Related Target Enhancers for Dorsal and NF-κB Signaling Pathways

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Drosophila dorsoventral (DV) patterning and mammalian hematopoiesis are regulated by related signaling pathways (Toll, interleukin-1) and transcription factors (dorsal, nuclear factor–κB). These factors interact with related enhancers, such as the *rhomboid* NEE and κ light chain enhancer, that contain similar arrangements of activator and repressor binding sites. It is shown that the κ enhancer can generate lateral stripes of gene expression in transgenic *Drosophila* embryos in a pattern similar to that directed by the *rhomboid* NEE. *Drosophila* DV determinants direct these stripes through the corresponding mammalian cis regulatory elements in the κ enhancer, including the κB site and κE boxes. These results suggest that enhancers can couple conserved signaling pathways to divergent gene functions.

There are numerous parallels between the regulation of gene expression along the DV axis of the *Drosophila* embryo and the regulation of lymphoid-restricted expression in the mammalian immune system (1–4). DV patterning is controlled by a signal transduction pathway that is initiated by the localized activation of the Toll receptor in the ventral region of early embryos (5). Toll activation triggers an intracellular signaling cascade in which a kinase, pelle (6), is required for the dissociation of an inactive cytoplasmic complex that includes the *rel*-containing transcription factor, dorsal (dl) (1), bound to an inhibitor, cactus (cact) (7). Once dl is released to the nucleus (8), it interacts with the promoter regions of a number of tissue determinants (3, 9). Mammalian hematopoiesis is regulated by various cytokines, including interleukin-1 (IL-1), which trigger the dissociation of nuclear factor–κB (NF-κB) from its inhibitor, IκB (10). The Toll and IL-1 receptors share a related intracytoplasmic domain (5), whereas NF-κB is related to dl by virtue of the rel homology domain (1). The inhibitory proteins that retain dl and NF-κB in the cytoplasm, cact and IκB, contain a series of related ankyrin repeats (7, 10).

We present evidence that conservation of the Toll–IL-1 pathway extends into the nucleus. The dl target gene *rho* is regulated by an enhancer (the NEE) (3) that is related to the κ immunoglobulin light chain enhancer (4). The *rho* gene is expressed in lateral stripes within the embryonic neuroectoderm in response to dl and other DV determinants (3, 11). The κ enhancer mediates B cell–specific expression in the mammalian immune system in response to NF-κB and various helix-loop-

helix (HLH) proteins, including E12 and E47 (12). We show that the κ enhancer directs stripes of gene expression in the neuroectoderm of transgenic *Drosophila* embryos, suggesting that the evolutionary conservation of the Toll and IL-1 pathways extends from the cell surface to related target enhancers in the nucleus.

Two and four tandem copies of the minimal mouse κ enhancer were inserted

upstream of a fusion gene containing the *hsp70* basal promoter attached to a *lacZ* reporter gene (13). The κ-*lacZ* fusion genes were introduced into the *Drosophila* germ line by P element–mediated transformation. The activity of the enhancer was assayed by in situ hybridization with the use of a *lacZ* antisense RNA probe on whole mount preparations of embryos derived from P-transformed strains (14).

