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Involvement of RecB-mediated (but not RecF-mediated) repair of DNA double-strand breaks in the γ-radiation production of long deletions in *Escherichia coli*

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Summary

Experiments were designed to determine the association between the repair of γ-radiation-induced DNA double-strand breaks (DSB) and the induction of 700–1000 bp long deletions ($Lac^- \rightarrow Lac^+$), base substitutions (*leuB19* → Leu^+), and frameshifts (*trpE9777* → Trp^+) in *Escherichia coli* K-12. Over the range of 2.5–20 krad, deletions were induced with linear kinetics, as has been shown for the induction of DSB, while the induction kinetics of base substitutions and frameshifts were curvilinear. Like the repair of DSB, deletion induction showed an absolute requirement for an intact *recB* gene as well as a dependency on the type of preirradiation growth medium; these requirements were not seen for base substitutions or frameshifts. In addition, about 80% of the spontaneous deletions were absent in the *recB21* strain. A *recC1001* mutation, which confers a 'hyper-Rec' phenotype, increased the rate of γ-radiation-induced deletions as well as the low-dose production of base substitutions and frameshifts. A *recF143* mutation increased the yield of γ-radiation-induced deletions without increasing base substitutions or frameshifts. A *mutS* mutation markedly enhanced the γ-radiation induction of frameshifts, and had a slight effect on base substitutions, but did not affect the induction of deletions. Resistance to γ-irradiation and the capacity to repair DSB (albeit at about half the normal rate) were restored to the radiosensitive *recB21* strain by the addition of the *sbcB215* and *sbcC201* mutations. However, the radioresistant *recB sbcBC* strain, which is recombination proficient via the RecF pathway, was still grossly deficient in the ability to produce deletions. A model for deletion induction as a by-product of the *recB*-dependent (Chi-dependent) repair of γ-radiation-induced DSB is discussed, as is the inability to detect deletions in cells that use only the *recF*-dependent (Chi-independent) mechanism to repair DSB.

Deletions and other genomic rearrangements are produced at a higher frequency (vs. base substitutions and frameshifts) by DNA strand-breaking agents such as ionizing radiation and bleomycin, compared to nonstrand-breaking

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agents such as UV radiation, ethyl methanesulfonate, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [cf., studies involving *Salmonella typhimurium* (Hartman et al., 1971; Alper and Ames, 1975; Levin et al., 1984), *Escherichia coli* (Schwartz and Beckwith, 1969), and Chinese hamster cells (Thacker, 1986; Tindall et al., 1984; Stankowski and Hsie, 1986)]. Such data have led to the suggestion that DNA single-strand gaps or breaks (e.g., Conkling and Drake, 1976; Levin et al., 1984) or double-strand breaks (DSB) (e.g., Chadwick and Leenhouts, 1978; Gibbs et al., 1987; Meuth, 1990) are somehow involved in the production of deletions.

One approach to deducing the nature of the causative lesion(s) for deletions is to compare the ability of various ionizing radiations to produce deletions with their ability to produce DNA single-strand breaks or DSB. For instance, Nelson et al. (1989) showed for *Caenorhabditis elegans* that recessive lethal mutations, of which 76% or more are known to be deletions or other chromosomal rearrangements (Rosenbluth et al., 1985), are produced at a rate that increases directly with the linear energy transfer (LET) coefficient of the radiation. Whaley and Little (1990) found a similar correlation for deletions in mammalian cells, although the correlation held only at higher doses of radiation. Since the rate of production of DSB, but not of single-strand breaks, also increases with LET (e.g., Munson and Bridges, 1969; Christensen et al., 1972), these data suggest that DSB are the causative lesions for deletions.

A second approach to determining the lesions that induce deletions has been to show that deletions as well as other chromosomal aberrations are induced when mammalian cells are electroporated with DNA restriction endonucleases (Bryant and Christie, 1989; Winegar and Preston, 1988). Similarly, *E. coli* has been transformed with single copies of a linearized plasmid with blunt, cohesive, or radiolytically-produced ends. These plasmids are recircularized at a low frequency and occasionally with deletion formation, and this DSB repair (highest for cohesive ends) and deletion mutagenesis (highest for blunt ends) is *recA*- and *recB*-independent (Bien et al., 1988). While these plasmid data appear to model DSB repair and deletion mutagenesis in the nonreplicated portion of the *E. coli* chromosome, their relevance to bacterial repair and mutagenesis is unclear, since the repair of DSB in the bacterial chromosome is believed to require duplicated regions of the chromosome and an intact *recA* gene (Krasin and Hutchinson, 1977; Wang and Smith, 1983; Sargentini and Smith, 1986b). Together, these data for mammalian and bacterial cells suggest that DSB may give rise to deletions, and that the *recA* dependence of this process in bacteria may depend on the location of the DSB relative to the replication fork.

A third approach to determining the lesions that induce deletions has been to compare the genetic control of the repair of DNA damage with the genetic control of deletion induction. Since the repair of γ-radiation-induced DSB requires the *recA* gene (Krasin and Hutchinson, 1977; Wang and Smith, 1983; Sargentini and Smith, 1986b), it would be valuable to know whether ionizing radiation-induced deletions are *recA*-dependent. The only previous work on this subject is that of Ishii and Kondo (1972, 1975), however, the X-ray-induced multiple deficiencies that they studied as indicative of multi-locus deletions are now known to arise by changes at a single locus, *tonB* (cf., Bachmann, 1990).

In this work, we use the genetic control approach to study the mechanism of deletion mutagenesis, but we rely on an assay for deletions that has been validated by sequencing analysis (Albertini et al., 1982). Since the *recB21* mutation blocks the repair of X-ray-induced DSB in *E. coli* (Sargentini and Smith, 1986b), but has no effect on the repair of X-ray-induced single-strand breaks (i.e., Type II repair, Town et al., 1973; Sargentini and Smith, 1986a), a testable hypothesis is suggested. If the *recB21* mutation blocks the formation of deletion mutations, then it is likely that the repair of DSB plays a central role in γ-radiation deletion mutagenesis. Similarly, since preirradiation growth conditions affect the capacity of *E. coli* to repair X-ray-induced DSB, but not single-strand breaks (Sargentini and Smith, 1985, 1986b), if the repair of DSB is important to deletion mutagenesis, then preirradiation growth conditions ought to regulate deletion mutagenesis. These and other experiments were performed here to determine the role of the repair of γ-

radiation-induced DSB in the production of deletions.

Materials and methods

Bacteria

The bacterial strains used are listed in Table 1. Bacteriophage transductions were performed generally as described by Miller (1972). The parental strain for our studies, DB/P90C (SR2413 in Table 1), has been partially described (Albertini et al., 1982). Strain DB/P90C is derived from P90C (Miller et al., 1977; Cupples and Miller, 1989) and carries the episome F′128 (Low, 1972) with additional episomal *lac* mutations, IQ and L8 (Miller et al., 1977). This episome is roughly 191 kb in size, assuming 91.5 kb for the remnant of F (i.e., 94.5 minus 3 kb; Sharp et al., 1972) and 99.8 kb for the lac^+ $proA^+B^+$ chromosomal insert (i.e., 2.1 min × 47.5 kb/min; Low, 1972; Gho and Miller, 1974; Bachmann, 1990). This episome was further modified to include *Δ(lacI–lacZ)14* (reviewed in Calos and Miller, 1981), which fuses the end of the *lacI* gene to the beginning of the *lacZ* gene. These cells produce a hybrid LacI–Z protein that is missing four amino acids from the C-terminus of the normal LacI protein, and 23 amino acids from the *N*-terminus of the normal LacZ protein. This hybrid protein possesses enough β-galactosidase activity such that these cells grow well with lactose as the only source of carbon and energy. However, the β-galactosidase activity encoded by this episome has been inactivated (i.e., cells are Lac^-) by introducing the *378* mutation (+1 frameshift) early in the *lacI* gene, and by introducing the *S42* mutation (a 13-bp deletion) at the distal end of the *lacI* gene, 697 bp away from the *378* mutation (Albertini et al., 1982). We have modified the DB/P90C strain further to be Trp^- and Leu^- by introducing the *trpE9777* frameshift (an additional adenine inserted into a run of 5 adenines; Bronson and Yanofsky, 1974) and the *leuB19* missense (reverts by AT → GC; Sargentini and Smith, 1979) mutations into the chromosome, e.g., strain SR2474.

