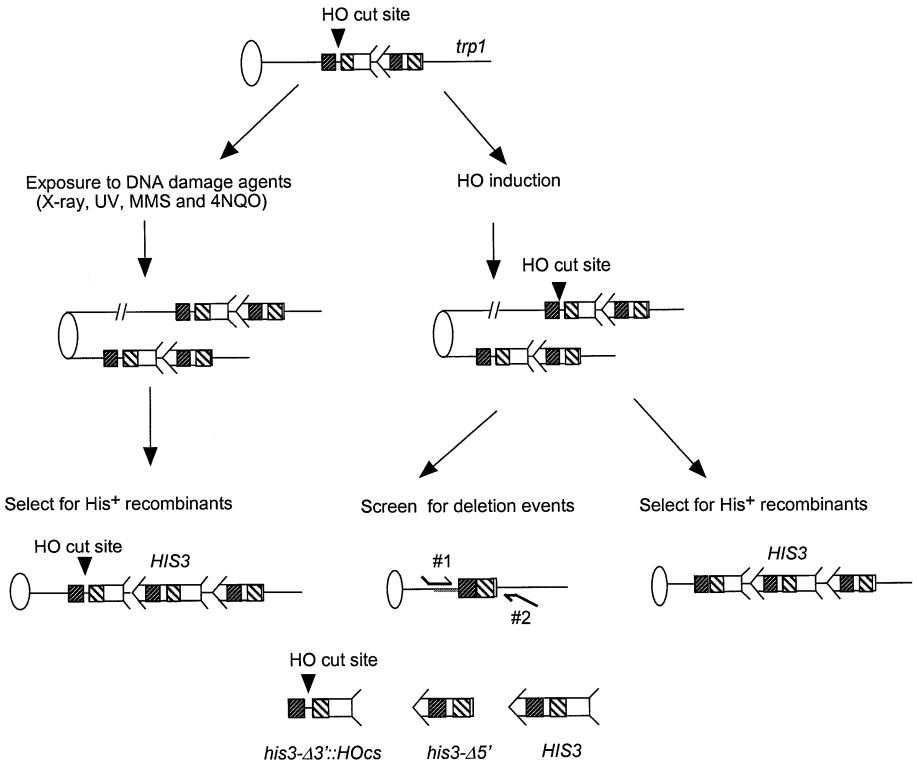

Figure 1. Recombination assays used in this study. Ovals represent centromeres and lines represent chromosomes. For simplicity, the left arms of chromosomes are not included. An arrow and feathers together denote *HIS3*. As indicated on the bottom of the figure, the 5′ deletion lacks the feather and the 3′ deletion lacks the arrow. The two regions of the sequence identity shared by the *his3* fragments are indicated by decorated boxes; dot-filled boxes indicate a region of 167 bp, and the hatched boxes indicate a region of ~300 bp. The 117 bp HO-cut site (*HOcs*), as indicated by an arrowhead, is located between these sequences within the *his3-Δ3′::HOcs* fragment. The *his3*-truncated fragments are integrated into the *trp1* locus on chromosome IV. His+ recombinants resulting from SCE were selected after exposure to DNA-damaging agents (left panel) or after the induction of HO endonuclease (right panel). The left panel indicates that the HO-cut site remains when there is no induction of HO endonuclease. The right panel indicates that HO endonuclease-induced DSB can be repaired by either the gap repair pathway, which generates SCE, or by the SSA pathway, which generates intrachromosomal deletions. The arrows indicate the sequences that are participating in gap repair. SCE events are measured by selecting for His+ recombinants while intrachromosomal deletions are screened, since the deletion renders cells unable to generate His+ recombinants. The primers are designed from regions flanking the *trp1* locus, as indicated by the thin curved lines labeled 1 and 2, and their sequences are identified in Materials and Methods.

compared with wild type, possibly resulting from the mutant's inability to excise potentially recombinogenic DNA adducts. The rate of spontaneous SCE in the *rad1 rad50* mutant was the same as in wild type, whereas the rate of spontaneous SCE in the *rad1 rad51* double mutant was reduced 10-fold compared with wild type. To determine whether *RAD51*-independent SCE requires other genes that participate in UV excision repair, spontaneous rates of SCE were measured in both *rad4* (YB225) and the *rad51 rad4* (YB226) double mutant; these rates were the same as those of wild type (Table 2). Because *RAD4* participates in the initial steps of UV excision repair (34,35), these data suggest that the *RAD1*-dependent recombination pathway does not require most genes in the UV excision repair pathway and might result from Rad1 nuclease activity. Thus, there is a strong requirement for *RAD1* in *RAD51*-independent SCE.

