Opposing Roles of the Holliday Junction Processing Systems of *Escherichia coli* in Recombination-Dependent Adaptive Mutation

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ABSTRACT

Aspects of the molecular mechanism of "adaptive" mutation are emerging from one experimental system: reversion of an *Escherichia coli lac* frameshift mutation carried on a conjugative plasmid. Homologous recombination is required and the mutations resemble polymerase errors. Reports implicating a role for conjugal transfer proteins suggested that the mutation mechanism is ordinary replication error occurring during transfer synthesis, followed by conjugation-like recombination, to capture the replicated fragment into an intact replicon. Whereas conjugational recombination uses either of two systems of Holliday junction resolution, we find that the adaptive *lac* reversions are inhibited by one resolution system and promoted by the other. Moreover, temporary absence of both resolution systems promotes mutation. These results imply that recombination intermediates themselves promote the mutations.

UNDERSTANDING the molecular mechanisms of spontaneous mutation is critical both to our understanding of the generation of genetic diversity that drives evolution, as well as the early events in cancer, in which mutagenesis underlies oncogenic transformation. For decades one mode of spontaneous mutation was believed to be exclusive. Spontaneous mutations were described as occurring before a cell experiences an environment in which the mutation might be useful, randomly in the genome, and were measured as mutations per cell per generation (LURIA and DELBRÜCK 1943; LEDERBERG and LEDERBERG 1952). The possibility of a fundamentally different mode of spontaneous mutation is emerging from studies of "adaptive" mutations in bacteria and yeast (*e.g.,* RYAN 1955; RYAN *et al.* 1961; CAIRNS *et al.* 1988; CAIRNS and FOSTER 1991; HALL 1992; JAYARAMAN 1992; STEELE and JINKS-ROBERTSON 1992; reviewed by FOSTER 1993). These occur only after exposure to a nonlethal genetic selection, in the apparent absence of cell division, and have been detected so far only in the genes whose functions were selected (references above but see HALL 1990). These characteristics suggested that adaptive mutations might represent an example of Lamarckian evolution (CAIRNS *et al.* 1988). Whether or not this is the case will be easier to discern once the molecular mechanisms of adaptive mutagenesis are understood.

It is already clear that there is more than one molecular mechanism by which adaptive mutations form (see DRAKE 1991; FOSTER 1993). Although little is known about the mechanism in most of the adaptive mutation assay systems, in one system significant molecular information exists. That system is reversion of a *lac* frameshift mutation carried on an F′ episome in *Escherichia coli* cells (CAIRNS and FOSTER 1991). In this system the following is known. First, the RecBCD system of homologous genetic recombination participates in adaptive but not growth-dependent Lac reversion (HARRIS *et al.* 1994). Second, because RecBCD enzyme loads onto DNA only at double-strand breaks (DSBs) (TAYLOR 1988), DSBs are implicated as a molecular intermediate in the adaptive mutagenesis (HARRIS *et al.* 1994; see ROSENBERG 1994; ROSENBERG *et al.* 1995a,b). Third, the adaptive reversions of this +1 frameshift allele are nearly all −1 deletions in small mononucleotide repeats, whereas the growth-dependent Lac$^+$ reversions are highly heterogeneous (FOSTER and TRIMARCHI 1994; ROSENBERG *et al.* 1994). Such simple repeat instability is characteristic of DNA polymerase error (RIPLEY 1990) thought to be caused by a template slippage mechanism (STREISINGER *et al.* 1966). The adaptive reversion sequences resemble the simple repeat instability seen in hereditary nonpolyposis colon cancer (reviewed by MODRICH 1994) and other cells that lack postsynthesis DNA mismatch repair (LEVINSON and GUTTMAN 1987; CUPPLES *et al.* 1990; STRAND *et al.* 1993). Fourth, the hypothesis that the absence of functional mismatch repair is responsible for the unique sequence spectrum of the adaptive Lac$^+$ reversions is supported by the finding that mismatch repair-defective cells produce a growth-dependent Lac$^+$ reversion spectrum that is indistinguishable from that of the adaptive reversions (LONGERICH *et al.* 1995).

These data have suggested a model (HARRIS *et al.* 1994; see ROSENBERG 1994; ROSENBERG *et al.* 1995a,b) in which the stressed, starving cells generate DNA DSBs that promote RecBCD-mediated homologous recombi-

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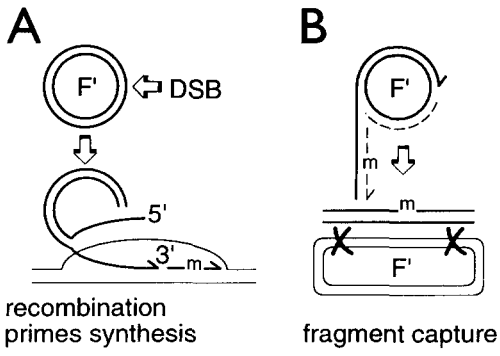

FIGURE 1.—Two models for recombination-dependent mutations using polymerase errors. (A) From HARRIS *et al.* (1994). The DSB is proposed to occur at the origin of transfer as a consequence of single-strand nicking by the transfer proteins (discussed in the text). (B) From PETERS and BENSON (1995); GALITSKI and ROTH (1995); FOSTER and TRIMARCHI (1995). Dashed lines represent newly synthesized DNA; m, a polymerase mistake that becomes a mutation. DSB, DNA double-strand break. Large Xs in B signify crossover recombination whole reactions. Models containing aspects of both models shown here are also possible. In both models, a DNA homology with the F′ is required for recombination, and in both, that homology is imagined to be a sister replication product. Sister molecules might be infrequent in starving cells. However, the occurrence of adaptive revertants is also infrequent and so is not discouraged by this concern.

nation (see Figure 1A). An invading 3′ end in a RecA/RecBCD-promoted strand exchange intermediate was suggested to prime DNA synthesis during which polymerase errors are made. The errors might escape mismatch repair due to insufficient mismatch repair activity in these cells. The failure to detect mutations in unselected genes could be caused by DSB-mediated killing of cells that do not become Lac$^+$, and thus do not escape the starvation stress that promotes DSBs. [Note that F plasmid loss causes death of the host cell (JENSEN and GERDES 1995)]. Alternatively, perhaps unselected mutations would be found if other loci on the *lac*-bearing F′ replicon were tested. This replicon might be particularly active in the recombination-dependent adaptive mutation mechanism.