Media

YENB is yeast extract (Difco, 0.75%) supplemented nutrient broth (Difco, 0.8%). SMM is a 0.4% glucose–salts medium (Ganesan and Smith, 1968), supplemented with L-arginine (if needed), L-leucine, and L-tryptophan at 1 mM. Plates contain 27 ml of media solidified with Bacto agar (Difco) at 1.5%. Leu-0 and Trp-0 plates are SMM plates deficient in leucine or tryptophan, respectively. Lac plates are SMM plates containing lactose in place of glucose. MacConkey plates contain MacConkey Agar (Difco) at 50 g/l. PB is Na_2HPO_4 at 5.83 g/l and KH_2PO_4 at 3.53 g/l, pH 7.0.

Preparation and irradiation of cells

Logarithmic-phase cells were prepared by diluting YENB or SMM overnight cultures 1:500 or 1:100, respectively, into homologous medium and shaking them at 37°C until an optical density at 650 nm (OD_{650}) of 0.4 was attained. Cultures were pelleted by centrifugation (6 min, 6000 × *g*), washed twice, and resuspended in PB at an OD_{650} of 10, which corresponds to about 4×10^9 (YENB-grown cells) or 1×10^{10} (SMM-grown cells) colony-forming units (CFU)/ml. ^{137}Cs γ-irradiation was accomplished using a Mark I, model 25 irradiator (J.L. Shepherd and Associates). X-Irradiation (50 kVp) was as described in Sargentini and Smith (1986b). Cells were bubbled with air for 3 min before and during the irradiation in a 25 × 100-mm Corex tube (mounted in a Lucite support) (for γ-irradiation) or simply in a Lucite chamber (for X-irradiation). The dose rate was 3.8 krad/min (γ) or 2.8 krad/min (X-ray), as determined by a ferrous sulfate method (Bonura et al., 1975).

Assays for survival and for the repair of DNA double-strand breaks

Assays have been described (Sargentini and Smith, 1986b).

Mutation assays

To determine their surviving fraction, irradiated cells were plated on YENB agar. To determine their mutant frequency, irradiated cells (7 ml) were added to 200 ml of warm YENB in a 1-liter erlenmeyer; replicate cultures were inocculated when needed to attain the goal of a total CFU/dose of $\geqslant 5 \times 10^9$ (see below)]. [When cells were grown in SMM, all platings and subsequent

TABLE 1

E. coli K12 STRAINS USED

Stanford Radiobiology No.	Genotype [a]	Source, reference or derivation [b]
SR248	F$^-$ *leuB19 metE70 thyA36 deo(C2?) bioA2 lacZ53 malB45 rha-5 rpsL151* IN(*rrnD-rrnE*) λ^-	KH21, R.B. Helling
SR716	F$^-$ *trpE9777*	W3110 *trpE9777*, C. Yanofsky
SR749	F$^-$ *argE3 hisG4 leuB6 Δ(gpt−proA)62 thr-1 thi-1 ara-14 galK2 lacY1 mtl-1 xyl-5 tsx-33 rfbD1 mgl-51 kdgK51 supE44 rpsL31 rac* λ^-	AB1157, ECGSC
SR853	F$^-$ *argH1 metA90 proA44 thi-1 pps-4 aceA4 rpsL9* λ^-	PA505-7-5, ECGSC
SR884	*recF143* Mtl$^+$ Xyl$^+$ *tnaA300*::Tn*10* Su$^-$, otherwise as SR749	JC12334, A.J. Clark
SR962	F$^-$ *hisG*::Tn*5 thyA deo*	CBK236, K.J. Shaw
SR1159	*recB21*, otherwise as SR749	Sargentini and Smith, 1986b
SR1181	F$^-$ *pyrF1189*::Tn*1 rpsL*	TH1161, S. Harayama
SR1286	F$^-$ *trpE65 recB*$^+$, otherwise as SR1159	Sargentini and Smith, 1987
SR1388	*recB21 recC22 sbcB15*, otherwise as SR749	JC7623, A.J. Clark
SR1454	*uvrA6 recB21 recC22 sbcB15 sbcC201* His$^+$, otherwise as SR749	Wang and Smith, 1985
SR1689	*mutS215*::Tn*10* F$^-$ *metB1 thy lacY14*	ES1481, R.G. Fowler
SR1691	F$^-$ *his-4 pro trpC3 thyA*::Tn*5 deo thi ara-9 galK2 lac-114 malA1 mtl-1 ton rpsL*	KD2173, H. Nakayama
SR2120	F$^-$ *Δ(argF−lac)*205 *deoC1 araD139 malE52*::Tn*10* flbB5310 *ptsF relA1 rpsL150* λ^-	TST1, ECGSC
SR2413	F$^-$ *Δ(lacI−lacZ)14 lacI378 lacIS42 proA*$^+$ *B*$^+$/*Δ(lac−proB)*XIII *thi ara* λ^-	DS/P90C, J.H. Miller
SR2414	*pyrF1189*::Tn*1*, otherwise as SR2413	SR2413 × P1 *vira*·SR1181, Ap^r
SR2415	*trpE65*, otherwise as SR2413	SR2414 × P1 *vira*·SR1286, Pyr$^+$
SR2419	trpE9777, otherwise as SR2413	SR2414 × P1 *vira*·SR716, Pyr$^+$
SR2421	F′ *argH1 metA90 proA44 thi-1 malE52*::Tn*10 pps-4 aceA4 rpsL9* λ^-	SR853 × P1 *vira*·SR2120, Tc^r
SR2427	HfrCavalli *cysG303 metB1 Δ(gpt−lac)5 car-96*::Tn*10 relA1? spoT1?* λ^-	EG333, ECGSC
SR2429	*metA90 trpE9777 malE52*::Tn*10*, otherwise as SR2413	SR2419 × P1 *vira*·SR2421, Tc^r
SR2431	*argE3 trpE9777 malE52*::Tn*10*, otherwise as SR2413	SR2429 × P1 *vira*·SR749, Met$^+$
SR2433	*argE3 trpE9777*, otherwise as SR2413	SR2431 × T4GT7·SR2413, Mal$^+$
SR2437	*sbcC201 zaj*::Tn*10* Su$^-$, otherwise as SR749	FS2821, S. Kulkarni
SR2448	*sbcC201 argE3 trpE9777 zaj*::Tn*10*, otherwise as SR2413	SR2433 × P1 *vira*·SR2437, λSKK9^s
SR2449	*sbcC201 argE3 hisG*::Tn*5 trpE9777 zaj*::Tn*10*, otherwise as SR2413	SR2448 × P1 *vira*·SR962, Kn^r
SR2452	*trpE9777 car-96*::Tn*10*, otherwise as SR2413	SR2419 × P1 *vira*·SR2427, Tc^r
SR2453	*leuB19 trpE9777*, otherwise as SR2413	SR2452 × T4GT7·SR248, Car$^+$
SR2468	*sbcB15 sbcC201 argE3 trpE9777 zaj*::Tn*10*, otherwise as SR2413	SR2449 × T4GT7·SR1454, His$^+$
SR2469	*sbcB15 sbcC201 argE3 trpE9777 thyA*::Tn*5 zaj*::Tn*10*, otherwise as SR2413	SR2468 × P1 *vira*·SR1691, Kn^r
SR2471	*recB21 sbcB15 sbcC201 argE3 trpE9777 zaj*::Tn*10*, otherwise as SR2413	SR2469 × P1 *vira*·SR1159, Thy$^+$
SR2472	*leuB19 trpE9777 thyA*::Tn*5*, otherwise as SR2413	SR2453 × P1 *vira*·SR1691, Kn^r
SR2474	*leuB19 trpE9777*, otherwise as SR2413	SR2472 × P1 *vira*·SR1159, Thy$^+$
SR2475	*recB21 leuB19 trpE9777*, otherwise as SR2413	SR2472 × P1 *vira*·SR1159, Thy$^+$
SR2507	*mutS215*::Tn*10 leuB19 trpE9777*, otherwise as SR2413	SR2474 × P1 *vira*·SR1689, Tc^r
SR2509	*sbcB15 sbcC201 recB21 argE3 trpE9777*, otherwise as SR2413	SR2471, Tc^s
SR2522	*recF143 leuB19 trpE9777 tna*::Tn*10*, otherwise as SR2413	SR2474 × P1 *vira*·SR884, Tc^r
SR2525	*recC1001 recF143* F′ *argA his-4 met Δ(rac)* λ^-	V73, A.J. Clark
SR2526	*recC1001 argA leuB19 trpE9777*, otherwise as SR2413	SR2472 × P1 *vira*·SR2525, Thy$^+$, Arg$^-$
SR2527	*sbcB15 sbcC201 recB21 argE3 trpE9777 car-96*::Tn*10*, otherwise as SR2413	SR2509 × P1 *vira*·SR2427, Tc^r
SR2528	*sbcB15 sbcC201 recB21 argE3 leuB19 trpE9777*, otherwise as SR2413	SR2527 × T4GT7·SR248, Car$^+$
SR2529	*sbcB15 sbcC201 recB21 mutS215*::Tn*10 argE3 leuB19 trpE9777*, otherwise as SR2413	SR2528 × P1 *vira*·SR1689, Tc^r
SR2531	*sbcB15 sbcC201 recB21 argE3 leuB19 trpE9777 thyA*, otherwise as SR2413	SR2528, Tm^r
SR2532	*sbcB15 sbcC201 argE3 leuB19 trpE9777*, otherwise as SR2413	SR2531 × T4GT7·SR2413, Thy$^+$