Although the *rad50* mutation conferred a modest decrease in SCE when combined with *rad1*, *rad51*, *rad50* and *rad59* mutations, the *rad1 rad51 rad50* triple mutant exhibited a 25-fold decrease in spontaneous SCE compared with wild type. These data indicate that in the absence of both *RAD51* and *RAD1*, a *RAD50*-dependent pathway, such as BIR, might play a major role in spontaneous SCE.

X-ray-associated SCE requires *RAD50*, *RAD54, RAD55* and *RAD57* and is reduced in the *rad59* mutant

Previous studies have demonstrated that chemical agents and radiation, including 4-NQO, MMS, UV and X-rays, stimulate SCE by *RAD51*-dependent (1) and *RAD52*-dependent recombination mechanisms (1,29,30). We investigated whether other genes within the *RAD52* epistasis group are also required for the stimulation of SCE after exposure to DNA-damaging agents. Because all the genes within the *RAD52* epistasis group initially were defined as conferring haploid resistance to ionizing radiation (7), it would be expected that all of the corresponding *rad* mutants would also be defective in stimulating SCE after X-ray exposure. As in previous studies (1), we observed a significant increase in SCE frequencies after wild-type cells were exposed to 4, 6 or 8 krads of X-rays (Fig. 2); a maximum 5-fold increase in SCE frequencies was observed after 8 krad exposure in the wild-type strain. However, no significant increase in SCE

Table 2. Spontaneous rates of SCEs in *S.cerevisiae rad* mutants

Genotype (strains)[a]	Rate of recombination ($\times 10^6$)[b]	Ratio[c]
RAD (YB163)	1.2 ± 0.2	1
RAD51 group		
rad51 (YB177)	1.5 ± 0.2	1.3
rad54 (YB222)	0.9 ± 0.1	0.8
rad55 (YB206)	1.2 ± 0.2	1.0
rad57 (YB208)	1.3 ± 0.2	1.1
rad55 rad57 (YB211)	1.3 ± 0.2	1.1
rad51 rad55 rad57 (YB212)	1.1 ± 0.1	0.9
RAD3 group		
rad1 (YB219)	2.6 ± 0.2	2.2
rad4 (YB225)	1.4 ± 0.1	1.1
rad1 rad50 (YB220)	1.8 ± 0.1	1.5
rad4 rad51 (YB226)	2.1 ± 0.1	1.6
rad1 rad51 (YB221)	0.1 ± 0.02	0.1
RAD50 and *RAD59*		
rad50 (YB215)	0.7 ± 0.1	0.6
rad59 (YB216)	1.3 ± 0.2	1.1
rad50 rad59 (YB218)	0.6 ± 0.2	0.5
rad50 rad54 (YB223)	0.6 ± 0.1	0.5
rad50 rad51 (YB214)	0.6 ± 0.1	0.5
rad51 rad59 (YB217)	0.5 ± 0.06	0.4
rad50 rad51 rad1 (YB224)	0.05 ± 0.01	0.04

[a]All strains are S288c(s) background. *RAD* disruptions are indicated. For full genotype, see Table 1.
[b]Rate, number of events per cell division; $n = 3$.
[c]Ratio, rate of SCE in mutant/rate of recombination in wild type.

frequencies was observed after the *rad55*, *rad57*, *rad54* (data not shown) and *rad50* mutants were exposed to X-rays (Fig. 2). All these mutants were very X-ray sensitive. The modest increase in SCE frequencies in the *rad59* mutant after exposure to X-rays also correlates with the moderate X-ray sensitivity of the *rad59* mutant (Fig. 2). These results indicate that there is a correlation between X-ray resistance and X-ray-associated SCE in *rad* mutants defective in recombinational repair.

UV-associated SCE requires *RAD54*, *RAD55*, *RAD57* and *RAD50*, but not *RAD59*

Whereas recombinational repair mutants are only modestly sensitive to UV, the *rad51* mutant exhibits reduced levels of UV-associated SCE (1). We therefore measured UV-associated SCE in *rad50*, *rad59*, *rad54*, *rad55* and *rad57* mutants. After exposure to UV, we observed a maximum 5-fold increase in SCE frequencies in wild type. No significant increase in SCE frequencies was observed in the *rad54*, *rad55*, *rad57* (data not shown) and *rad50* mutants after exposure to UV (Fig. 2). However, frequencies of SCE were increased in the *rad59* mutant at all levels of UV exposure (Fig. 2). Thus, there are different genetic requirements for UV-associated SCE and X-ray-associated SCE.