A fifth piece of information from this system has provided a possible source of the DSBs, has encouraged the idea that the F′ replicon is special, and is suggesting a different molecular mechanism: the F′-encoded proteins responsible for conjugative transfer of the plasmid (but not actual transfer) are necessary for high frequency Rec-dependent Lac$^+$ reversion, such that both transfer-defective F′s and a chromosomal *lac* gene appear mutationally inactive (FOSTER and TRIMARCHI 1995; GALITSKI and ROTH 1995; RADICELLA *et al.* 1995). These authors hypothesize that conjugative transfer replication could be the source of the DNA polymerase errors that lead to adaptive mutation. Recombination is not usually required for transfer of conjugative plasmids or for transfer replication (FROST *et al.* 1994) but could be necessary if the transfer replication were incomplete such that the newly synthesized fragment must be recombined into an intact replicon in order to preserve the mutation (Figure 1B) (GALITSKI and ROTH 1995; FOSTER and TRIMARCHI 1995; PETERS and BENSON, 1995). In this model, recombination acts after the polymerase error. A whole recombination reaction should be required to capture the fragment containing the error (see Figure 1B). This would seem to be indistinguishable from bacterial conjugational recombination. This contrasts with the mechanism discussed previously (see Figure 1A), in which the recombinational strand exchange intermediate itself primes the DNA synthesis during which the errors occur. If the strand exchange intermediates themselves are mutagenic (*i.e.*, if only partial recombination reactions are necessary), then the recombination proteins required might differ from those for conjugational recombination whole reactions in that failure to resolve intermediates might promote mutation.

In this paper, we investigate the role of recombination and recombinational strand exchange intermediates in adaptive Lac$^+$ reversion by manipulating the enzymes that process strand exchange intermediates into recombinant products. In *E. coli* conjugational recombination, strand exchange intermediates are resolved to products by either of two Holliday junction resolution systems (LLOYD 1991; see WEST 1994). We find that this is not the case for Lac$^+$ reversion. First, the two resolution systems, RecG and RuvABC, which appear redundant for conjugational recombination, affect Lac adaptive mutation in opposite ways, one inhibiting and the other promoting mutation. Second, delaying the action of both resolution systems causes hypermutation. These results imply that recombination intermediates themselves promote Lac$^+$ adaptive mutation.

MATERIALS AND METHODS

Bacterial strains: Strains used in this work are listed in Table 1. All new genotypes were made by standard P1 transduction methods. Throughout the work, unless otherwise specified, the *ruvA* alleles used were *ruvA59::*Tn*10*, which is polar on *ruvB* creating RuvAB-deficiency, and a streptomycin-resistant derivative of this allele, *ruvA76::*Tn*10Sm*. *ruvA76::*Tn*10Sm* was constructed by disruption of the tetracycline resistance gene of Tn*10*, inserting the streptomycin-resistance cassette, using the method of FRANÇOIS *et al.* (1987).

Mutation assays: Adaptive reversion assays were performed as described previously using the same media and conditions (HARRIS *et al.* 1994), except that the *ruv recG* mutants were constructed and grown at ≤32° to avoid accumulation of growth-defect-suppressing mutations and were then assayed for Lac$^+$ reversion at 37°. These procedures are as follows. Each strain to be assayed for adaptive mutation is taken directly from the original stock that was constructed and tested and frozen at −80°. The strain is streaked for single colonies on M9 minimal medium containing vitamin B1 and 0.1% glycerol. Four to 12 independent cultures used in each experiment are inoculated, each from one whole (separate) single colony from the plate and grown to saturation in M9 B1 0.1% glycerol.

TABLE 1

***E. coli* K12 strains**

Strain	Relevant genotype	Reference
Frameshift-bearing cells		
FC40	*ara* Δ*(lac-proB)*$_{XIII}$ *thi* Rif^r [F′ *lacI33*]	CAIRNS and FOSTER (1991)
SMR624	FC40 Δ*(srlR-recA)306::*Tn*10*	HARRIS *et al.* (1994)
RSH38	FC40 *ruvC53 eda51::*Tn*10*	This work
RSH45	FC40 *ruvC53 eda51::*Tn*10 recG258::*Tn*10miniKan*	This work
RHS152	FC40 *ruvA200 eda51::*Tn*10*	This work
RSH154	FC40 *ruvA59::*Tn*10*	This work
RSH155	FC40 *ruvB9 zea3::*Tn*10*	This work
RSH159	FC40 *ruvA200 eda51::*Tn*10 recG258::*Tn*10miniKan*	This work
RSH160	FC40 *ruvA59::*Tn*10 recG258::*Tn*10miniKan*	This work
RSH161	FC40 *ruvB9 zea3::*Tn*10 recG258::*Tn*10miniKan*	This work
RSH275	FC40 *ruvA76::*Tn*10Sm* Δ*(srlR-recA)306::*Tn*10 recG258::*Tn*10miniKan*	This work
RSH316	FC40 *recG258::*Tn*10miniKan*	This work
RSH326	FC40 *recG258::*Tn*10miniKan* Δ*(srlR-recA)306::*Tn*10*	This work
Scavenger cells		
FC29	*ara* Δ*(lac-proB)*$_{XIII}$ *thi* [F′ Δ*(lacI-lacZ)*]	CAIRNS and FOSTER (1991)
RSH9	FC29 Δ*(srlR-recA)306::*Tn*10*	ROSENBERG *et al.* (1995a)
RSH353	FC29 *ruvC53 eda51::*Tn*10*	This work
RSH355	FC29 *ruvC53 eda51::*Tn*10 recG258::*Tn*10miniKan*	This work

The saturated cultures are washed twice in M9 B1 and resuspended to a concentration of viable cells that gives an assayable number of Lac$^+$ colonies over the duration of the experiment when 50–200 μl are mixed with an eight- to 40-fold excess of scavenger cells (grown up using the same procedure as just described), plated in M9 B1 0.1% lactose top agar on M9 B1 0.1% lactose agar plates and incubated at 37°. Two different dilutions of each separate culture are plated. These same saturated cultures are assayed for the number of viable cells on LB plates and are tested for the presence of *rec* or *ruv* mutations. Because poorly viable genotypes such as all of the *ruv recG* combinations accumulate high frequencies of growth-defect-suppressor mutations and true reversion mutations (discussed below), we have found that it is imperative both to minimize growth of the cultures used in the experiments (*i.e.*, avoid diluting and regrowing saturated cultures as in the procedure of FOSTER 1994) and to test each of the actual cultures used in the adaptive reversion experiments for presence of *ruv* and *recG* alleles and for the absence of suppressor mutations (described below and further in the text).