incubations used SMM]. After shaking the liquid cultures 16–18 h for mutation fixation and expression, they were centrifuged (6 min, $6000 \times g$), washed twice, and the cells were resuspended in PB at an OD_{650} of 50. Cells at various dilutions were spread at 0.2 ml/plate on Lac, Leu-0, Trp-0, or SMM plates to determine Lac^+, Leu^+, Trp^+ or CFU per ml. Plates were incubated two days (three for *recB21* strains) at 37°C. To determine Lac^+ mutants, the total colonies per Lac plate were counted and at the same time a random sample of 100 or more colonies were patched onto Lac plates (50/plate) and incubated overnight at 37°C. Then, the Lac^+ patches were patched onto MacConkey plates and incubated 8–12 h at 37 °C. Only the patches that caused a deep-red precipitate to form external to the patch were counted as Lac^+ patches (Lac^- patches varied from 1 to 10% depending on the strain and experiment), and their ratio was used to determine the total Lac^+/ml in the 16–18 h cultures. Albertini et al. (1982) used a similar procedure to select the Lac^+ mutations that they showed to be deletions (700–1000 bp long) by a DNA-sequencing analysis. Induced deletion (Lac^+) mutant frequencies were calculated as the mutant frequencies in the 16–18-h cultures for irradiated cells minus the mutant frequencies for nonirradiated cells.

The value of 5×10^9 initial CFU/ml that was used in our experimental protocol was selected from an analysis of the observed Lac^+ mutant frequencies when graded numbers of γ-irradiated wild-type cells (SR2415) were added to fixed volumes of warm YENB for a 16-h incubation. Since an initial CFU of 5×10^9 was about 8-fold more than was needed to observe the maximum mutant frequency induced by 20 krad (data not shown), we concluded that this value would also give reliable mutant frequency data at doses as small as 2.5 krad.

Results

To test for a requirement for the repair of DSB in the production of deletion mutations by ionizing radiation, a *recB21* mutation, which completely blocks the repair of X-ray-induced DSB (Sargentini and Smith, 1985), was tested for its effect on deletion mutagenesis. This mutation was found to completely block the γ-radiation production of deletions (Fig. 1a), while base substitutions (Fig. 1b) and frameshifts (Fig. 1c) were hardly affected.

Since the *recB* gene product might be required for processes other than the repair of DSB, we also used a 'physiological' approach to determine whether the repair of DSB is required in the production of deletion mutations. When grown in rich medium (e.g., YENB) rather than minimal medium (e.g., SMM), DNA-repair-proficient *E. coli* K-12 cells are more resistant to ionizing radiation (Fig. 2, and Sargentini et al., 1983) and show more repair of X-ray induced DSB, although this effect on DSB repair is only observed at X-ray doses $\geqslant 8$ krad (cf., Fig. 3, and Sargentini and Smith, 1985). This growth medium-dependent effect on cell survival and DSB repair is thought to result from the greater number of sister-DNA duplexes and the more rapid postirradiation synthesis of RecA protein in cells grown in rich medium vs. minimal medium (Sargentini et al., 1983). In support of the hypothesis that deletions arise during the repair of DSB, the production of deletions by γ-irradiation shows a dependency on the richness of the growth medium and on radiation dose similar to that shown for the repair of X-ray-induced DSB (Fig. 4a), while

Notes to Table 1:

[a] Genotype nomenclature is that used by Bachmann (1990).

[b] Kn^r, Ap^r, Tc^r, and Tm^r indicate resistance to kanamycin, ampicillin, tetracycline, and trimethoprim, respectively. Tc^s indicates that a spontaneous tetracycline-sensitive substrain was obtained with the selection technique of Maloy and Nunn (1981) with modifications (S.R. Maloy, personal communication). $\lambda SKK9^s$ indicates that cells were selected for Tc^r and then screened for showing a 1000-fold increase in plating efficiency for bacteriophage λSKK9 (Kulkarni and Stahl, 1989). P1 *vira* was obtained from A.J. Clark (University of California at Berkeley) and is a reisolate of P1 *vir*. Bacteriophage T4GT7 is described by Wilson et al. (1979). ECGSC is the *E. coli* Genetic Stock Center.

base substitutions do not (Fig. 4b). In contrast, the induction of frameshifts (putative −1; Fig. 4c) was enhanced substantially by growing cells in minimal medium vs. rich medium. [Note that one should multiply 50 kVp X-ray doses by 1.5 when comparing their biological effects with those pro-

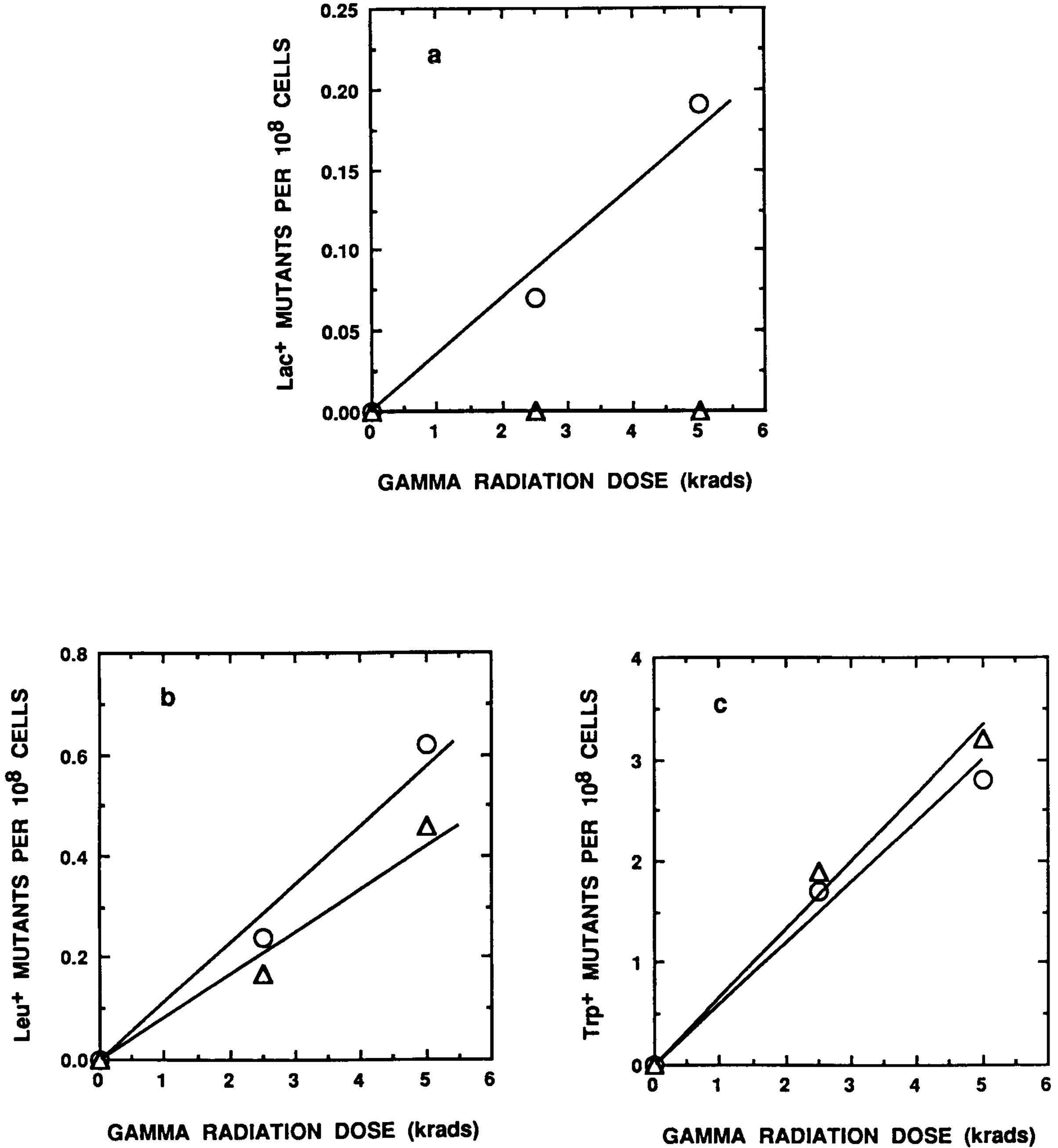


Fig. 1. γ-Radiation mutagenesis of wild-type (○, SR2474) and *recB* (△, SR2475) strains. Cells were grown in rich (YENB) medium, γ-irradiated in PB, and incubated 16–18 h in YENB before plating for mutants. Panels: (a), deletion mutants; (b), base substitution mutants; (c), frameshift mutants.