MMS- and 4-NQO-associated SCE requires *RAD54*, *RAD55*, *RAD57* and *RAD50*, but is modestly defective in the *rad59* mutant

4-NQO and MMS are considered to be DNA-damaging agents that are UV mimetic and X-ray mimetic, respectively (35,36).

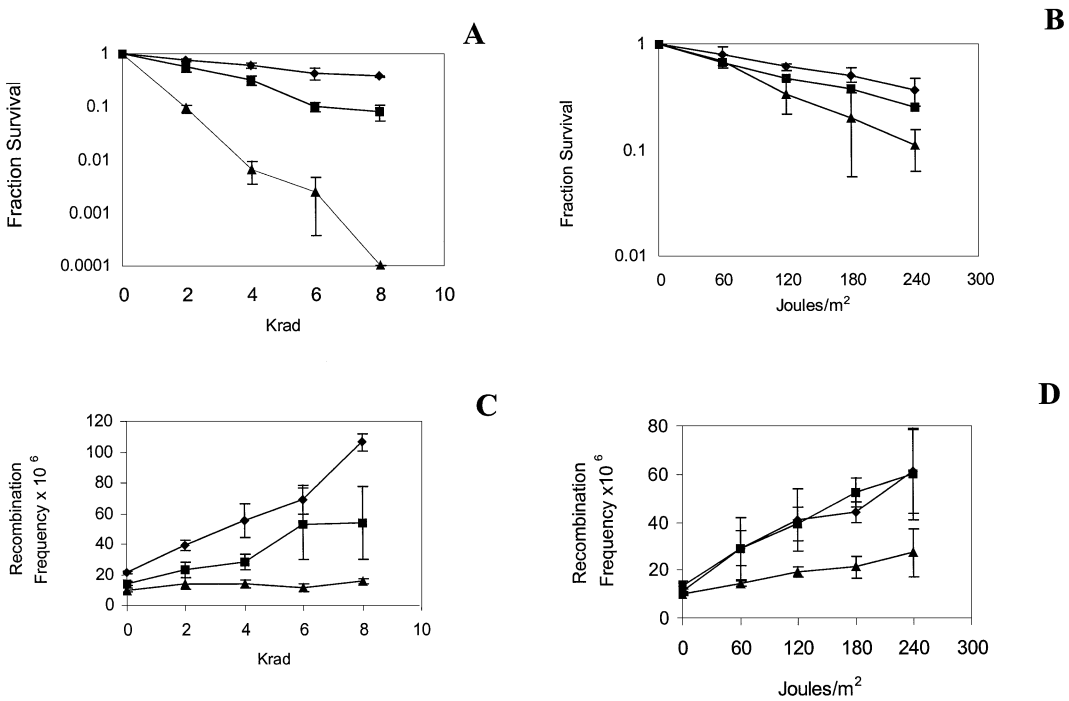

Figure 2. DNA damage-associated SCE after wild type, *rad59* and *rad50* strains were exposed to UV and X-rays. Data on the left were obtained when strains were exposed to X-rays. Data on the right were obtained when strains were exposed to UV. (**A**) Fraction survival after cells were exposed to X-rays. (**B**) Fraction survival after cells were exposed to UV. (**C**) Frequency of His^+ recombinants (number of His^+ recombinants/c.f.u.) plotted against the X-ray dose. (**D**) Frequency of His^+ recombinants (number of His^+ recombinants/c.f.u.) plotted against the UV dose. Filled diamonds = wild type (YB163); filled squares = *rad59* mutant (YB218); filled triangles = *rad50* mutant (YB213).

Table 3. Stimulation of SCE by 4-NQO in *rad50*, *rad54*, *rad51*, *rad55, rad57* and *rad59* mutant strains

