digoxygenin conjugated to alkaline phosphatase (*3*). The stained embryos were photographed with Nomarski optics.
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Recombination in Adaptive Mutation

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The genetic requirements for adaptive mutation in *Escherichia coli* parallel those for homologous recombination in the RecBCD pathway. Recombination-deficient *recA* and *recB* null mutant strains are deficient in adaptive reversion. A hyper-recombinagenic *recD* strain is hypermutable, and its hypermutation depends on functional *recA* and *recB* genes. Genes of subsidiary recombination systems are not required. These results indicate that the molecular mechanism by which adaptive mutation occurs includes recombination. No such association is seen for spontaneous mutation in growing cells.

Adaptive mutation is a process that appears to produce useful mutations only in the presence of selection for those mutations and in the absence of cell growth (*1–4*). Its molecular mechanism has not yet been elucidated, and because the properties of adaptive mutation challenge established dogma regarding the mechanisms by which mutations occur (*5*), the reality of adaptive mutation as a phenomenon distinct from spontaneous mutation in growing cells has been questioned (*6, 7*). In this report, we address the mechanism of adaptive mutation, which distinguishes adaptive mutation from mutation in growing cells. Unlike spontaneous growth-dependent mutations, adaptive reversion of a *lacZ* frameshift mutation (*2, 3*) in *Escherichia coli* requires genetic recombination genes of the bacterial RecBCD recombination system. These results indicate that recombination is part of the molecular mechanism by which adaptive mutations occur.

The system used to monitor adaptive mutation is that described in (*2, 3*). Samples of *E. coli* carrying a *lacI-lacZ* fusion gene with a frameshift mutation (*lac*$^-$) are plated on minimal lactose medium. Such cells cannot form colonies unless they mutate to *lac*$^+$. The *lac*$^+$ mutant colonies arise continuously during exposure to the selective medium (Fig. 1). No net increase occurs in the number of *lac*$^-$ cells that generate the *lac*$^+$ mutations. The number of *lac*$^-$ cells is assayed by the removal of a fixed volume of agar from the plate each day, suspension of the cells in liquid, and then an assay of the number of *lac*$^-$ viable cells on rich medium (Fig. 1). In all of the experiments reported here, with every recombination-mutant genotype examined, neither net growth nor death of the *lac*$^-$ cells was observed over the course of the experiment. The adaptiveness of the late-arising mutations described here has not been demonstrated by us but relies on previous results (*2*) with the same strain (Fig. 1) (*2*) and is not the subject of this study. This study addresses the mechanism by which the late-arising mutations form. The term adaptive mutation, used in (*2–4, 6, 7*), is used here to identify the phenomenon as that described previously (*2–4*) rather than as an assertion that we have demonstrated adaptiveness.

To test whether recombination is necessary for adaptive mutation (*1, 3, 8, 9*), recombination-defective mutant derivatives of the *lacZ* frameshift–bearing strain were examined for their ability to mutate adaptively. The primary RecBCD recombination system of *E. coli* requires RecA

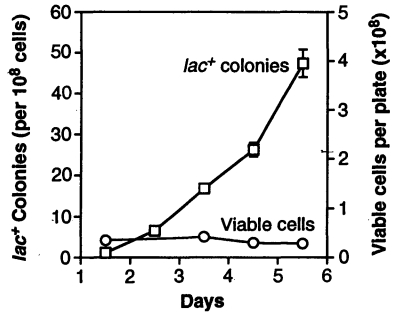

Fig. 1. Adaptive mutation of a *lac* frameshift mutation to *lac*$^+$. The *rec*$^+$ *lacIZ* frameshift-bearing strain (*2*) carries a fusion of the *lacI* and *lacZ* genes that is constitutively transcribed from the *lacI*^{q} promoter and that bears a CCC to CCCC frameshift in *lacI* that is polar on *lacZ*. This strain was plated as described in (*2*) on minimal lactose plates, in the presence of an excess of Δ*lac* scavenger cells (*26*). The scavenger cells do not mutate to *lac*$^+$ and are added to consume any residual nonlactose carbon sources that may be present. The *lac*$^+$ mutant colonies are allowed to accumulate over time. The scavengers are rifampicin-sensitive and the frameshift-bearing strain is rifampicin-resistant. Viable cell counts of the *lac*$^-$ frameshift cells were performed (*2*) by the removal of a plug of agar from between visible *lac*$^+$ colonies on the lactose plates, suspension in M9 broth, and plating on rich rifampicin medium (LB or MacConkey lactose) to determine the number of colony-forming units. No net increase of the *lac*$^-$ cells was observed during the period when increasing numbers of *lac*$^+$ revertant colonies were scored, in agreement with previous reports (*2, 3*). Error bars represent standard errors and, when not visible, are smaller than the symbols they bracket. Means were of counts from two cultures plated on six plates plus two cultures plated on five plates (n = 22).