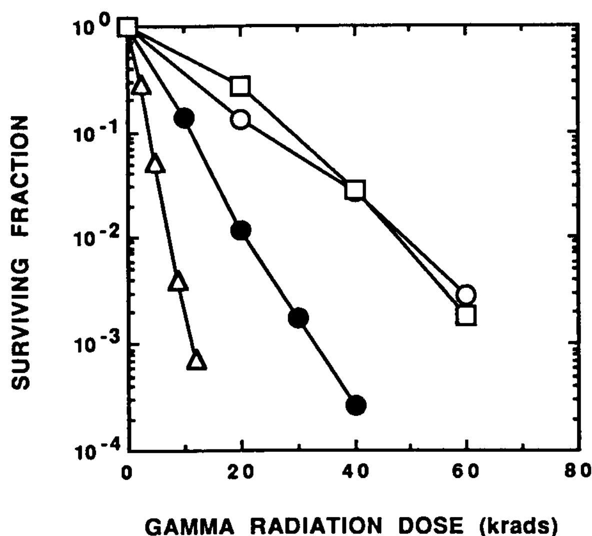


Fig. 2. γ-Radiation survival of wild-type, *recB* and *recB sbcBC* strains. Cells grown to log phase in rich medium (YENB) (○, △, □) or minimal medium (SMM) (●), irradiated in PB, and plated on homologous medium. Symbols: ○, ●, wild-type (SR2474); △, *recB* (SR2475); □, *recB sbcBC* (SR2528).

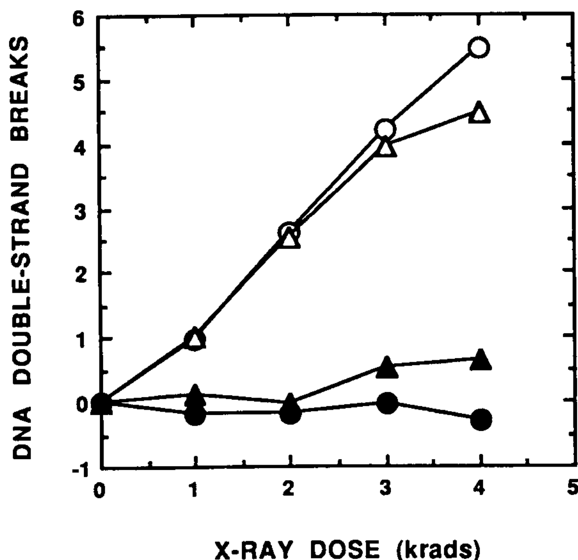
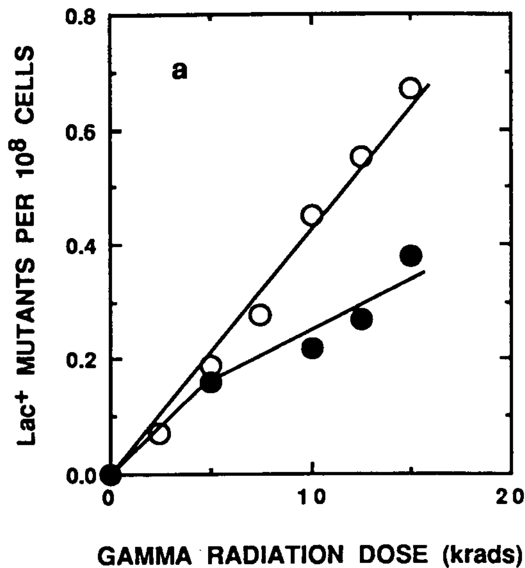
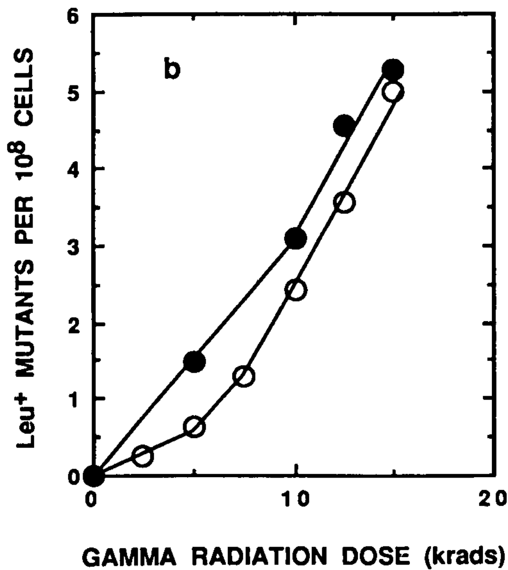


Fig. 3. X-Ray induction of DNA double-strand breaks and their repair in wild-type cells grown in rich or minimal medium. Cells (SR749) were grown to log phase in YENB, (○, ●) or minimal medium (△, ▲), X-irradiated in PB, and incubated in homologous medium at 37°C for either zero (○, △) or 120 (●, ▲) min before analysis for the presence of DNA double-strand breaks per double-strand genome.

duced by ^{137}Cs γ-irradiation (Bonura et al., 1975). Also, one can compare the yield of γ-radiation-induced deletions, i.e., 4.6×10^{-10} deletions per krad (cf., Fig. 1a), and the yield of DSB per F′ episome, i.e., 1.6×10^{-2} per krad (cf., Sargentini and Smith, 1986; Bonura et al., 1975), to calculate that one deletion was detected for every 3.5×10^7 DSB induced in F′ DNA.]

As suggested by the studies of Lloyd and Buckman (1985), the wild-type level of γ-radiation survival was restored to the *recB21* strain by introducing the *sbcB215* and *sbcC201* mutations (Fig. 2). The continued presence of the *recB* mutation was confirmed by being able to transduce an appropriate radiation-sensitizing phenotype into a wild-type strain 23% of the time when selecting for the *thyA*$^+$ marker (data not shown). The suppression of the *recB21*-associated γ-radiation sensitivity by the *sbcBC* mutations suggests that the *recB21*-associated deficiency in the repair of DSB (Sargentini and Smith, 1985) has also been suppressed. A comparison of the kinetics of the repair of γ-radiation-induced DSB between the wild-type strain and a *recB21 recC22 sbcB215 sbcC201* strain indicates that the repair of DSB does occur to the normal extent in the *recBC sbcBC* strain, but that it occurs at a slower rate. That is, the process of repairing the DSB produced by 20 krad of X-rays seems to take about 30 min longer than in the wild-type strain whether one compares the disappearence of the broken DNA that sediments near the top of the sucrose gradient (Fig. 5), or if one compares the kinetics of the formation of fast-sedimenting DNA, or the kinetics of the conversion of fast-sedimenting DNA to DNA that sediments like that from non-irradiated cells (data not shown; cf., Sargentini and Smith, 1985). While the *sbcBC* mutations seemed to restore the capacity for the repair of DSB in a *recBC* strain (Fig. 5), they did not restore the rate of γ-radiation induction of deletion mutations that was observed in a *rec*$^+$ *sbcBC* strain (Fig. 6a). Also, a *recB*$^+$ *sbcBC* strain seems to be slightly deficient in radiation-induced and spontaneous deletions when compared to the wild-type strain, SR2474 (Fig. 6a and Table 2). This suggests that the very low levels of spontaneous deletions in the *recB sbcBC* strains are due





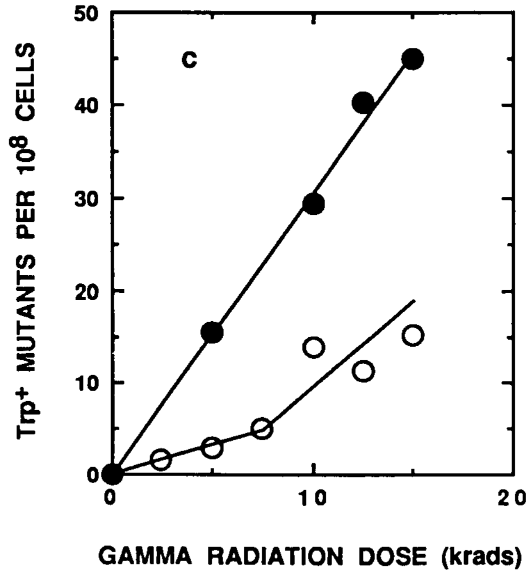


Fig. 4. γ-Radiation mutagenesis of wild-type cells grown in rich (YENB) or minimal medium (SMM). Cells (SR2474) were grown to log phase in YENB (○) or SMM (●), γ-irradiated in PB, and incubated 16–18 h in homologous medium before plating for mutants. Panels: (a), deletion mutants; (b), base substitution mutants; (c), frameshift mutants.