A severe growth defect is caused by the double mutant combinations *ruvA recG, ruvB recG* and *ruvC recG,* such that cells carrying these combinations are genetically unstable—they readily accumulate growth-defect-suppressing mutations and also true reversions of the transposon-insertion null alleles (LLOYD 1991; MANDAL *et al.* 1993; and R. S. HARRIS and S. M. ROSENBERG, unpublished observations). Both the suppressor mutations and the true reversion mutations can be distinguished from their *ruv recG* parents by their larger colony size and by their increased UV-resistance (LLOYD 1991; MANDAL *et al.* 1993; and R. S. HARRIS and S. M. ROSENBERG, unpublished observations). In addition to these phenotypes, we have found (reported below) that such suppressor and reversion strains behave differently in adaptive mutation experiments; they show severely decreased adaptive reversion whereas cultures of all of the *ruv recG* combinations that retain their extreme UV sensitivity and small colony size display adaptive hypermutation (see RESULTS AND DISCUSSION for details). To ensure that the independent cultures used in adaptive reversion experiments are free from growth-defect-suppressor mutations and true reversions, the procedures were modified as follows: first, on the streak plate from which colonies for the saturated cultures used in the experiments are obtained, small colonies are chosen. Some large suppressor and revertant colonies are usually present but these are avoided. Second, the saturated cultures are grown slowly at 30–32° rather than at 37°. We have found that this reduces the frequency of large colony-forming, UV-resistant cells in the final cultures. Third, the saturated cultures used in the experiments are tested for UV sensitivity and for the presence of the transposon associated with the *ruv* and *recG* alleles as described above. Fourth, we showed that adaptive mutation selection conditions do not promote accumulation of suppressor or reversion mutations; the ratio of large to small colonies present in cultures plated is the same as that observed in *lac*$^-$ cells recovered after 4 days incubation under adaptive reversion conditions. Finally, for each *ruv* allele used in each *ruv recG* double mutant combination, two to three strains were constructed independently and shown to give the same results in adaptive mutation experiments when the precautions and testing described here were done for each. The occasional suppressor and reversion strains that we obtained have UV-resistance levels that range from comparable with a *ruv* single mutant (which is more resistant than any of the *ruv recG* double mutants) to as resistant as *rec*$^+$ cells. Those that we examined, at several different UV-resistance levels, showed adaptive hypomutation, in contrast with their *ruv recG* parents (RESULTS AND DISCUSSION). One of them, with a UV-resistance level comparable with a *ruv* single mutant, was in fact a revertant of *recG,* presumably by precise excision of the transposon disrupting that gene. We did not map or characterize further the other, growth-defect-suppressor mutant strains. Similar suppressor mutants characterized by MANDAL *et al.* (1993) carry *rus* mutations, which activate an otherwise cryptic pathway of Holliday junction resolution.

The Lac$^+$ colonies arising over time are expressed per 10^8 viable cells plated (measured in the viable cell counts of the cultures to be plated). As previously, the number of viable frameshift-bearing cells was measured each day of the experiments and neither growth nor death was observed in any of

the experiments reported here (see RESULTS AND DISCUSSION for data). Therefore in all experiments, with all strains used here, the number of viable cells plated is the number of viable cells that remained on the plates throughout the course of the experiments. Thus, the different mutation phenotypes reported represent mutations per viable cell on the plates.

Growth-dependent mutation rates were measured as described previously (HARRIS *et al.* 1994). Mutation rates were calculated using the method of the median (LEA and COULSON 1949) as modified by VON BORSTEL (1978).

RESULTS AND DISCUSSION

Experimental system: The mutation assay system described by CAIRNS and FOSTER (1991) measures reversion of the *lacI33* +1 frameshift mutation carried on an F′ episome in cells with the chromosomal *lac* operon deleted. *lacI33* is a fusion of the *lacI* and *lacZ* genes such that the +1 frameshift mutation in *lacI* is polar on *lacZ* and the cells are Lac$^-$. Growth-dependent mutant colonies appear on the second day after plating on minimal lactose medium, and form independently of RecA and RecBCD proteins. Adaptive mutants arise continuously during the week after plating (CAIRNS and FOSTER 1991) and do not arise in *recA*, or *recB* null mutant cells (HARRIS *et al.* 1994). The *lac*$^-$ cells giving rise to the Lac$^+$ mutants are prevented from multiplying on the minimal lactose medium by the presence of an excess of nonrevertible, *lac*-deletion "scavenger" cells that consume any contaminating, nonlactose carbon sources that might be present. The absence of growth of the frameshift-bearing cell is confirmed by daily viable cell measurements in which a plug of agar from the plate is suspended in liquid and assayed for colony-forming units on rifampicin plates that let the frameshift-bearing cell, but not the scavenger, form colonies. Such measurements showed no net growth or death for all of the experiments reported here.

The scavenger cell is also male, carrying an F′ with no *lac* genes, in order to discourage transfer between the frameshift-bearing cell and the scavenger. In fact, about 8–10% of Lac$^+$ adaptive revertants have transferred their F′ into the scavenger (RADICELLA *et al.* 1995; ROSENBERG *et al.* 1995a). However, F′ transfer appears to be unnecessary for, and probably occurs after, adaptive Lac reversion as shown by the following observations: first, mutations that decrease transfer by 10^5-fold decrease Lac$^+$ adaptive reversion by only 10-fold (FOSTER and TRIMARCHI 1995); and second, the RecA protein is required in the frameshift-bearing cell and not in the scavenger (ROSENBERG *et al.* 1995a and data presented below). Thus, models in which transfer synthesis is thought to be critical for Lac$^+$ adaptive reversion suppose that the transfer synthesis occurs without actual transfer (FOSTER and TRIMARCHI 1995; GALITSKI and ROTH 1995).

The RuvABC and RecG Holliday junction resolution systems play opposing roles in Lac$^+$ adaptive reversion: Both the RecA and RecBCD proteins, which are necessary for Lac$^+$ adaptive reversion, function early in recombination to initiate formation of strand exchange intermediates (reviewed by ROSENBERG and HASTINGS 1991; WEST 1992; KOWALCZYKOWSKI *et al.* 1994). In conjugational recombination, the strand exchange intermediates are then resolved either by the RuvABC resolution system or by an alternative system that requires RecG (LLOYD 1991; WEST 1994). Thus, cells that carry *ruv* single mutations, or carry a *recG* mutation, are recombination-proficient. Only the *ruv recG* double mutant combinations produce recombination-deficiency (LLOYD 1991).