Genotype (strain)[a]	His$^+$/c.f.u. $\times 10^6$ (average)[b] Control	2 μM 4-NQO (survival%)	Fold increase[c]	10 μM 4-NQO (survival%)	Fold increase[c]
RAD (YB163)	15 ± 4	74 ± 20 (71)	5	164 ± 48 (30)	11
RAD51 group					
rad51 (YB177)	20 ± 4	19 ± 4 (54)	<1	22 ± 8 (13)	1.1
rad54 (YB216)	10 ± 2	13 ± 4 (53)	1.1	17 ± 2 (14)	1.7
rad55 (YB206)	15 ± 4	16 ± 1 (62)	1.1	23 ± 6 (10)	1.5
rad57 (YB208)	17 ± 10	14 ± 1 (70)	<1	24 ± 11 (10)	1.4
rad55 rad57 (YB211)	14 ± 3	23 ± 1 (70)	1.6	39 ± 5 (9)	2.8
rad55 rad57 (23°C)	9 ± 2	10 ± 3 (68)	1.1	11 ± 3 (10)	1.2
rad51 rad55 rad57 (YB212)	15 ± 2	22 ± 3 (39)	1.5	14 ± 4 (4)	<1
RAD50 and *RAD59*					
rad50 (YB213)	9 ± 4	17 ± 8 (52)	1.9	46 ± 19 (6)	5.1
rad59 (YB218)	16 ± 1	40 ± 10 (80)	4.0	64 ± 23 (21)	6.2

[a]For complete genotype, see Table 1.
[b]Frequency is events/viable cells; experiments were performed at 30°C unless otherwise stated; $n = 3$.
[c]His$^+$ frequency with agent/spontaneous His$^+$ frequency.

Table 4. Stimulation of SCE by MMS in *rad50*, *rad54*, *rad51*, *rad55*, *rad57* and *rad59* mutant strains

Genotype (strain)[a]	His$^+$/c.f.u. $\times 10^6$ (average)[b] Control	2 mM MMS (survival%)	Fold increase[c]	10 mM MMS (survival%)	Fold increase[c]
RAD (YB163)	15 ± 5	59 ±12 (92)	4	118 ± 27 (60)	8
RAD51 group					
rad51 (YB177)	21 ± 9	35 ± 10 (48)	1.7	37 ± 12 (4)	1.8
rad54 (YB216)	13 ± 2	19 ± 4 (50)	1.5	17 ± 1 (4)	1.3
rad55 (YB206)	10 ± 2	24 ± 6 (65)	2.6	31 ± 8 (11)	3.1
rad55 (23°C)	9 ± 2	15 ± 2 (64)	1.7	21 ± 4 (4)	2.3
rad57 (YB208)	17 ± 8	33 ± 9 (50)	1.9	22 ± 7 (11)	1.3
rad55 rad57 (YB211)	13 ± 3	39 ± 5 (64)	3	24 ± 5 (12)	1.8
rad55 rad5 (23°C)	9 ± 1	13 ± 4 (64)	1.4	12 ± 5 (6)	1.3
rad51 rad55 rad57 (YB212)	17 ± 4	25 ± 3 (39)	1.5	36 ± 16 (2)	2.1
RAD50 and *RAD59*					
rad50 (YB213)	10 ± 3	22 ± 13 (22)	2.2	38 ± 15 (1.3)	3.8
rad59 (YB218)	16 ± 1	76 ± 4 (66)	4.8	64 ± 23 (35)	4.0

[a]For complete genotype, see Table 1.
[b]Frequency is events/viable cells; experiments were performed at 30°C unless otherwise stated; $n = 3$.
[c]His$^+$ frequency with agent/spontaneous His$^+$ frequency.

We therefore investigated whether the chosen *rad* mutants exhibited higher SCE frequencies after exposure to 4-NQO and MMS. In wild type, the frequencies of His$^+$ recombinants increased a maximum of 11- and 8-fold above the spontaneous frequency after exposure to 4-NQO and MMS, respectively. *rad54*, *rad55* and *rad57* mutants are all defective in 4-NQO- and MMS-associated SCE compared with wild type (Tables 3 and 4). Compared with frequencies of spontaneous SCE (Tables 3 and 4), frequencies of DNA damage-associated SCE were 3-fold higher ($P < 0.05$) in the *rad55* mutant and in the *rad55 rad57* double mutant after exposure to 2 mM MMS, and 3-fold ($P < 0.05$) higher in the *rad55 rad57* double mutant after exposure to 10 μM 4-NQO. This slight increase is probably related to temperature because, when the *rad55* single mutant and the *rad55 rad57* double mutant were exposed to either MMS or 4-NQO at room temperature, DNA damage-associated SCE frequencies were increased <2-fold (Tables 3 and 4). No increase in DNA damage-associated SCE was observed when the *rad51 rad55 rad57* triple mutant was exposed to either MMS or 4-NQO (Tables 3 and 4). These results are consistent with the idea that DNA damage-associated SCE occurs by a gap repair mechanism that requires the *RAD51* subgroup (13) but, in the absence of *RAD55*, a modest stimulation of recombination is still possible.