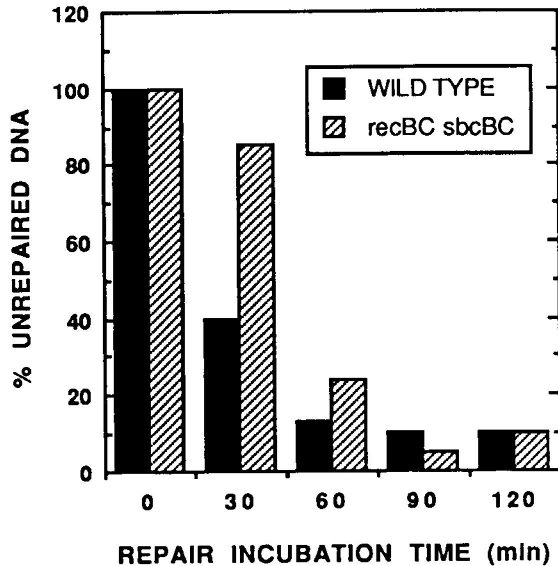


Fig. 5. Repair of DNA double-strand breaks in γ-irradiated wild-type (SR749) and *recBC sbcBC* (SR1388) cells. Cells were grown to log phase in YENB, γ-irradiated (20 krad) in PB, and incubated in YENB for 0–120 min before sampling for neutral sucrose gradient analysis. Since the DNA sedimentation profiles from repair-incubated γ-irradiated cells are complex (cf., Sargentini and Smith, 1985), here we compare only the disappearance of slow-sedimenting DNA from the top half of the gradient for the two strains (vs. the DNA profile for nonirradiated cells).

to a combined effect of the *recB* and *sbcBC* mutations (Table 2).

The recognition and/or repair of base-pair mismatches seems to be a limiting factor in the amount of nonhomologous recombination that can occur via the RecB mechanism, as evidenced by the enhanced nonhomologous recombination that occurs in *mutS* cells vs. wild-type cells (Shen and Huang, 1989). We reasoned that, since spontaneous deletion mutagenesis depends heavily on the homology of directly repeated terminal sequences (e.g., Albertini et al., 1982), *mutS*-dependent mismatch repair (reviewed in Radman and Wagner, 1986) might normally reduce the frequency of misalignments and deletions, and that their frequency would be enhanced in *mutS* and *recB sbcBC mutS* strains (vs. the respective $mutS^+$ parents). However, the *mutS* mutation had no effect on deletion mutagenesis in either the wild-type or the *recB sbcBC* strains (Fig. 7a and Table 2), while the production of frameshifts and, to some extent, base substitutions was significantly enhanced (Figs. 7b, 7c and Table 2).

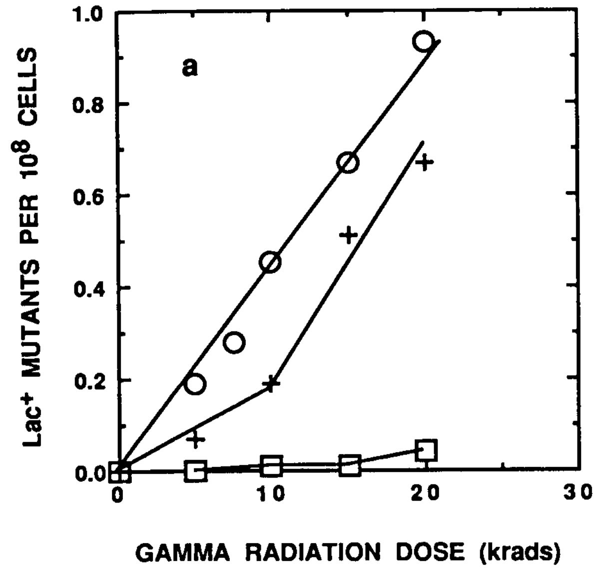
The RecF mechanism, i.e., the recombinogenic mechanism operating in *recB sbcBC* cells, seems to be relatively error-free in terms of spontaneous and induced deletion mutagenesis (Table 2 and Fig. 6a). This suggests that the DSB that are repaired by the RecF mechanism in a $recB^+$ strain are less likely to induce deletion mutations, and it suggests that the rate of production of γ-irradiation-induced deletions in a wild-type strain is the net result of the error-prone (RecB) and error-free (RecF) mechanisms. This hypothesis was tested by determining whether a *recF143* mutant, which can repair DSB only by the error-prone, RecB mechanism, would show a higher than normal rate of γ-irradiation-induced deletions. This predicted hypermutability was observed in the *recF143* strain at doses greater than 5 krad (Fig. 8a), and although the enhancement was not impressive, it is significant that the effect was seen only for deletions and not for base substitutions or frameshifts (Figs. 8b and 8c).

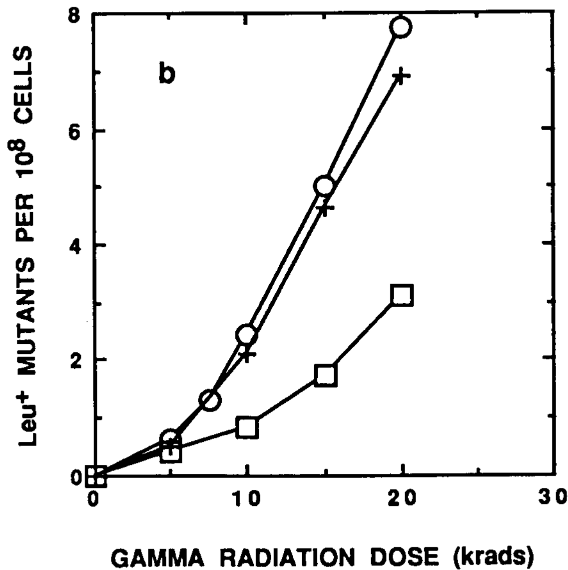
TABLE 2

SPONTANEOUS MUTANT FREQUENCIES IN STRAINS DEFICIENT IN DNA REPAIR [a]

Strain (relevant genotype)	Base substitutions $Leu^+/10^8$	Frameshifts $Trp^+/10^8$	Deletions $Lac^+/10^8$
SR2474 (+)	0.01	0.5	0.14
SR2474 (+), SMM-grown	0.02	1.0	0.54
SR2475 (*recB*)	0.01	0.8	0.04
SR2507 (*mutS*)	4.40	110.0	0.14
SR2522 (*recF*)	0.03	1.8	0.16
SR2526 (*recC*)	0.01	14.5	0.52
SR2528 (*recB sbcBC*)	0.02	1.9	0.01
SR2529 (*recB sbcBC mutS*)	4.40	476.0	0.00
SR2532 (*sbcBC*)	0.02	2.2	0.06

[a] Overnight yeast extract-supplemented nutrient broth or glucose minimal-medium (SMM if noted) cultures were washed twice and resuspended in PB at an OD_{650} of 50, and 0.2 ml of nondiluted or tenfold-diluted cells were spread on Leu-0, Trp-0, or Lac plates to determine Leu^+, Trp^+, or Lac^+ mutants, respectively, and diluted cells were also spread on SMM plates to determine total viable cells. Data are the median frequencies of mutants in the total viable population.





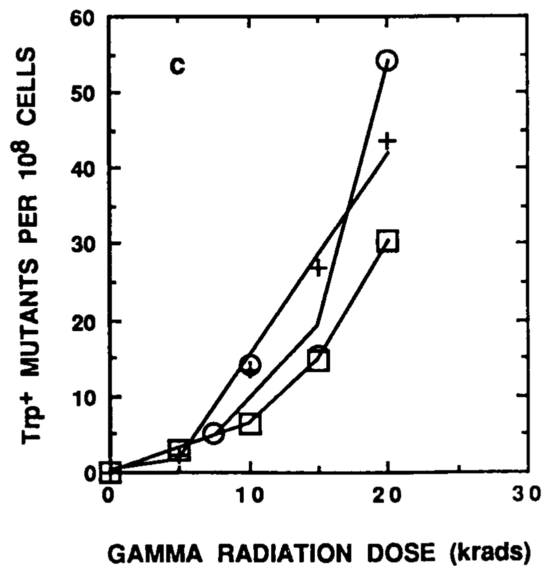


Fig. 6. γ-Radiation mutagenesis of wild-type (○, SR2474), *sbcBC* (+, SR2532), and *recB sbcBC* (□, SR2528) strains. Cells were grown in rich (YENB) medium, γ-irradiated in PB, and incubated 16–18 h in YENB before plating for mutants. Panels: (a), deletion mutants; (b), base substitution mutants; (c), frameshift mutants.