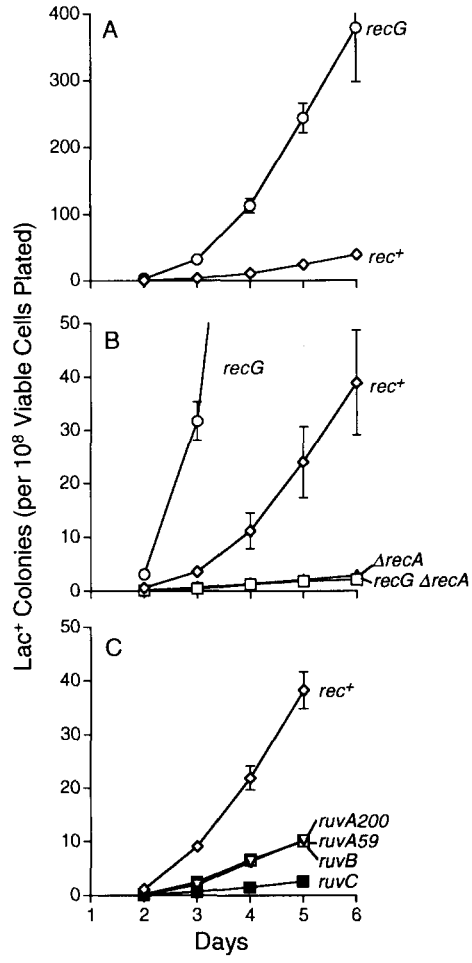

FIGURE 2.—Opposite roles of the RuvABC and RecG resolution systems on Lac$^+$ adaptive reversion. Error bars represent one standard error of the mean.

To ask whether the genetic requirements for Lac$^+$ adaptive reversion parallel those for conjugational recombination, we examined the effects of single mutations in the RecG and RuvABC systems on postplating Lac$^+$ reversion. In Figure 2A, a *recG* null mutant shows greatly elevated postplating Lac$^+$ reversion. This contrasts with the phenotype of *recG* deficiency in conjugational recombination, in which a very small depression is seen (LLOYD 1991). The hypermutation in a *recG* strain could be an elevation of genuine RecABC-dependent Lac$^+$ adaptive reversion, but could also have been

caused by the activation of some other, new, RecA-independent route to mutation. To distinguish these possibilities, a *recG recA* double mutant was tested. In Figure 2B, the hypermutation in a *recG* strain is shown to be completely RecA-dependent. Thus, the presence of the RecG protein appears to antagonize RecA-dependent Lac$^+$ adaptive reversion.

ruvA, ruvB, and *ruvC* mutations have little effect on conjugational recombination in the presence of a functional *recG*$^+$ gene (see LLOYD 1991). This is not the case for Lac$^+$ adaptive reversion. In Figure 2C, the data show that postplating Lac$^+$ reversion is strongly inhibited in *ruvA* and *ruvB* null mutants and is abolished in a *ruvC* null mutant strain. Two different *ruvA* alleles show the same effect: *ruvA200,* which blocks only RuvA function, and *ruvA59::*Tn*10,* which is also polar on the *ruvB* gene. We conclude that the RuvABC system is necessary for Lac$^+$ adaptive reversion even in the presence of functional RecG. This is unlike normal conjugational recombination. A possible similarity between Lac$^+$ adaptive reversion and two unusual recombination assay systems that show *ruv*-dependence in the presence of RecG (LLOYD 1991; MATIC *et al.* 1995) is discussed below.

A possible biochemical basis for the opposite effects of the two resolution systems on Lac$^+$ adaptive reversion is considered below. For now we wish to conclude, first, that the genetic requirements of Lac$^+$ adaptive reversion and conjugational recombination are different. This discourages fragment capture models for Lac$^+$ adaptive reversion. Second, the enzymes in these resolution systems are well defined biochemically: RuvAB and RecG proteins bind to, and perform branch migration of, Holliday junctions and other strand exchange intermediates; RuvC endonuclease binds to and then cleaves such intermediates, assisted by RuvAB (WEST 1994). The involvement of all of these proteins provides evidence that strand exchange intermediates are also intermediates in Lac$^+$ adaptive reversion. The data in Table 2 show that growth-dependent, RecA-independent Lac$^+$ reversion rates are unaffected by these proteins.

Temporary absence of both the RuvABC and RecG resolution systems promotes Lac$^+$ adaptive hypermutation: Conjugational recombination is blocked by the absence of RuvA, RuvB, or RuvC, and RecG proteins simultaneously, presumably because both routes to resolution of strand exchange intermediates are blocked (LLOYD 1991). Under this situation, the strand exchange intermediates should accumulate but should not produce recombinant products. If strand exchange intermediates themselves were responsible for priming the DNA synthesis that leads to recombination-dependent Lac$^+$ adaptive mutation (HARRIS *et al.* 1994; Figure 1A), then it is possible that blocking both resolution pathways in *ruv recG* double mutants might cause an increase in Lac$^+$ adaptive reversion. This is seen for a *ruvC recG* double mutant and a *ruvA recG* double mutant in Figure 3, A and B, respectively. The *ruvA* allele is polar on *ruvB.* We also observe this effect with a different *ruvA* allele, *ruvA200,* a nonpolar allele, in combination with *recG,* and with the *ruvB recG* double mutant combination (data not shown). The hypermutation in *ruvA recG* and *ruvC recG* is completely RecA-dependent (*e.g.,* see Figure 3B) and thus represents enhancement of normal recombination-dependent Lac$^+$ adaptive reversion, not creation of a novel mutagenic route. The *ruv recG* resolution-defective mutation combinations do not affect growth-dependent Lac$^+$ reversion rates (Table 2).

All of the *ruv recG* double mutant combinations used

TABLE 2

Mutation rates in growing cultures

Genotype	Experiment	Number of cultures	Rate of mutation to Lac$^+$ (mutations per cell per generation)
rec$^+$	1	40	4.7×10^{-10}
	2	40	7.2×10^{-10}
	3	40	9.6×10^{-10}
	4	40	6.4×10^{-10}
	5	40	7.5×10^{-10}
recG	3	40	20×10^{-10}
	4	40	15×10^{-10}
	5	40	22×10^{-10}
ruvA	1	39	4.5×10^{-10}
	2	40	5.7×10^{-10}
	5	40	4.0×10^{-10}
ruvC	1	40	4.0×10^{-10}
	2	40	3.3×10^{-10}
	5	40	2.6×10^{-10}
ruvA recG	1	26	3.6×10^{-10}
	2	34	8.0×10^{-10}
	5	40	16×10^{-10}
ruvC recG	1	33	9.4×10^{-10}
	2	31	28×10^{-10}
	5	40	5.5×10^{-10}
rec$^+$	6	10	4.7×10^{-10}
	7	10	4.0×10^{-10}
recG	6	10	29×10^{-10}
	7	9	61×10^{-10}
recG Δ*recA*	6	10	3.0×10^{-10}
	7	4	1.7×10^{-10}