We next measured SCE frequencies in *rad50* and *rad59* after exposure to 4-NQO and MMS and compared them with those obtained after UV and X-ray exposure. DNA damage-associated SCE was defective in *rad50* after mutants were exposed to low concentrations of MMS or 4-NQO, which is consistent with the observations that *rad50* mutants are defective in UV- and X-ray-stimulated recombination (Tables 3 and 4). After *rad59* cells were exposed to low concentrations of MMS (2 mM) and 4-NQO (2 μM), we

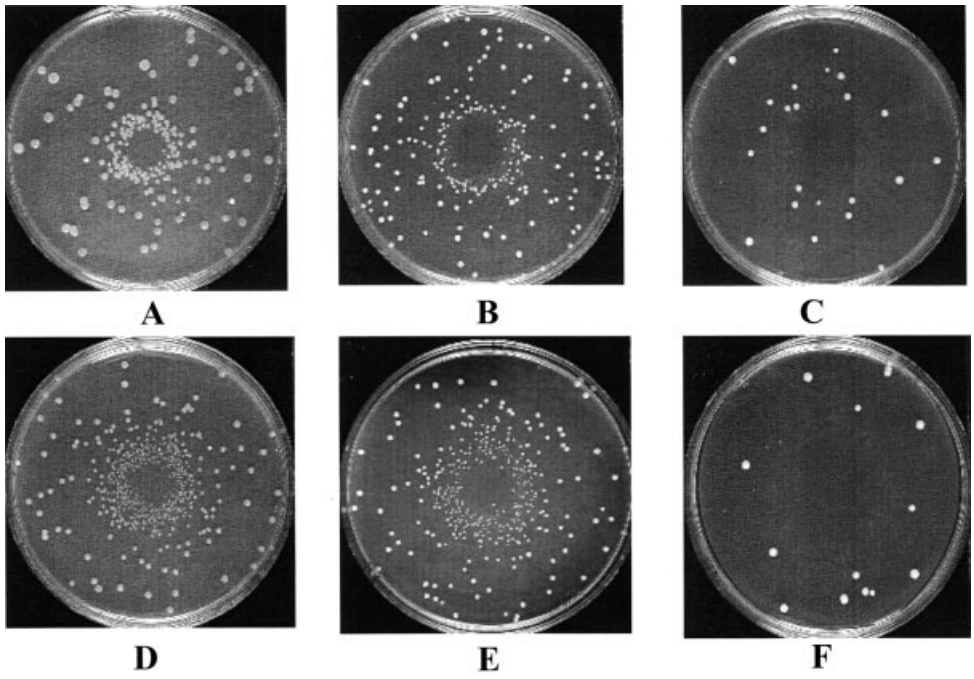

Figure 3. Plate assay demonstrating the DNA damage inducibility of SCE in wild-type and *rad59* strains but not in *rad50* strains. Each SC-HIS plate contains a lawn of 10^7 cells, and the DNA-damaging agent was placed in the center. (**A**) Wild-type strain (YB163) + 0.2 µl of 4-NQO (15 mM). (**B**) *rad59* mutant + 0.2 µl of 4-NQO (15 mM). (**C**) *rad50* mutant + 0.2 µl of 4-NQO (15 mM). (**D**) Wild-type strain + 0.2 µl of MMS (10.5 M). (**E**) *rad59* mutant + 0.2 µl of MMS (10.5 M). (**F**) *rad50* mutant + 0.2 µl of MMS (10.5 M).

observed SCE frequencies that were 5- and 4-fold higher, respectively, than spontaneous SCE frequencies, similar to increases that are observed in wild type (Tables 3 and 4). However, after *rad59* cells were exposed to high concentrations of 4-NQO (10 µM) and MMS (10 mM), a more modest increase was observed compared with wild type. Thus, *RAD50* is required for all DNA damage-associated recombination; however, DNA damage-associated SCE can be detected in *rad59* mutants.

To visualize the 4-NQO- and MMS-associated SCE events in *rad59* mutants, we performed a simple plate assay. Wild type, *rad50* and *rad59* cells were plated on SC-HIS plates, and the chemical compound was diffused from the center of the plate. We observed a halo of His^+ recombinants on plates inoculated with wild type or *rad59* cells, but not on plates containing *rad50* cells (Fig. 3), even when lower amounts of 4-NQO (<3 nmol) and MMS (<22 nmol) were diffused from the center of the plates (data not shown). The halo of recombinants is observed when there are more viable recombinants per total number of inoculated cells, not when there are simply more recombinants per viable cell. These results support the idea that exposure to MMS and 4-NQO can stimulate SCE events in *rad59* but not in *rad50* mutants.