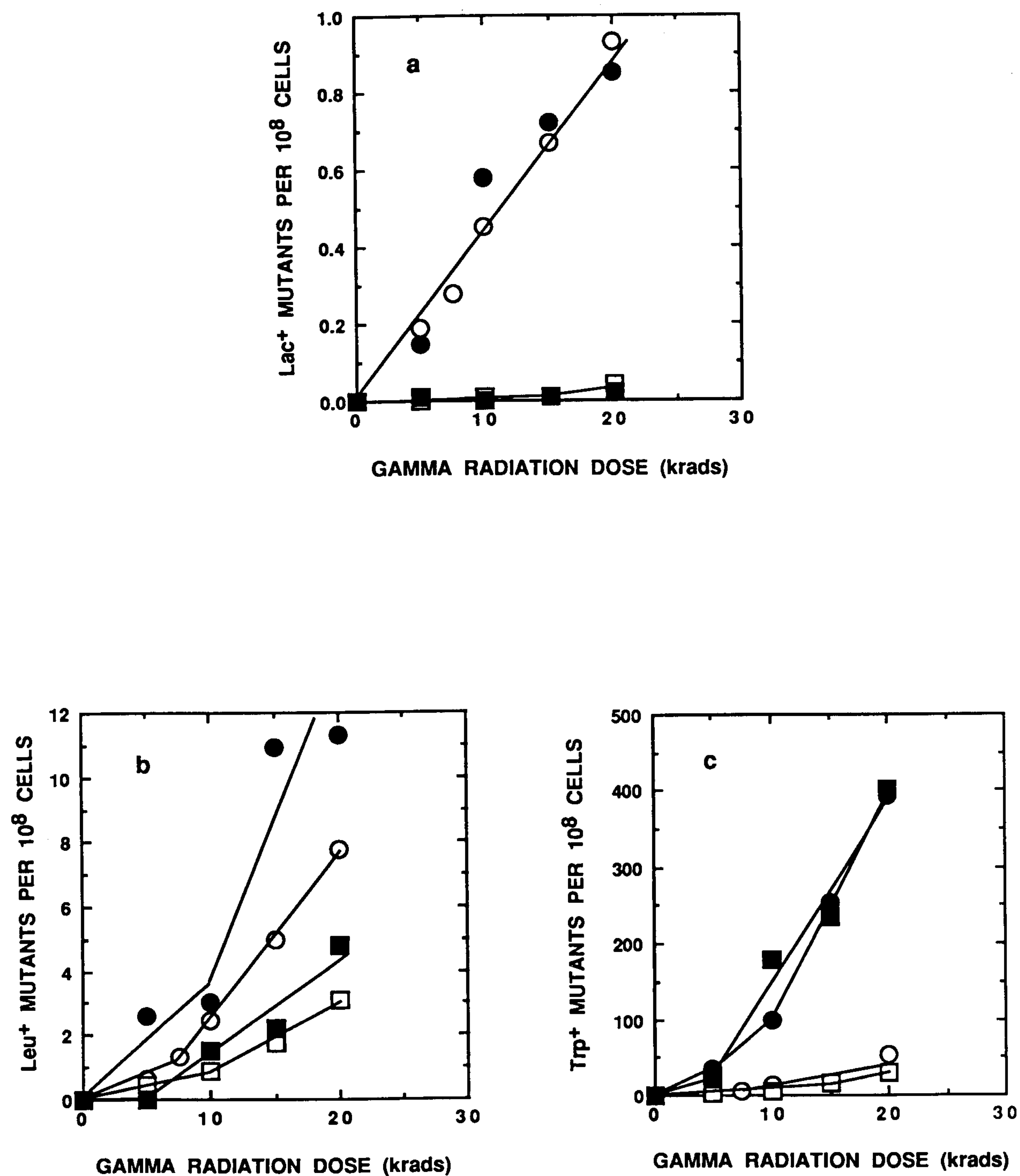


Fig. 7. γ-Radiation mutagenesis of wild-type (○, SR2474), *mutS* (●, SR2507), *recB sbcBC* (□, SR2528), and *recB sbcBC mutS* (■, SR2529) strains. Cells were grown in rich (YENB) medium, γ-irradiated in PB, and incubated 16–18 h in YENB before plating for mutants. Panels: (a), deletion mutants; (b), base substitution mutants; (c), frameshift mutants.

An essential requirement of the RecBC mechanism of recombination is the 8-bp sequence in DNA called Chi (Admundsen et al., 1990). A *recC1001* mutation prevents the recognition of the Chi sequence by the RecBCD enzyme, but since *recC1001* strains show enhanced levels of

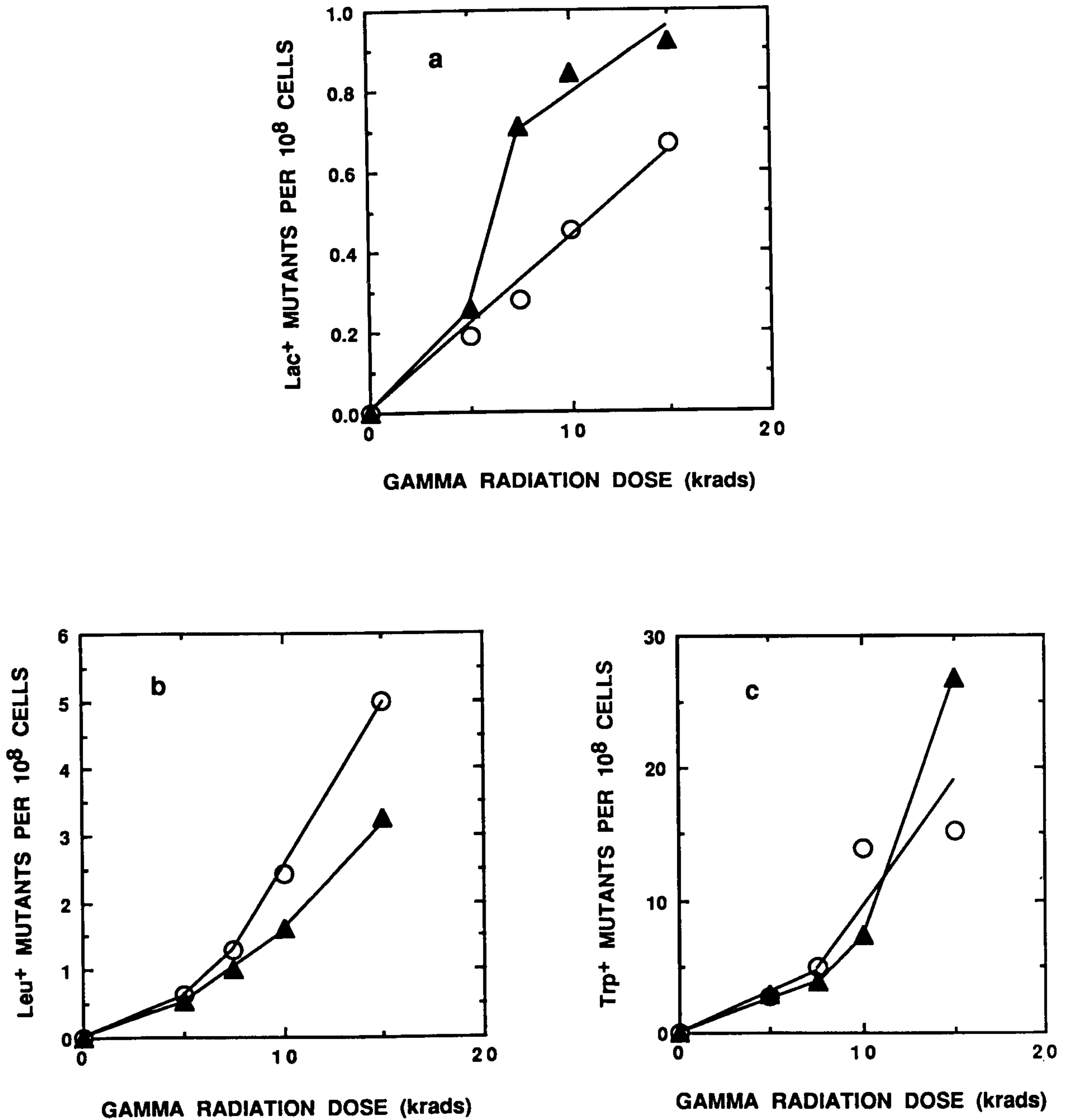


Fig. 8. γ-Radiation mutagenesis of wild-type (○, SR2474) and *recF* (▲, SR2522) strains. Cells were grown in rich (YENB) medium, γ-irradiated in PB, and incubated 16–18 h in YENB before plating for mutants. Panels: (a), deletion mutants; (b), base substitution mutants; (c), frameshift mutants.