Strains *rec*$^+$, *recG, ruvA, ruvC, ruvA recG, ruvC recG* and *recG* Δ*recA* are FC40, RSH316, RSH154, RSH38, RSH160, RSH45 and RSH326, respectively (Table 1). Mutation rates are calculated by the method of the median (LEA and COULSON 1949 as modified by VON BORSTEL 1978) and are measured as determined previously (HARRIS *et al.* 1994). The *recG* strain displays extreme Lac$^+$ adaptive hypermutation (Figure 2) and also appears hypermutable in growth-dependent Lac$^+$ reversion here. The apparent elevation of growth-dependent mutation might be due to contamination of the preplating revertants with postplating, RecA-dependent adaptive revertants. This possibility is supported by the finding that the increase in *recG* is entirely *recA*$^+$-dependent (experiments 6 and 7). The RecA-independent, growth-dependent Lac$^+$ reversion rate is unaffected by *recG.*

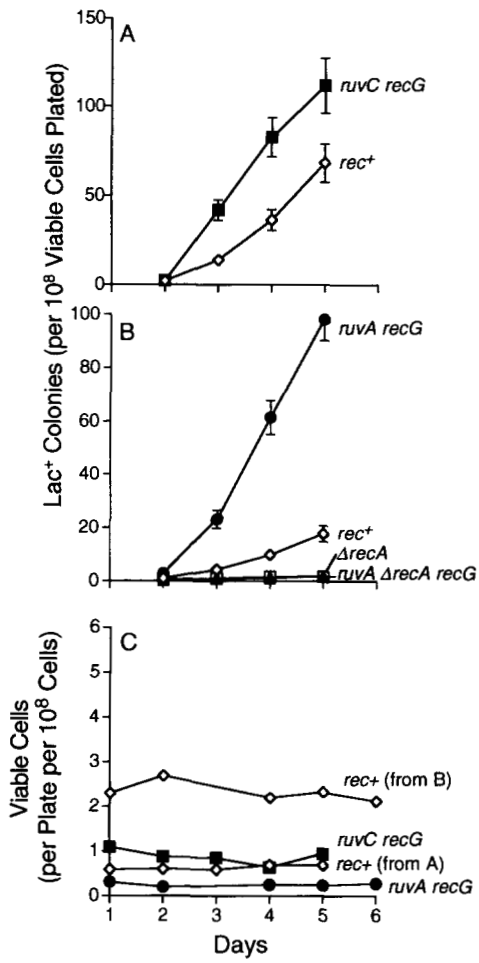

FIGURE 3.—Lac$^+$ Adaptive hypermutation in cells defective for both RuvABC and RecG resolution systems. (A) Hypermutation in a *ruvC recG* strain. (B) Hypermutation in a *ruvA recG* strain. Strains RSH160 and RSH275 (Table 1) carry *ruvA* polar mutations that also create *ruvB*-deficiency (MATERIALS AND METHODS). (C) Viable cell measurements of the *lac*$^-$ rifampicin-resistant frameshift-bearing cells during the experiments displayed in (A) and (B). Error bars represent one standard error of the mean.

here have impaired growth compared with *rec*$^+$ and with *ruv* and *recG* single mutant cells, and all of them readily accumulate growth defect-suppressing mutations and true reversion mutations (LLOYD 1991; MANDAL *et al.* 1991; R. S. HARRIS and S. M. ROSENBERG, unpublished observations). The suppressor-containing strains and revertants are distinguishable from true *ruv recG* strains by their increased colony size and increased UV resistance. Special precautions were taken here to avoid accumulation of such mutants and to verify that every culture used in adaptive mutations experiments was free from such mutations (see MATERIALS AND METHODS). Our procedure uses cultures derived each from a single (small) colony and grown to saturation (MATERIALS AND METHODS). The procedure of FOSTER (1994), which involves growth of a saturated culture, dilution and regrowth to saturation, resulted in cultures with increased UV resistance. These behaved differently in adaptive reversion experiments, showing very low levels of reversion comparable with those seen for *ruv* single mutant strains (data not shown). Although one culture that we tested was a *recG* true revertant (*i.e.*, a *ruv* single mutant), this phenotype of depressed mutation was also seen for cultures carrying growth defect-suppressor mutations, as evidenced by their UV-resistance level, which was higher than *ruv* single mutants. MANDAL *et al.* (1993) characterized suppressor mutants arising in *ruv recG* strains as mutations in *rus* that activate an otherwise cryptic Holliday junction resolution system.

We have found that *ruvA recG*, *ruvB recG*, and *ruvC recG* strains manifest *recA*-dependent adaptive hypermutation. An obvious conclusion is that, again, Lac$^+$ adaptive reversion has different genetic requirements from conjugational recombination. Fragment capture models for the mutagenesis are not supported, and in this case it appears that the idea that intermediates themselves are mutagenic is supported by these data. The data imply that accumulation of strand exchange intermediates in the doubly resolvase-defective cells causes increased RecA-dependent Lac$^+$ reversion.

A somewhat less obvious consideration is that, taken at face value, these data would seem to imply that it is possible to recover viable mutant colonies without ever resolving the strand exchange intermediates that promoted the mutations. This perplexing possibility will be disputed by the data to follow, which, in summary, will indicate that resolution is actually required but that when cells are *ruvA* or *ruvB* or *ruvC* and *recG* defective, the resolution occurs after the intermediate is transferred into the *rec*$^+$ scavenger cells. Although transfer of recombination intermediates was not expected by us, the following lines of evidence lead us to suggest this possibility.

First, we noted that the magnitude of the hypermutation effect caused by *ruv recG* double mutations varies from experiment to experiment. This can be seen, for example, by comparing the different magnitudes of the *ruvC recG* and *ruvA recG* hypermutation effects relative to *rec*$^+$ in Figure 3, A and B, and also varies between experiments with a single strain. For this reason, a quantitative comparison of hypermutation between *recG* and *ruv recG* strains has not been done. We have not observed such variability with any other *rec* or single *ruv* mutations tested here or previously (HARRIS *et al.* 1994; ROSENBERG *et al.* 1995a). We have determined that this variability is caused by small variations in the proportion of the *ruv recG* frameshift-bearing cells relative to the *rec*$^+$ scavenger cells. When varied systematically, we find that decreasing the number of *rec*$^+$ scavenger cells relative to *ruv recG* frameshift-bearing cells greatly increases the amount of adaptive Lac$^+$ reversion caused by *ruv recG*. In Figure 4, experiments conducted in parallel using 1×10^8 frameshift bearing cells mixed with either 8×10^8 or 4×10^9 scavenger cells show high and normal levels of adaptive mutation, respectively. These data are