Expression from the transgene is first detected during nuclear cleavage cycle 13 in a homogeneous pattern of expression throughout the embryo (Fig. 1A). This initially broad pattern is probably driven by ubiquitously distributed HLH proteins, including *daughterless* (*da*) (15) and gene products of the *achaete-scute* complex (ASC) (16). During cellularization, lacZ staining becomes augmented in two ventrolateral stripes that extend along the length of the embryo in the presumptive neuroectoderm (Fig. 1B). The gap in expression seen in posterior regions might involve repression by the segmentation gene *knirps* (*kni*) (17). The lateral stripes are similar to those specified by the *rho* NEE (Fig. 1C) (3). Both the mouse κ enhancer and the *Drosophila* NEE are completely inactive in ven-
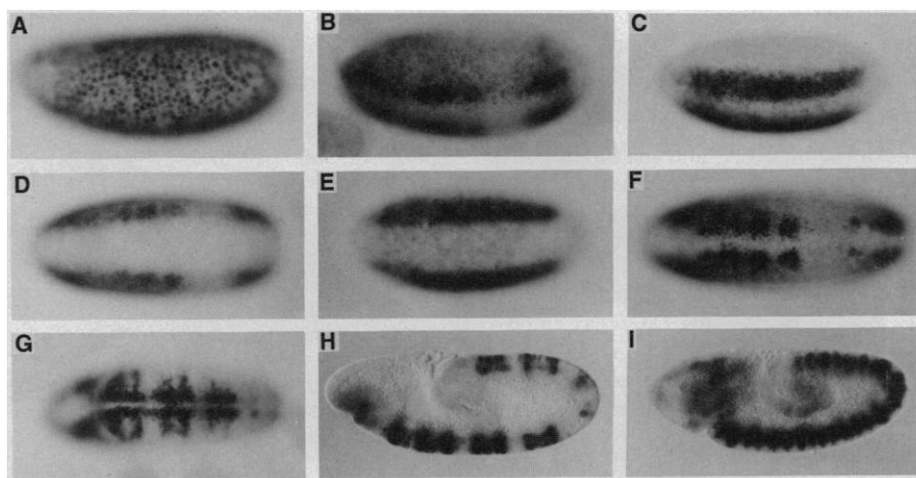

Fig. 1. Expression of the κ enhancer in transgenic *Drosophila* embryos. Embryos were collected from P transformants that contain four tandem copies of the minimal κ enhancer attached to a *lacZ* reporter gene, unless otherwise indicated. Expression patterns were visualized by in situ hybridization with the use of a digoxygenin-U–labeled antisense *lacZ* RNA probe. Embryos are oriented with anterior to the left. (**A**) Precellular embryo at nuclear cleavage cycle 13. Staining is detected throughout the embryo. (**B**) Lateral view of a cellularizing embryo. The strongest staining is observed in lateral stripes within the neuroectoderm, with weaker staining in dorsal regions. Expression is excluded from the presumptive mesoderm in ventral regions. There is a gap in the lateral stripes within the presumptive abdomen. (**C**) Similar to (B) except that the embryo contains a *rho* NEE-*lacZ* fusion gene. Staining is restricted to lateral stripes in the neuroectoderm. (**D**) Ventral view of a cellularized embryo carrying a κ-*lacZ* fusion gene. Staining is excluded from the ventral mesoderm. (**E**) Ventral view of a cellularizing embryo containing a *rho* NEE-*lacZ* fusion gene. (**F**) Ventral view of a gastrulating embryo carrying a κ-*lacZ* fusion gene. The staining pattern is subdivided into patches that encompass the presumptive proneural clusters. (**G**) Ventral view of an embryo undergoing germ band elongation. Staining is restricted to the ventral-most one to three cells of the neuroectoderm and also includes transverse lines that extend into more lateral regions. (**H**) Elongated embryo showing κ-*lacZ* expression in proneural clusters. (**I**) Similar to (H) except that the embryo was stained to show the expression pattern of the endogenous *T3* gene. Staining is restricted to proneural clusters.

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tral regions of the embryo, which correspond to the presumptive mesoderm (Fig. 1, D and E).

After invagination of the mesoderm, expression of the κ-lacZ fusion gene is restricted to ventral regions of the neuroectoderm (Fig. 1F). The pattern becomes modulated along the anteroposterior axis such that expression is restricted to patches of five to six cells; these patches are separated by two to three nonexpressing cells (Fig. 1G). Similar segmental patches are also observed for rho and the neuronal regulatory gene, T3 (16), and probably depend on regulation by pair-rule genes. After the rapid phase of germ band elongation, the κ-lacZ fusion gene is expressed in proneural clusters (Fig. 1H), similar to the T3 pattern (Fig. 1I).