recombination they are presumed to have their recombination stimulated by non-Chi DNA sequences that are more numerous than Chi (Schultz et al., 1983). To further test for the similarities between the RecB mechanism for recombination and for deletion mutagenesis, we

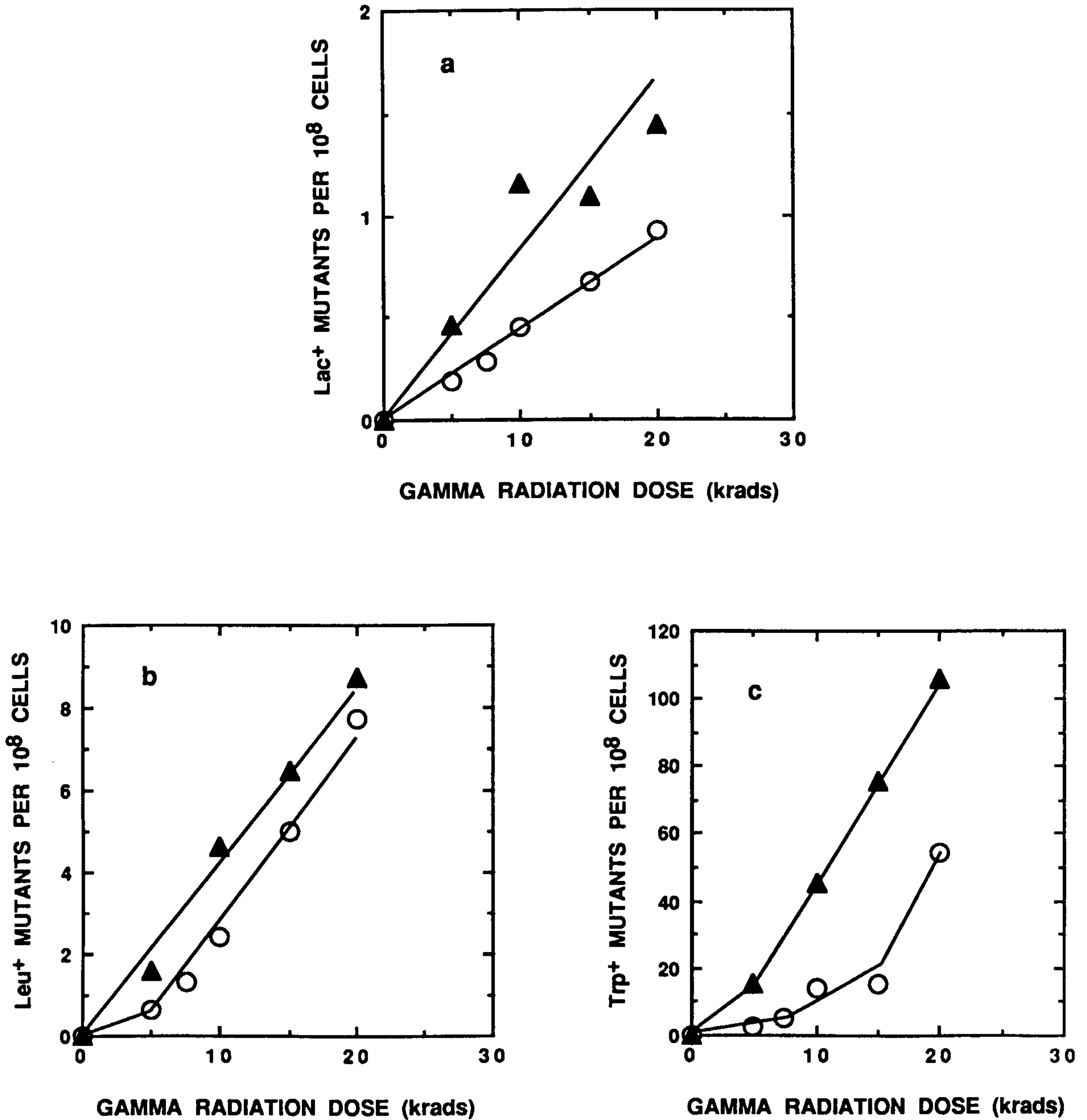


Fig. 9. γ-Radiation mutagenesis of wild-type (○, SR2474) and *recC* mutants (▲, SR2526). Cells were grown in rich (YENB) medium, γ-irradiated in PB, and incubated 16–18 h in YENB before plating for mutants. Panels: (a), deletion mutants; (b), base substitution mutants; (c), frameshift mutants.

measured the γ-radiation production of deletions in a *recC1001* strain. As predicted, the hyper-Rec character associated with the *recC1001* mutation is also manifested as a higher rate of deletion mutagenesis (Fig. 9a and Table 2). However, the concomitantly increased rates for spontaneous frameshifts (Table 2) and for γ-irradiation-induced base substitutions and frameshifts (Figs. 9b and 9c) suggest that the *recC1001* strain may also be constitutively induced for SOS-dependent mechanisms.

Discussion

Sequencing analyses of deletion mutations at many sites in both procaryotes and eucaryotes show that deletions occur almost uniformly at sites where a DNA segment possesses terminal direct repeats, and that the deletion results from the loss of the intervening DNA segment and one of the terminal direct repeats (e.g., Farabaugh et al., 1978; Albertini et al., 1982; Singer and Westlye, 1988; Yusaki et al., 1989; Keyeux et al., 1989; Stoppa-Lyonnet et al., 1990; Mita et al., 1990; and refs. therein).

That the aforementioned direct repeats play a critical role in deletion mutagenesis is supported by the results of two experiments. In one experiment involving a deletion hotspot with terminal direct repeats, the substitution of just one of the 14 homologous bases (in a 17-bp sequence) was sufficient to effect a dramatic decrease in the frequency of deletions at the hotspot site (Albertini et al., 1982). In the second experiment, an additional direct repeat was placed at one end of the deletion target (thereby doubling the chances for misalignment) and a 100-fold increase in the deletion rate was realized (Balbinder et al., 1989).

Such data led Efstratiadis et al. (1980), Albertini et al. (1982), and others (reviewed in Mita et al., 1990) to propose a 'slipped-mispairing' mechanism of intragenic recombination that occurs during DNA synthesis; a model derived from that proposed by Streisinger et al. (1966) as the mechanism for frameshift mutagenesis. Additional support for DNA replication-facilitated slipped mispairing being the mechanism for deletion mutagenesis was provided by Kunkel (1985), who showed that deletions arise between repetitive DNA sequences in an in vitro DNA synthesis system requiring only eucaryotic DNA polymerase β.

In spite of the evidence for a slipped-mispairing replication mechanism, a mechanism relying on direct-repeat stimulated strand misalignment during the recombinational repair of DSB seems more appropriate for ionizing radiation-induced deletions (Chadwick and Leenhouts, 1978). We make three observations that support this model. *First*, the process for the γ-radiation induction of deletion mutations in *E. coli* shows the same genetic (*recB*) and physiological requirements (Figs. 1a and 3a) as does the process for the repair of X-ray-induced DSB. Our results are in concert with those showing that endonucleolytically-induced DSB can produce deletions in mammalian cells and bacteria (Bien et al., 1988; Winegar and Preston, 1988; Bryant and Christie, 1989), and with those data suggesting that DSB production and deletion mutagenesis show the same LET dependence (cf., Nelson et al., 1989; Rosenbluth et al., 1985; Whaley and Little, 1990; Munson and Bridges, 1969). On the other hand, since the repair of X-ray-induced DNA single-strand breaks and UV radiation-induced DNA daughter-strand gaps is essentially normal in *recB21* mutants (Town et al., 1973; Wang and Smith, 1983), our results appear to contradict previous suggestions (Levin et al., 1984; Conkling and Drake, 1976) that the repair of these lesions might be important to deletion mutagenesis.