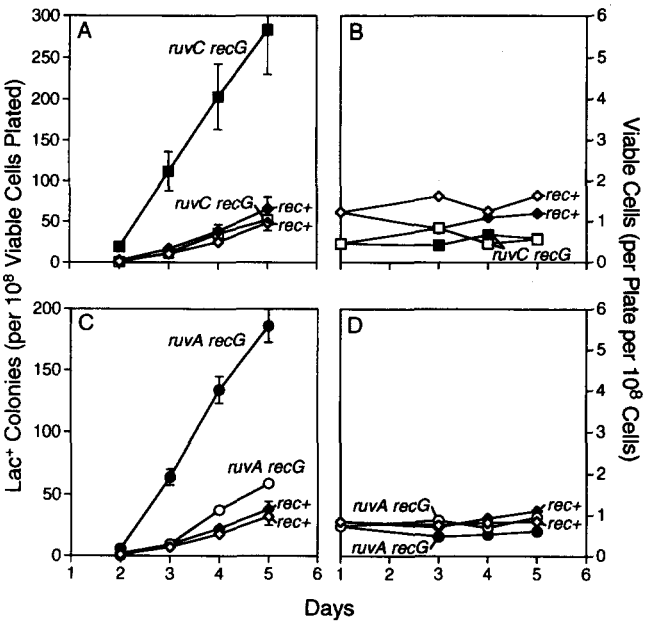

FIGURE 4.—Hypermutation of *ruv recG* double mutants varies inversely with the number of *rec*$^+$ scavenger cells. (A and C) Open symbols represent an experiment with a 40:1 ratio of *rec*$^+$ scavenger cells to *ruv recG* frameshift-bearing cells (4×10^9 scavengers + 1×10^8 frameshift-bearers); closed symbols indicate an 8:1 ratio (8×10^8 scavengers + 1×10^8 frameshift-bearers). (B and D) Viable cell measurements of the *lac*$^-$ rifampicin-resistant frameshift-bearing cells during the experiments displayed in A and C, respectively. These show that decreasing the number of scavengers does not promote hypermutation by allowing growth of the frameshift-bearing cell. Under extremely hypermutagenic conditions (filled symbols), the frameshift-bearing cells do not multiply. Error bars represent one standard error of the mean.

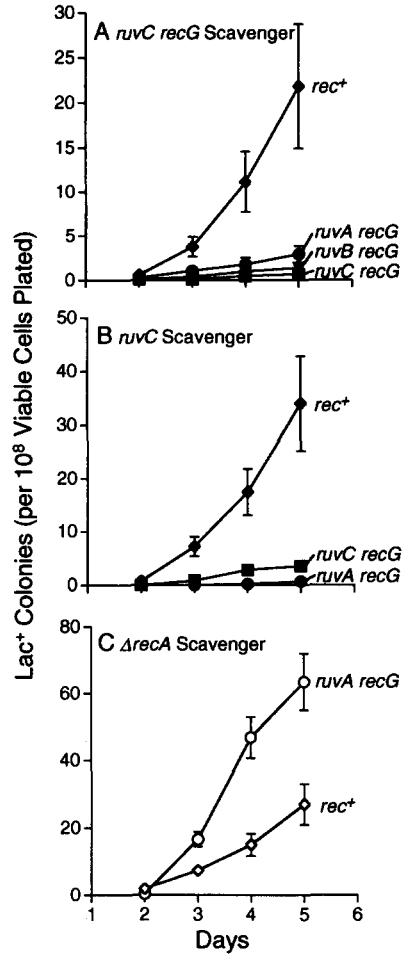

FIGURE 5.—Hypermutation of *ruv recG* double mutants requires functional resolution proteins in the scavenger cells. Discussed in the text. (A) Adaptive hypermutation of *ruvA recG*, *ruvB recG*, and *ruvC recG* cells is abolished by plating with a *ruvC recG* scavenger cell, and, (B) by plating with a *ruvC* scavenger cell. This indicates that functional resolution proteins of the RuvABC system are needed in the scavenger cell for recovery of the Lac$^+$ adaptive mutants from these doubly resolvase-defective strains. (C) Functional *recA* is not required in the scavenger cell for recovery of adaptive mutants from a *ruvA recG* strain. Error bars represent one standard error of the mean.

highly repeatable and the elevated mutation is not caused by growth of the frameshift-bearing cells in the presence of fewer scavengers (Figure 4, B and D, also Figure 3C). These data suggest the following hypothesis: perhaps strand exchange intermediates must eventually be resolved to recover viable cells and perhaps this resolution occurs after transfer of the F′, with its unresolved recombination intermediate, into a *rec*$^+$ scavenger cell. If the persistence of the unresolved strand exchange intermediate is mutagenic, then a delay in finding a *rec*$^+$ scavenger cell with which to mate would increase mutation. Thus, we hypothesize that the fewer scavenger cells plated, the longer the *ruv recG* frameshift cell waits to transfer into a *rec*$^+$ scavenger cell, and the more mutations are promoted, though, ultimately, the intermediates promoting them must be resolved.

The idea that the *ruv recG* hypermutation events must resolve eventually in the *rec*$^+$ scavenger cell is supported by the following observations. First, we find that nearly all of the Lac$^+$ revertants isolated from *ruv recG* experiments contain the rifampicin-sensitivity marker and wild-type *ruv* and *recG* genes that are present on the scavenger cell chromosome but not on the chromosomes of the frameshift-bearing strains (CAIRNS and FOSTER 1991). The numbers of isolated Lac$^+$ revertants carrying the scavenger cell's chromosomal markers were 15/15 (*ruvC recG*), 13/15 (*ruvA59 recG*), 16/16 (*ruvA200 recG*), and 14/16 (*ruvB recG*). This is not the case for *recG* and *rec*$^+$ frameshift-bearing cells, which produced only three out of 14 (*recG*, this work), and nine out 116 (*rec*$^+$) (ROSENBERG *et al.* 1995a) Lac$^+$ adaptive revertants carrying the chromosomal rifampicin-sensitivity marker from the scavenger cell. Therefore, in the *ruv recG* experiments, most surviving Lac$^+$ revertants transferred into the scavenger cell.