Stimulation of SCE by HO endonuclease-induced DSBs requires *RAD51*, *RAD55* and *RAD57*, but not *RAD50* and *RAD59*

Because exposure to UV, X-ray, 4-NQO and MMS generates a variety of DNA lesions and confers significant lethality in all *rad* mutants, we examined whether stimulation of SCE by a site-specific DSB generated by an inducible HO endonuclease would be defective in *rad* mutants. The DSB could be repaired by SSA, SCE or NHEJ (Fig. 1). All the *rad* strains contain the *MAT***a**-inc allele so that there is no HO endonuclease digestion at the *MAT* locus.

We observed an ~13-fold increase in the SCE frequency in wild type after HO induction, but no significant increases in *rad54*, *rad55* and *rad57* mutants (Table 5). For all these mutants, the percentage viability (mean, 77 ± 4%) after HO induction is similar. Thus, similar to *rad51* mutants, stimulation of SCE by HO-induced DSBs is defective in *rad55*, *rad54* and *rad57* mutant strains.

Equivalent lethality of HO-induced DSBs in wild type and in *rad54*, *rad55* and *rad57* mutants suggests that alternative pathways for repair of the DSB at the HO-cut site, such as NHEJ or SSA, are the preferred repair pathways. SSA would generate a deletion and a single *his3* fragment (Fig. 1) that could be confirmed by PCR; thus, His^- colonies would not be able to generate His^+ recombinants. We therefore determined whether unselected Trp^+ His^- c.f.u.s arising after HO induction could generate His^+ recombinants, and we observed that ~77% (average) could not generate His^+ recombinants (Table 6). Thus, SSA was stimulated in both wild type and in *rad54*, *rad55* and *rad57* mutants. These data suggest that the HO-induced DSB at *trp1::his3-Δ3′* is repaired by similar mechanisms in *rad54*, *rad55*, *rad57* and *rad51* mutants.

Although both *rad59* and *rad50* mutants are defective in the X-ray stimulation of SCE, we found that HO-induced DSBs stimulated SCE in both *rad50* and *rad59* mutants. In *rad59* and *rad50* mutants, there was a 19- and 16-fold increase, respectively, in SCE frequencies after HO induction (Table 5). Of the unselected Trp^+ His^- colonies that appeared after HO induction in *rad59* and *rad50* mutants, 77% and 89% of them, respectively, did not generate His^+ recombinants (Table 6).

Table 5. Stimulation of SCE by HO-induced DSBs in *rad50*, *rad54*, *rad55*, *rad57* and *rad59* mutants

Genotype (strain)[a]	% viability after HO induction[b]	His+ recombinants/Trp+ c.f.u. × 10^5			Trp+ c.f.u./total c.f.u. (%)	
		Before HO induction[c]	After HO induction[d]	Fold increase[e]	Before HO induction	After HO induction
RAD (YB163)	84 ± 18	5.8 ± 1	76 ± 13	13	94 ± 3	91 ± 6
RAD59 and *RAD50*						
rad59 (YB218)	70 ± 13	7.4 ± 1	137 ± 21	19	95 ± 1	92 ± 3
rad50 (YB213)	41 ± 3	14 ± 4	217 ± 25	16	96 ± 1	83 ± 2
RAD51 group						
rad51 (YB177)	77 ± 14	8.2 ± 2	7 ± 3	<1	83 ± 8	75 ± 6
rad54 (YB216)	77 ± 17	8.2 ± 2	7 ± 3	<1	93 ± 2	91 ± 1
rad55 (YB206)	84 ± 14	1.1 ± 0.2	1.3 ± 0.5	1.2	93 ± 5	90 ± 8
rad57 (YB208)	75 ± 25	1.5 ± 0.4	1.3 ± 0.3	<1	96 ± 3	92 ± 5

[a]For complete genotype, see Table 1.
[b]Trp+ c.f.u. after HO induction/Trp+ c.f.u. before HO induction × 100%.
[c]His+ recombinants before HO induction/Trp+ c.f.u. before HO induction.
[d]His+ recombinants after HO induction/Trp+ c.f.u. after HO induction.
[e]His+ frequency after HO induction/His+ frequency before HO induction.

This finding suggests that HO-induced DSBs can be repaired by SSA in both mutants. However, the level of viability after HO induction in the *rad50* mutant decreased significantly, while the *rad59* mutant exhibited a level of viability similar to *rad51*, *rad55*, *rad57* and *rad54* mutants. We suggest that this higher lethality of HO-induced DSBs in *rad50* mutants may result from the *rad50* defect in NHEJ, as previously documented (37).