The *second* observation supporting the role of a DSB-repair-stimulated recombinational process, rather than a Streisinger-type slipped-mispairing misreplication process, in γ-radiation deletion mutagenesis is that the γ-radiation-induced reversion of the *trpE9777* mutation, which is believed to occur by the Streisinger mechanism (Sargentini and Smith, 1987), is in several ways different from γ-radiation-induced deletion mutagenesis. Unlike the production of deletions, the reversion of *trpE9777* (+1 frameshift) to Trp$^+$ is not blocked by the *recB21* mutation (Fig. 1c), nor is it reduced by growing cells in minimal medium vs. rich medium, in fact, it was increased (Fig. 4c). Conversely, frameshift production was significantly enhanced by inactivating mismatch repair with a *mutS* mutation, while deletion production

was not affected (cf., Figs. 7a and 7c). Also, the production of deletions in rich medium-grown cells was linear with dose [as is the ionizing radiation production of DSB (cf., Bonura et al., 1975; Sargentini and Smith, 1986b)], while the production of frameshifts was curvilinear (cf., Figs. 6a and 6c). One must conclude that there is a very clear differentiation between the induction of very short deletions (i.e., commonly called frameshifts) and longer deletions, that has important mechanistic implications.

The *third* observation supporting a recombinogenic mechanism in the γ-irradiation induction of deletions is simply that the only known function of the RecB protein, a subunit of exonuclease V, is in genetic recombination (e.g., reviewed in Smith, 1989).

One may note that some mutagens that efficiently produce DNA–DNA crosslinks have been shown to produce a good yield of deletions, e.g., nitrous acid (reviewed in Zimmerman, 1977) and 8-methoxypsoralen plus near-UV radiation (Yatagai and Glickman, 1986), but it is clear that DSB and homologous recombination are essential steps in the repair of DNA–DNA crosslinks (e.g., Sinden and Cole, 1978; Cupido and Bridges, 1985).

For the mechanism of the γ-radiation induction of deletions, we propose a model derived from models for RecBCD-dependent recombination (e.g., Smith, 1989) and from models for the repair of DSB (Resnick, 1976; Krasin and Hutchinson, 1977; Szostak et al., 1983). Briefly, we propose that the RecBCD enzyme attaches to the end of the DSB and unwinds (and rewinds) the DNA duplexes until a Chi site is encountered. Since the unwinding action is more rapid than the rewinding action, single-stranded DNA loops are formed. Cutting of the single-stranded DNA loop near the Chi site allows the invasion of the 3′ end into a sister-DNA duplex where it can search for a homologous DNA sequence. The proper alignment of the invading strand with its complementary homologue can lead to the accurate repair of DSB either by noncrossover or crossover recombination (depending on how the Holliday structure is resolved). However, misalignment of the invading strand (with or without the aid of an inverted-repeat stimulated hairpin structure; Glickman and Ripley, 1984) at another locus, either with a partially replicated sister duplex or downstream with the same duplex, would lead to a loop of nonhomologous single-stranded DNA that could be lost by excision, or through bypass replication, to produce a deletion.

Since the *recA* gene is known to be essential for the repair of DSB (Krasin and Hutchinson, 1977; Wang and Smith, 1983; Sargentini and Smith, 1986b) and for genetic recombination (reviewed in Clark, 1973), this suggests that most of the spontaneous deletions noted here (Table 2) and by Albertini et al. (1982) arose by the inaccurate recombinational repair of DSB of unknown origin. On the other hand, there are several reports of *recA*-independent spontaneous deletions (e.g., Franklin, 1967; Inselburg, 1967; Levin et al., 1984) with other deletion assays, and a significant fraction of the spontaneous deletions measured here were *recA* and *recB* independent (Table 2, and Albertini et al., 1982). Thus, it seems likely that a DNA synthesis-stimulated slipped-mispairing mechanism may be the source of these *recA*-independent deletions.

Transposon Tn*10* insertion produces a 9300-bp structure with terminal 9-bp direct repeats and subterminal 1329-bp inverted repeats. Deletion involving the terminal direct repeats leads to precise excision of Tn*10*, while deletion involving a pair of subterminal direct repeats leads to excision of the transposon, but leaves a 50-bp insertion (Lundlblad et al., 1984; and refs. therein). Both types of transposon excision rely on terminal direct repeats but are independent of *recA*, *recB*, *recC* and any transposon encoded functions, and seem to be dependent on DNA gyrase (Miura-Masuda and Ikeda, 1990). This argues that the *recA recB*-independent spontaneous deletion mechanism may only be efficient in the presence of subterminal inverted repeats. In fact, evidence for this mechanism was found in most of the spontaneous *lacI* deletion mutants analyzed by Glickman and Ripley (1984), who showed that subterminal inverted repeats could stabilize a looped-out segment of single-stranded DNA. Thus, the *recA* dependence or independence of spontaneous deletions may indicate whether DSB or subterminal inverted repeats are the critical factors in the induction of specific deletions. Since

transposon excision has not been reported to be enhanced by DNA-damaging agents, one must consider either that subterminal inverted-repeat-mediated loop-formation may interfere with DNA repair-induced deletion formation, or that previous studies have not involved transposon insertions near a Chi site (see below).

Three possible explanations come to mind to explain the poor yield of γ-radiation induced deletions detected in the *recB sbcBC* strain. (i) While the repair of chromosomal DSB occurs, albeit it at a slightly slower rate (e.g., Fig. 5), and a wild-type level of radiation resistance is observed (Fig. 2), it may be possible that the RecF mechanism (the only recombination mechanism operating in *recB sbcBC* cells, e.g., Smith, 1989) does not repair DSB in the F′ episome, which is essential for the production of Lac^+ mutants. This point is difficult to check, and it does not seem likely since the episome shows the same supercoiled folding, its initiation of replication is under the same stringent control, and it exists in the same intracellular environment as the bacterial chromosome (e.g., Kline and Miller, 1975). (ii) γ-Radiation deletion mutagenesis is proposed here to be the result of a faulty attempt at the repair of DSB. If recombination via the RecF pathway more stringently excludes misalignment than does recombination via the RecBCD pathway, then one would expect to find fewer deletions by this repair mode. In support of this notion, conjugational recombination using the RecF pathway shows a higher incidence of mismatch correction than does that by the RecBCD pathway (Mahajan and Datta, 1979). Perhaps related to this finding, the *recB sbcBC* strain requires 2–3-fold greater minimum homology to initiate recombination (Shen and Huang, 1986), it repairs DSB more slowly (Fig. 5), and it shows a slower rate of integration and segregation of conjugated DNA than do rec^+ recipient strains (Mahajan and Datta, 1979; Bresler et al., 1981; Lloyd and Thomas, 1983). It should be noted that the recombination efficiency of the *recB sbcBC* strain vs. $recB^+$ strains varies from 70 to 100% (Lloyd et al., 1987; Zieg and Kushner, 1977) for conjugation assays, from 63 to 100% for transduction assays (Zieg and Kushner, 1977; Crawford and Preiss, 1972), and was 3-fold higher for an assay based on recombination between nontandem chromosomal duplicated sequences (Zieg and Kushner, 1977). Thus, differences in recombination efficiency between the RecF and RecBCD mechanisms do not seem to explain the huge difference in their ability to produce deletions, however, differences in recombination accuracy may be relevant to the different deletion rates by these mechanisms. This conclusion is supported by the enhanced deletion rates observed here for *recF143* cells and for *recC1001* cells, both of which may be assumed to be using the RecBCD mechanism more often than would be the case for wild-type cells. (iii) We predict that the major difference between RecBCD- and RecF-mediated DSB repair is that with the RecF mechanism, the 3′ end of the DNA strand that invades an unbroken sister duplex is closely associated with the DSB site, while in the RecBCD mechanism, the 3′ end is always associated with a Chi (or Chi-like) site. Thus, the RecBCD enzyme effectively transfers the DSB from its original site to the Chi site. Depending on the proximity and orientation of the Chi site relative to a potential deletion site, the probability of a deletion being induced may be much higher or much lower than if the probability of deletion induction depended on the distance between the deletion site and the original DSB (as in the *recB sbcBC* strain). While there are no Chi sequences (GCTGGTGG 3′; e.g., Smith et al., 1989) in the *lacI* gene [cf., Farabaugh, 1978 (the one that is listed at bp 894 is incorrect according to the amino acid sequence that is shown)], and the sequence on either side of the episomal *lacI–Z* fusion gene is not known, there is a Chi site in the *lacZ* gene at bp 2229 (Triman et al., 1982; Kalnins et al., 1983). Even though this Chi site is about 2.3 kb away from the potential deletion site, it should still exert about 50% of its normal enhancing effect on recombination (Ennis et al., 1987). There are three Chi-like sites in the *lacI–Z* fusion (G*A*TGGTGG at bp 1962 in *lacZ*, and GCTGGT*T*G at bp 753 and GCTGGTG*A* at bp 569 in *lacI*), but these are thought to be inactive (Smith et al., 1981). The huge difference in the yield of γ-radiation-induced deletions between the wild-type and *recB*

sbcBC strains (e.g., 36-fold at 20 krad) most likely reflects a combination of explanations (ii) and (iii).

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