Second, when *ruvA recG*, *ruvB recG*, or *ruvC recG* frameshift-bearing cells are plated with scavenger cells that are either *ruvC recG* (Figure 5A) or *ruvC* (Figure

5B), Lac$^+$ adaptive reversion is abolished. This demonstrates a requirement for RuvC-dependent resolution functions in the scavenger cell when the frameshift-bearing cell is unable to resolve recombination intermediates, *i.e.*, is *ruv recG*. The scavenger cell genotype is irrelevant to mutation levels observed in *rec*$^+$, *recA*, *ruvA*, *ruvB*, *ruvC*, or *recG* cells (ROSENBERG *et al.* 1995a; data in Figure 5; and data not shown). We favor the hypothesis that it is resolution that must occur in the scavenger, rather than an entire, normal conjugational recombination reaction, occurring perhaps after a single strand is transferred, for two reasons. First, a *ruvC* single mutation in the scavenger also abolishes Lac$^+$ adaptive reversion with *ruv recG* frameshift-bearing cells (Figure 5B). This is unlike conjugational recombination, in which *recG*$^+$ substitutes for *ruvC*$^+$ (LLOYD 1991), and is like the requirement for *ruv* genes seen in Figure 2C. Second, there is no requirement for RecA protein in the scavenger cell (Figure 5C). Thus, it appears that it is not necessary to initiate strand exchange in the scavenger, but merely to resolve an already-formed intermediate.

An alternative explanation might be that the *ruv recG* scavengers are simply poor recipients of conjugation and thus do not admit the transferred F′. LLOYD (1991) observed a 10-fold decrease the ability to act as a transfer recipient in a *ruvC recG* strain. However, this explanation cannot explain the requirement for *ruvC*$^+$ function in the scavenger cells (Figure 5B). *ruvC* cells are reasonably proficient recipients of transfer, showing only a twofold decrease with respect to *rec*$^+$ (LLOYD 1991), but yet a *ruvC* mutation in the scavenger cell completely blocks adaptive reversion of *ruv recG* strains plated with it. Thus the idea that the resolution functions of the RuvABC system are required in the scavenger is supported.

The results presented above imply that when Holliday junction resolution is completely blocked the accumulation of strand exchange intermediates is hypermutagenic. This supports the idea that strand exchange intermediates prime DNA synthesis during which polymerase errors occur. Second, the data imply that although mutagenic, strand exchange intermediates must be resolved to recover viable mutant colonies, and that this resolution occurs after transfer into the scavenger cell.

FURTHER DISCUSSION

Conclusions from the data reported here can be summarized as follows. First, the presence of RecG protein inhibits Lac$^+$ adaptive reversion. Second, the RuvABC proteins are required for Lac$^+$ adaptive reversion. Involvement of these junction-specific proteins implies that strand exchange intermediates are also intermediates in Lac$^+$ adaptive reversion. Third, accumulation of strand exchange intermediates in doubly resolvase-defective cells is hypermutagenic, but requires eventual exposure to the RuvABC proteins, presumably for eventual resolution. Apparently, that resolution can occur after transfer of the presumed unresolved intermediate into a scavenger cell. It seems as though delaying that transfer by making the scavengers few increases Lac$^+$ adaptive reversion. These results support models in which the recombinational strand exchange intermediate is mutagenic. One such model envisions that a 3′ end that has invaded a homologous duplex directly primes the DNA synthesis in which polymerase errors occur and that these become the mutations (Figure 1A). Other models are possible.

The idea that strand exchange intermediates might be transferred into another cell was very surprising to us in view of conventional assumptions that only single-strand DNA is transferred (see FROST *et al.* 1994). However, L. FROST (Edmonton) made us aware that the idea is not without precedent. WONG and PARANCHYCH (1976) found evidence for transfer of RNA molecules containing secondary structures through pili.

The results of FOSTER *et al.* (1996) were kindly shared with us before publication, during the preparation of this manuscript. They obtain results similar to ours for *ruv* and *recG* singly mutant strains. However, with *ruvA recG*, *ruvB recG* and *ruvC recG* double mutants, they report depressed adaptive reversion which they argue is not the result of growth-defect-suppressor or reversion mutations. We report adaptive hypermutation of such double mutants, which is demonstrated not to result from growth-defect-suppressor or reversion mutations, and which depends on successful transfer of the F′ into a *ruv*$^+$ scavenger cell. It is possible that the absence of hypermutation in their experiments may be caused by experimental conditions that are not favorable for transfer. Ultimately, both labs find that the recombination intermediates must be resolved for recovery of viable Lac$^+$ revertants. Because our conditions allow recovery of transferred molecules, we were able to observe the hypermutation that appears to result when resolution is delayed until transfer into a *ruv*$^+$ scavenger cell.

Opposing roles of the RuvABC and RecG systems: A possible explanation for why RecG protein inhibits Lac$^+$ adaptive reversion, whereas the RuvABC system promotes it, is suggested by the biochemistry of these proteins. These resolution systems each consist of a branch-migration component plus a resolution component. The branch-migration components are an association of RuvA and RuvB proteins (RuvAB) for the RuvABC system, and the RecG protein for the RecG system (see WEST 1994). Branch-migration must precede resolution. The RuvABC system's resolvase is RuvC, and the resolvase for the RecG system has not yet been identified. Biochemically, the branch-migration proteins are helicases (see WEST 1994; WHITBY *et al.* 1994) and, like many DNA helicases, they have preferred strand polari-