Deoxyribonuclease I footprint assays (18) indicate that increasing concentrations of a GST-dl fusion protein result in complete protection of the authentic κB site and of a second, weaker site containing a partial match to the consensus κB motif (Fig. 2A). In addition, a GST-sna fusion protein protects the κE2 site and a neighboring sequence (Fig. 2B). This observation is consistent with the finding that the κE2 site can mediate repression in fibroblast cell lines (19). Moreover, a number of HLH proteins, such as da and T4, are ubiquitously distributed throughout the early embryo, and several of these have been shown to bind with high affinity to synthetic oligonucleotides containing the κE2 motif (20). The locations of the dl, HLH, and sna binding sites in the κ enhancer are summarized (Fig. 2C). This arrangement of binding sites is quite similar to the organization of the rho NEE (Fig. 2C) (3).

To determine whether dl and HLH proteins present in the early Drosophila embryo are directly responsible for the regulation of the κ enhancer in the neuroectoderm, we created point mutations in the κB and E box binding sites in the context of otherwise normal κ-lacZ fusion genes (21). Disruption of the κB site completely abolished lateral neuroectoderm stripes (Fig. 3B), although the initially broad pattern observed in precellular embryos appeared normal (Fig. 3A). Essentially identical results were obtained when both the κB site and the second, more proximal dl site were disrupted (22).

Mutations in the κE2 site also abolish neuroectoderm-specific expression. During precellular stages, there are somewhat stronger levels of expression in ventral as opposed to dorsal regions (Fig. 3, C and D). This weak ventral staining persists during cellularization, but disappears by the onset of gastrulation (22). It seems likely that the transient ventral pattern is mediated by dl because previous studies have shown that dl binding sites are sufficient to drive weak

ventral expression from minimal promoters (11). Similar results were obtained when the κE1 and κE3 sites were mutagenized (22).

To determine whether sna might repress the κ enhancer in the ventral mesoderm, we crossed the κ-lacZ fusion gene into sna mutants (sna^IIG05 homozygotes) (23). In these mutants there is a severe ventral derepression of the pattern, so that lacZ staining is found in both the ventral neuroectoderm and in the entire presumptive mesoderm (Fig. 4, A and B). This pattern is similar to that observed for rho and T3 in sna mutants (24). Despite the altered DV limits, the pattern is normally modulated along the anteroposterior axis. For example, the gap in the expression pattern in the presumptive abdomen is clearly evident (Fig. 4B), and the pattern is refined into a series of bands that encompass the proneural clusters (Fig. 4C).

The posterior gap in the κ-lacZ stripes

(Fig. 1B) coincides with the site of kni expression (17); and in kni mutant embryos (kni^IID48 homozygotes) (23) the gaps disappear so that the lateral stripes are continuous along the anteroposterior axis (Fig. 4D). The continuity of the pattern is maintained during gastrulation (Fig. 4E) and germ band elongation (Fig. 4F). These results suggest that sna and kni might function as repressors of the κ enhancer.

We have shown that the κ enhancer functions in a manner analogous to the rho NEE to integrate the dl and HLH activators, and the sna repressor, to generate neuroectoderm stripes in the Drosophila embryo. The arrangement of binding sites in the κ enhancer permits local factor-factor interactions required for activating gene expression in the lateral neuroectoderm, including cooperative DNA binding interactions between neighboring dl and HLH proteins (11, 25). The rel-HLH interactions are also important for B cell differen-