DISCUSSION

SCE has been suggested to occur by several mechanisms, including template switching, gap repair and BIR. In this study, we defined genetic pathways for spontaneous and DNA damage-associated SCE by measuring rates of spontaneous SCE and frequencies of DNA damage-associated SCE in *S.cerevisiae rad* mutants. We suggest that DNA damage-associated SCE occurs mainly by gap repair and requires genes within the *RAD51* group, but the requirement for *RAD50* or *RAD59* depends on the DNA-damaging agent. Although genes in the *RAD51* group are not required for spontaneous SCE, *rad1 rad51* double mutants showed a synergistic decrease in recombination, whereas a *rad59* deletion conferred only a modest reduction in *RAD51*-independent SCE. Together, these results suggest that BIR is not a major mechanism for spontaneous SCE, and we speculate that spontaneous SCE may involve as yet undefined mechanisms involving template switching (Fig. 4).

The *RAD50* and *RAD59* requirements for DNA damage-associated SCE depend on the DNA-damaging agent and might reflect the requirement for different nucleases to process HO-induced DSBs (38), X-ray-induced DNA damage (39) and UV-induced DNA damage for recombinogenic repair. Because UV does not induce DSBs directly, we speculate that DSBs are not obligate intermediates for UV-associated SCE. A DNA replication-dependent SCE pathway involving template switching has been proposed previously (5) and could be consistent with the *RAD51*-dependent recombination pathway that we propose for spontaneous SCE (Fig. 5).

Table 6. Stimulation of intrachromosomal deletions by HO-induced DSBs in *rad50*, *rad51*, *rad54*, *rad55*, *rad57* and *rad59* mutant strains

Genotype (strain)[a]	His− c.f.u. containing deletions (%)[b] (total His− c.f.u./ total Trp+ c.f.u.)	Fold difference[c]
RAD (YB163)	75 (124/165)	1.0
RAD59 and *RAD50*		
rad59 (YB218)	77 (381/499)	1.0
rad50 (YB213)	89 (371/416)	1.2
RAD51 group		
rad51 (YB177)	76 (93/123)	1.0
rad54 (YB216)	79 (244/309)	1.1
rad55 (YB206)	68 (137/202)	0.9
rad57 (YB208)	73 (87/119)	1.0

[a]For complete genotype, see Table 1.
[b]Deletion events after HO induction/total Trp+ c.f.u. before HO induction × 100%.
[c]Deletion % in mutant strain/deletion % in wild-type strain.

Genetic differences between UV-associated SCE and DSB-initiated recombination are observed in *Escherichia coli*, in which the RecO, RecR and RecF proteins promote UV-associated SCE; whereas RecBC functions in DSB repair (40).

Genetic requirements for spontaneous SCE are different from those for either DSB-initiated gap repair or BIR. DSB-initiated gap repair requires all the genes in the *RAD51* group (13), whereas BIR initiated at the *MAT* locus requires *RAD59* and *RAD50* but not *RAD1* (4,9). However, we observed a 10-fold decrease in rates of spontaneous SCE in the *rad1 rad51* but not in the *rad59 rad51* or *rad50 rad51* double mutants. The minor role of BIR in spontaneous SCE agrees with observations that cell cycle checkpoints prevent replication fork collapse and thus minimize BIR (41).

The genetics of spontaneous SCE are consistent with a template switch mechanism, first proposed for mammalian