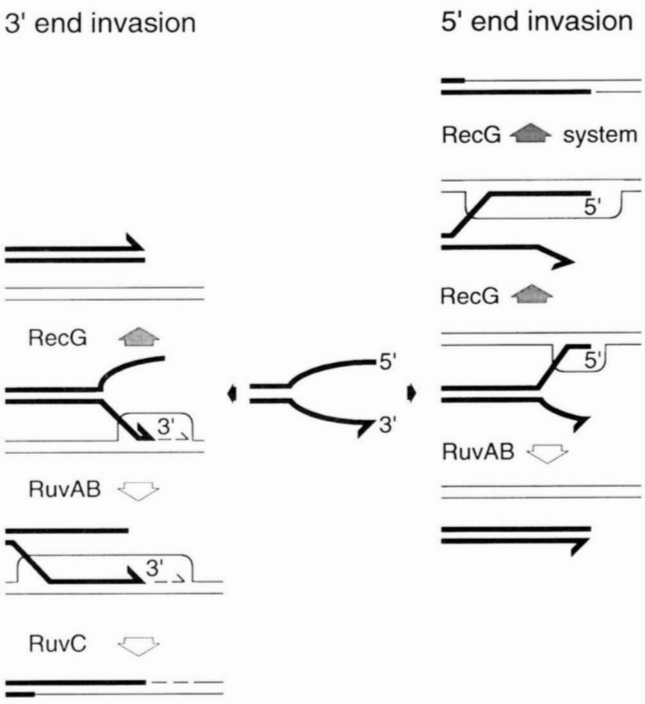

FIGURE 6.—Opposite polarities of the RuvAB and RecG branch migration components can explain opposing roles of the two resolution systems in Lac$^+$ adaptive mutation. Parallel lines represent strands of DNA. Dashed lines indicate newly synthesized DNA. The central molecule is unwound by RecBC protein (not drawn) and the strand invasions to the left and right of it are catalyzed by RecA (not drawn) (ROSENBERG and HASTING 1991). The model suggests that each resolution system treats the two invasion intermediates in opposite ways, such that the RuvABC system resolves 3′ end invasions but undoes 5′ end invasions, whereas the RecG system resolves 5′ end invasions but undoes 3′ end invasions. "RecG system" is indicated at the resolution step in the upper right to denote a Holliday junction resolvase that works with RecG helicase. The resolvase of the RecG system has not yet been identified (see WEST 1994). Both systems function in conjugational recombination (LLOYD 1991), in which both 3′ and 5′ ends are proposed to contribute to products (ROSENBERG and HASTINGS 1991). In adaptive mutation, only the 3′ ends are proposed to be active because only these can prime DNA synthesis. Thus, RecG is inhibitory and RuvABC is necessary for Lac reversion. The specific polarities suggested are opposite those proposed by WHITBY and LLOYD (1995). Because circular single-strand DNAs, not linear molecules, were used in their assay, it seems possible that our model is not inconsistent with their data; circular and linear DNAs have given apparently different polarities in strand exchange assays before (KONFORTI and DAVIS 1987). For clarity, only one of the possible recombination products is shown.

ties. For the RuvAB and RecG branch migration helicases, their polarities are detectable on RecA-coated DNA substrates, and on such substrates the two have opposite strand polarities (WHITBY *et al.* 1993; WHITBY and LLOYD 1995). We will suggest a model that uses the opposite strand polarities of the RuvAB and RecG branch migration components to explain the following facts: first, RecG inhibits, whereas RuvABC facilitates Lac$^+$ adaptive reversion; second, the functions of these systems are redundant and necessary for conjugational recombination.

The model is presented in Figure 6. First, in RecABCD-mediated recombination, there is evidence that both 5′ and 3′ single-strand DNA ends may form RecA-promoted strand exchange intermediates with a homologous duplex (DUTREIX *et al.* 1991; ROSENBERG and HASTINGS 1991; RINKEN *et al.* 1992; MEISEL and ROTH 1996; RAZAVY *et al.* 1996). Both intermediates are presumed to lead to recombination products (top right and bottom left of Figure 6), but only the 3′ end invasions are suggested to lead to adaptive mutation, because only the 3′ ends can prime the DNA synthesis during which polymerase errors occur (Figure 6, lower left). We suggest that a 3′ end invasion intermediate is extended by RuvABC, but is unwound and undone by RecG (Figure 6, left); and that the converse happens to a 5′ end invasion intermediate (Figure 6, right), which is extended and resolved by the RecG system but is undone by RuvAB. That is, resolution of the intermediates of each polarity is proposed to be specific to the resolvase system. This can explain why these systems have opposite effects on Lac$^+$ reversion (proposed to be active with 3′ ends only) and redundant effects on conjugational recombination (occurs well enough with either intermediate). See Figure 6 for further discussion and an alternative view of these enzymes.

Two cases of recombination are known in which the RuvABC system is necessary in the presence of functional RecG: recombination of ColE1-based plasmids (LLOYD 1991) and conjugational recombination between the 85% identical (homeologous) DNAs of *E. coli* and Salmonella (MATIC *et al.* 1995). We suggest that in both cases, only 3′ end invasions will work. For plasmid recombination, this could be because it is RecBCD independent and uses components of another *E. coli* recombination pathway, RecF (LUISI-DELUCA *et al.* 1989). The RecF pathway may use 3′ invasions exclusively because of its use of a 5′ exonuclease, RecJ. In the case of homeologous recombination, perhaps only 3′ ends work because DNA synthesis primed at the joint is a necessity for achieving a long, stable heteroduplex junction with no DNA mispairs in it. The mispairs, we suggest, destabilize the junctions because of the many proteins that interact with such DNA distortions (see also PRIEBE *et al.* 1994).

How much mutation results from blocking Ruv and RecG resolution routes? Previous measurements of the number of transfers occurring between frameshift-bearing cells and scavengers estimate that only 8–10% of Lac$^+$ revertants had transferred into the scavenger (RADICELLA *et al.* 1995; ROSENBERG *et al.* 1995a). It seems reasonable that the same percent of transfers may occur in *ruvC recG* cells. If so, the hypermutation events scored in our experiments with *ruv recG* cells plated with *rec*$^+$ scavenger cells may be only 10% of the hypermutation that occurred. The rest of the muta-

tional events would be lost because they are not transferred and not resolved into viable molecules.

Molecular mechanism of Lac$^+$ adaptive mutation: Our results support models in which strand exchange intermediates somehow promote mutation, and almost certainly, more models are possible than have been considered here. One version of our suggestion that strand exchange intermediates prime the DNA synthesis that leads to the mutation was considered by KUZMINOV (1995; see also FOSTER *et al.* 1996). In this version, branch migration is used to migrate the newly synthesized, error-containing DNA into a region where its complementary strand is also new and thus unmethylated. This would prevent mismatch repair from correcting the error properly because its strand discrimination would be lost. This version is inconsistent with our unpublished and FOSTER *et al.*'s (1995) observation that overexpression of mismatch repair proteins decreases adaptive reversion. This could occur only if the polymerase errors were correctable, *i.e.*, present in hemimethylated DNA.

Why are the conjugational transfer proteins required for Lac$^+$ adaptive reversion, whereas transfer itself is not (FOSTER and TRIMARCHI 1995; GALITSKI and ROTH 1995; RADICELLA *et al.* 1995)? Perhaps their action at the origin of transfer, *oriT*, on the F′ leads to the required DSB (ROSENBERG *et al.* 1995a,b). The transfer proteins make a single-strand nick at *oriT* (see FROST *et al.* 1994) which could lead to a DSB by any of several mechanisms. If the sole function of the transfer proteins is to generate single-strand nicks that lead to DSBs that serve as RecBCD loading sites, then we expect that there will be chromosomal locations that can utilize the Rec-dependent mutation mechanism being uncovered in this system. Although much of the bacterial chromosome is cold for RecBCD-promoted recombination, and so presumably has few DSBs, there are sites that are hot (LOUARN *et al.* 1991; ASAI *et al.* 1993) and these may be mutationally active.

Tests of the hypotheses presented here will be revealing. In this system, and for others in which recombination is implicated in formation of mutations (DEMEREC 1962; MAGNI and VON BORSTEL 1962; PASZEWSKI and SURZYCKI 1964; ESPOSITO and BRUSCHI 1993; STRATHERN *et al.* 1995), further work on the molecular mechanisms will be informative.

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