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protein and RecBCD enzyme (*10*). A partial-function, recombination-impaired *recA* point mutant shows decreased adaptive mutation (Fig. 2A) (*2*). In a null *recA* deletion strain, adaptive mutation is abolished (Fig. 2, A and B) (*4*). Because RecA protein functions in processes other than recombination (*11*), these results do not exclude nonrecombinational models for the mechanism of adaptive mutation.

RecBCD enzyme is a heteromultimer with subunits encoded by the *recB*, *recC*, and *recD* genes (*12*). Null mutations in *recB* or *recC* destroy all function of the enzyme and render cells recombination-deficient. A *recB* null mutant is deficient in adaptive mutation (Fig. 2A). RecD functions as a negative effector of the recombination activity of RecBCD (*10, 13*) such that *recD* null mutants are hyper-recombinagenic (*13–15*). We find that a *recD* null mutant strain is adaptively hypermutable (Fig. 2, A and B).

We tested whether the hypermutation observed in *recD* cells is the result of hyper-recombination. Just as the hyper-recombination in *recD* cells depends on functional *recA* (*14, 15*), the hypermutation in *recD* also requires functional *recA* (Fig. 3). The hyper-recombination in *recD* mutants is the result of a hyper-recombining RecBC(D^-) enzyme and requires functional *recB* (*14, 15*). Hypermutation in *recD* cells also requires functional *recB* (Fig. 3). Because the hypermutation in *recD* requires genes of the RecBCD recombination pathway, like adaptive mutation in rec^+, it is unlikely that the *recD* mutation activates a different route to the formation of adaptive mutations. These data indicate that the same pathway is used to form lac^+ revertants in *recD* cells and in rec^+ cells.

DNA can be recombined by *E. coli* with the use of subsidiary recombination pathways called RecE and RecF, which require different *rec* genes, including *recJ* and *recQ* (*16*). These genes, which are not necessary for RecBCD-mediated recombination, are also not necessary for adaptive reversion to lac^+ (Fig. 4).

The genetic requirements for adaptive mutation described are identical to those for recombination in the RecBCD system. Such requirements are not observed for spontaneous reversion of the same *lacZ* mutation in growing cultures (Table 1) (*2*). These results indicate a mechanistic difference between the two sorts of mutation (*2, 7*) and indicate that recombination is part of the molecular mechanism of adaptive mutation but not part of the mechanism of mutation in growing cells.

Several different recombinational models for the molecular mechanism of adaptive mutation are tenable. All of them can be classified as either templated or nontemplated. In the templated mutation models, the mutant sequence or sequences that restore lac^+ information preexist in the genome. These sequences are transferred into the mutating gene by recombination or gene conversion (*8, 9*). In the nontemplated mutation models, although recombination is required for the formation of the adaptive mutation, the mutant sequence is formed de novo (*1, 3*).

We previously suggested a model in which adaptive mutations are templated from partially homologous (homeologous) sequences elsewhere in the genome (*8*). The normal barriers to homeologous recombination (*17*) were proposed to be relaxed during stress, so that homeologies could recombine.