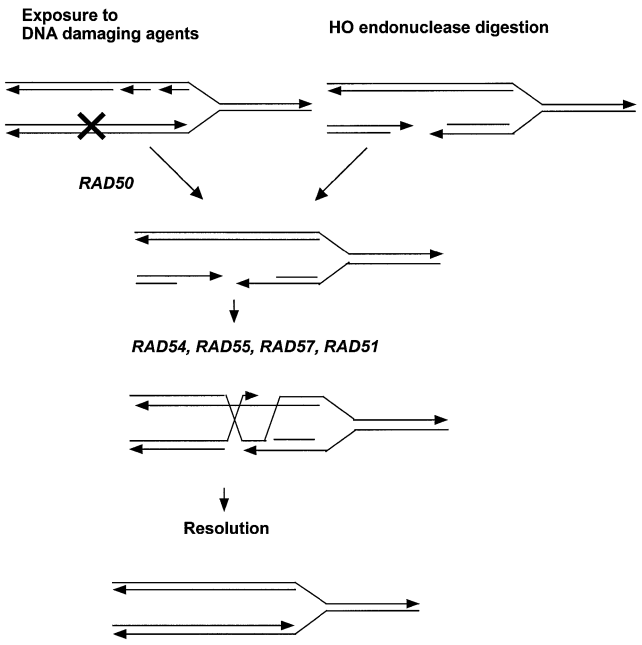

Figure 4. A model for the *RAD51*, *RAD54*, *RAD55* and *RAD57* pathway for DNA damage-associated SCE. Each line represents a single strand of DNA, and the arrow designates the 3′ end. A DNA lesion occurs after progression of the DNA replication fork. Left: in radiation-associated SCE, *RAD50* is required to process the site of DNA damage to reveal 3′ single-strand tails. Right: HO-induced DSBs already have 3′ overhangs and are not *RAD50* dependent. *RAD51*, *RAD54*, *RAD55* and *RAD57* are then required for gap repair of both types of DSBs.

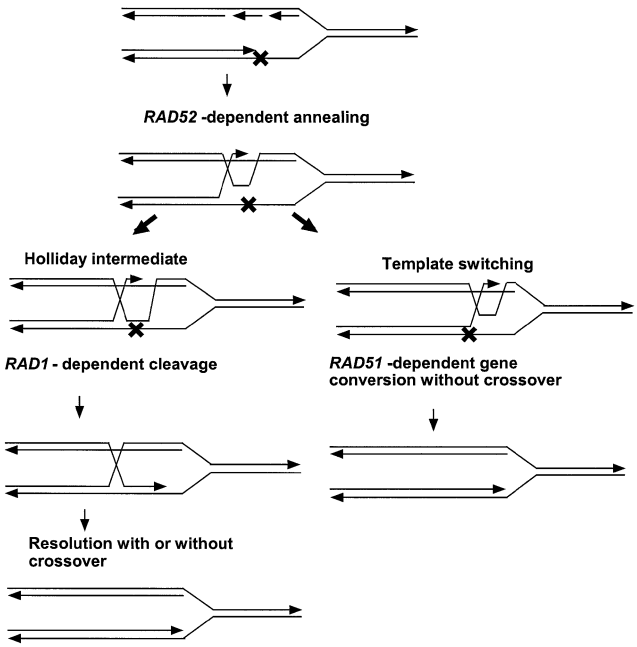

Figure 5. A model of the *RAD1* and *RAD51* pathways for spontaneous SCE. Each line represents a single strand of DNA, and the arrow designates the 3′ end. A DNA lesion blocks DNA polymerase progression that results in a recombinogenic single-strand gap. Rad52 then catalyzes the initial strand invasion of the sister chromatid, forming a transient replication intermediate that can bypass the replication block. Left: Rad1 and associated proteins (Rad10) cleave the transient replication intermediate resulting in the stable formation of the Holliday structure, which can be resolved either with or without crossing over. Right: in the absence of Rad1, the Holliday structure is unstable, but replication can continue and branch migration of the replication intermediate is Rad51 dependent. This process may be similar to synthesis-dependent strand annealing and will generate gene conversion events.

cells (42). In our model (Fig. 5), recombination is initiated by a single-strand gap after a replication fork stalls. Recombination forms a transient replication intermediate that allows polymerase progression after the replication block. A stable Holliday intermediate is then generated by the Rad1/Rad10 nuclease and is resolved to generate either crossovers or non-crossovers. The *RAD1*-independent pathway is *RAD51* dependent and cannot result in crossovers. Although we could not measure crossovers directly, Kadyk and Hartwell (5) did demonstrate that most UV-associated SCE in *rad1* mutants occurs by gene conversion not associated with crossing over. The synergistic decrease in SCE observed in *rad1 rad51* mutants is consistent with a proposed *RAD1*-dependent pathway for plasmid integration (43). The initiation of SCE by single-strand gaps has also been used to explain the phenotypes of *sgs1* and *srs2* yeast mutants (44).

Considering that yeast and mammalian *RAD* genes have significant similarities, our studies may reveal new insights into the genetics of SCE in mammalian cells. For example, when Rad51 protein is inhibited by a dominant negative, the effect on the frequency of spontaneous SCE is variable and depends on the cell type (45,46). We speculate that the variable effect of inhibiting *RAD51* gene expression may reflect the requirement for *RAD51*-independent recombination on *ERCC1* (*RAD10*), which is required for crossing over in gene targeting experiments (13,47).

In summary, the genetic requirements of both spontaneous and DNA damage-associated SCE indicate that there are multiple pathways for SCE. Additional studies in mammalian cells are needed to find similar genetic relationships.

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