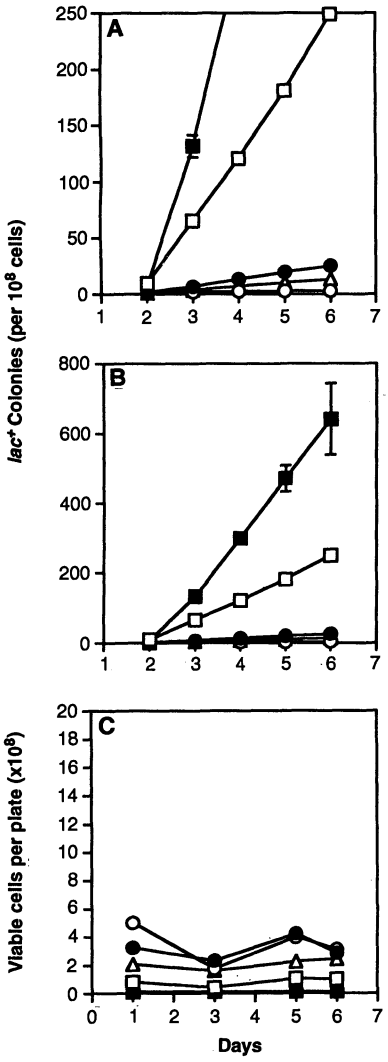

Fig. 2. Ability of *rec* mutants to mutate adaptively. Experiments are as in Fig. 1. (**A**) Reversion of *lac* in different *rec* mutant derivatives of the *lacIZ* frameshift-bearing strain: rec^+ (open squares); *recA430* (filled circles), a point mutation (*2*). All other *rec* mutant strains were constructed for this work with the use of standard P1 transduction methods and carry *rec* null alleles: *Δ(srlR-recA)306::Tn10* (open circles), a *recA* deletion that confers recombination deficiency. The *recB21* allele (open triangles) abolishes all function of RecBCD recombination enzyme and thus is also recombination-deficient. The *recD* null allele used here, *recD6001::mini-Tet::Kan* (filled squares), was constructed for this work (*28*). Null mutations of *recD* confer a hyper-recombinagenic phenotype (*10, 13–15, 24*). (**B**) Data shown in (A) expressed on an expanded ordinate in which *recD* results are visible. For rec^+, n = 24 on all days except 5 and 6, on which n = 12; *recA430*, n = 18; *ΔrecA*, n = 12; *recB*, n = 24; *recD*, n = 29 except on day 6, on which n = 14. (**C**) Viable cell measurements of the various *rec* strains over the course of the experiment illustrated in (A) and (B).

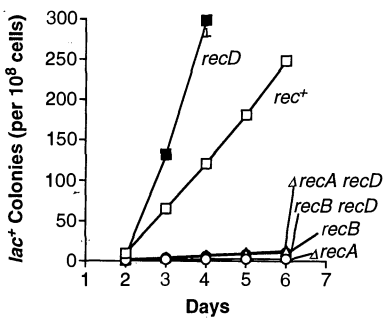

Fig. 3. Functional *recA* and *recB* genes required for adaptive hypermutation in *recD* mutants. Experiments and data presentation are as in Figs. 1 and 2. Data and symbols for single *rec* mutants are the same as those shown in Fig. 2. Filled circles, *Δ(srlR-recA)306::Tn10 recD6001::mini-Tet::Kan* (n = 17). Filled triangles, *recB21 recD6001::mini-Tet::Kan* (n = 12).

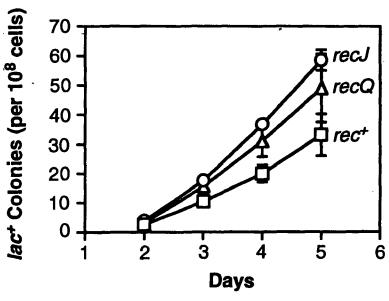

Fig. 4. Genes required for alternative recombination pathways RecF and RecE are not required for adaptive mutation to lac^+. Experiments are as in Figs. 1 to 3. The *recJ284::Tn10* and *recQ61::Tn3* alleles were transduced into the *lacIZ* frameshift strain (*2*) by standard methods. For rec^+, n = 44 except for day 5, on which n = 22; for *recJ* and *recQ*, n = 36 except for day 5, on which n = 18. If it is significant, the slight increase in adaptive mutation in *recJ* and *recQ* strains could be explained as follows: Perhaps only RecBCD-mediated recombination can participate in the formation of adaptive mutations, and perhaps the debilitation of the RecF and RecE recombination pathways shunts more recombination into the RecBCD pathway. This effect would elevate mutation, just as a RecBCD pathway-specific hyper-rec mutation (*recD*) does.

Table 1. Mutation rates in growing cultures of *rec* mutant strains (*29*).

rec Genotype	Experiment	Number of cultures	Number of revertants in median culture	Median number of revertants (per 10^{10} cells)	Rate of mutation to *lac*$^+$ (mutations per cell per generation)
rec$^+$	1	37	4	42.1	5.7×10^{-10}
	2	59	4	41.2	5.7×10^{-10}
Δ*recA*	1	38	1	23.3	3.6×10^{-10}
	2	59	1	7.2	1.5×10^{-10}
recB	1	38	2	117	13×10^{-10}
	2	59	1	43.9	5.9×10^{-10}
recD	1	40	2	25.4	3.9×10^{-10}
	2	59	2	16.9	2.8×10^{-10}

An example of a recombinational mechanism in which mutations are not templated is mutagenic recombination. In yeast mitotic recombination, new mutations are found near recombination junctions (*18*) [foreshadowed by work in *Salmonella* (*19*), yeast (*20, 21*), and filamentous fungi (*22*)]. Formation of the mutations is thought to result from an error-prone repair synthesis associated with recombination. If this faulty synthesis also occurs in *E. coli*, then any mechanism that increases recombination could increase mutation. Adaptive mutations could result from high-frequency recombination, even between identical molecules (sister chromosomes or intrachromosomal duplicated regions). The sequencing of adaptive mutations will help to distinguish models in which mutations are templated from those in which they are not.

In both templated and nontemplated mutation models, the failure to find selection-induced irrelevant mutations (*1*) could be achieved by invoking a hypermutable state that a subpopulation of cells enters and that cells either die from or exit only by generating an adaptive mutation (*4*). In such models irrelevant genes mutate, but because the cells die if they do not become *lac*$^+$, nonadaptive mutations are not observed. Our results suggest the molecular basis of such a state.

The recombination genes necessary for adaptive reversion to *lac*$^+$ are those of the RecBCD system. Although RecBCD is used for recombination in conjugation, recombination of the vegetative bacterial chromosome is mostly RecBCD-independent (*23*). This independence can be understood upon consideration that RecBCD enzyme loads onto DNA at double-strand breaks (DSBs) (*24*), which should be present during conjugation, but are not expected in the vegetative chromosome. We suggest that during stress, DSBs are created in the bacterial chromosome and allow RecBCD to load, thereby elevating recombination dramatically. For example, 10^5-fold more recombination is seen at the terminus of replication, where DSBs are thought to occur (*25*). Double-strand ends could form at paused replication forks by annealing of the new strand-ends with each other instead of with the old strands (*25*). Other means of DSB formation are also possible. Double-strand break formation, allowing high-level recombination, could be the molecular basis of the hypermutable state (*4*) that either kills or is stopped when an adaptive mutation (templated or nontemplated) rescues the cell. Failure to make the adaptive mutation allows continued double-strand breakage, which kills.

Gene duplication or amplification (*3*) could provide a means by which the DSBs postulated here could be repaired. Survivors of selection would be those cells that (i) form a DSB that allows RecBCD loading; (ii) contain a preexisting duplication of the DNA segment in which the DSB forms, which allows recombinational repair of the DSB; and (iii) recombine the *lac* gene with either the identical duplicated *lac* gene or a partially homologous *lac* region elsewhere to form nontemplated or templated mutations, as described above.

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26. Selective plates were overlayed with M9 proline, thiamine, and 0.1% lactose top agar containing 10^{10} scavenger cells (*2*) per plate, incubated for 1 day at 37°C, and then overlayed with *lac* frameshift cells plus 10^9 scavenger cells on day 0 of each experiment. This procedure gave consistent inhibition of growth of the frameshift cells on the minimal lactose plates. Media are as described (*2*) and contained 0.1% lactose.
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28. The Tet cassette of *recD1903::mini-Tet* was disrupted with a kanamycin-resistance cassette by the method of V. Francois, J. Louarn, J. Patte, and J.-M. Louarn [*Gene* **56**, 99 (1987)]. A *recD*$^-$, *recBC*$^+$ isolate was screened, as a kanamycin-resistant, tetracycline-sensitive strain that permits large plaque formation of phage λ*red gam* Chi°, and it is ultraviolet-resistant.
29. Independent cultures of each strain were grown from single colonies to saturation in minimal (M9, proline, thiamine) 0.1% glycerol liquid medium, washed in the same medium without glycerol, and concentrated and plated on minimal lactose (*26*) to measure *lac*$^+$ revertant colonies and on rich (LB) plates to assay viable cells. To obtain counts only for mutants that preexisted in the liquid cultures rather than adaptive mutants that arise after plating, experiments were performed with two separate *lac*$^+$ revertants of each *rec* genotype to determine the earliest time after plating that colony counts could be taken accurately on the selective plates. These times (30 hours for *rec*$^+$ and *recD*, 36 hours for *recA*, and 40 hours for *recB*) were then used for scoring the presence of *lac*$^+$ revertants that arose during the growth of the liquid cultures. Mutation rates are calculated by the method of the median (*27*).

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