Fig. 2. The dl and sna proteins bind the mouse κ enhancer. A 400-bp Alu I–Alu I genomic DNA fragment spanning the minimal κ enhancer (4) was labeled with ^{32}P, incubated with increasing amounts of either GST-dl (A) or GST-sna (B) fusion protein, digested with deoxyribonuclease I, and fractionated on a polyacrylamide-urea gel. The G+A lanes show the sequence of the labeled DNA fragment. Lanes labeled 0 show the deoxyribonuclease I digestion pattern without added protein. (**A**) Increasing amounts of the GST-dl protein give complete protection of the κB sequence (upper nucleotide sequence to the right of the autoradiogram; κB site underlined). There is an additional region of protection that contains a weak match to the NF-κB consensus sequence (lower sequence to the right). (**B**) Increasing amounts of the GST-sna protein protect two tandem sequences in the κ enhancer (see sequence to the right of the autoradiogram). The upper region contains the κE2 sequence (underlined). Both regions of protection contain a match to the sna consensus sequence (3). (**C**) Summary of dl and sna binding sites in the minimal κ enhancer. The black rectangles correspond to the E box sequences; the κE2 site has been previously shown to bind da-T3 and da-T4 heterodimers (20). Below is a summary of the factor binding sites in the 300-bp rho NEE (3).
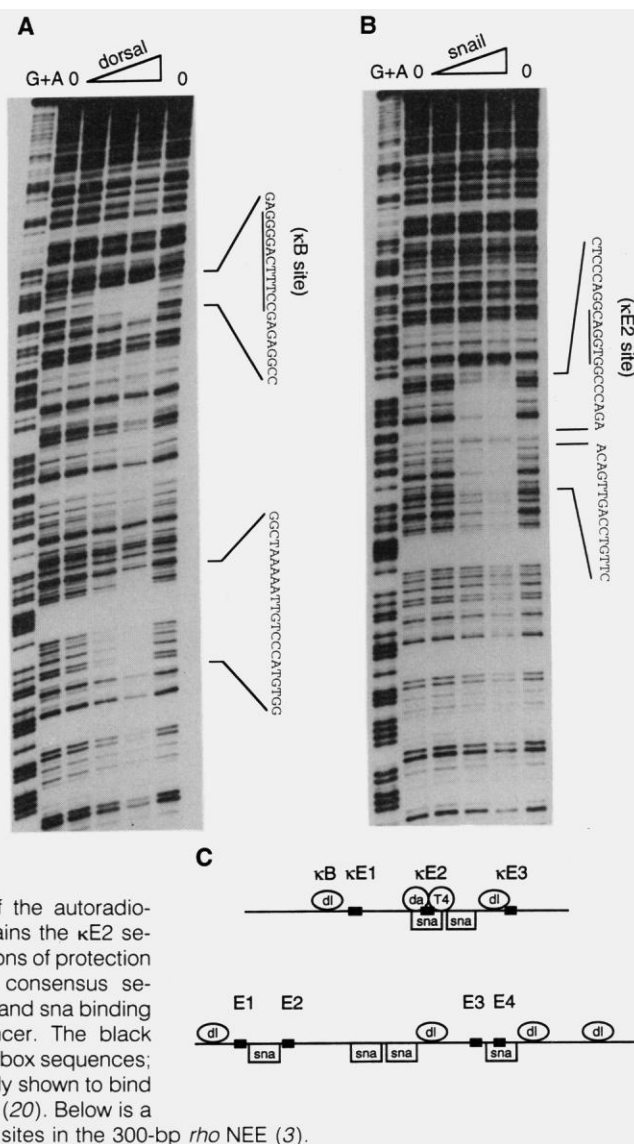
tiation (12). The κ enhancer, like the rho NEE (3) and the even-skipped stripe 2 enhancer (26), functions as an integrating pattern element that generates a relatively sharp pattern of expression in response to gradients of transcriptional activators and repressors in the early embryo.

It is conceivable that mammalian homologs of the Drosophila sna and kni repressors participate in B cell–specific expression of the κ enhancer. The expression pattern of a mouse sna gene suggests that it might mediate multiple functions in development after implantation, including B cell differentiation (27). Moreover, kni is a member of the steroid receptor superfamily, and a

recent study has shown that the chicken thyroid hormone receptor (cT3R-alpha1) can bind the κB site in the human immunodeficiency virus long terminal repeat and block activation by NF-κB (28). It has been shown that evolutionarily conserved enhancers regulate alcohol dehydrogenase in the Drosophila fat body and mammalian liver (29). This study provides evidence that conserved enhancers can also regulate distinct target genes.

Recent studies suggest that fly DV patterning and mammalian hematopoiesis share a common evolutionary origin. A gene related to dl, called dif (dl-related immunity factor), is not involved in embryonic pat-

terning, but instead mediates an immune response in Drosophila larvae (30). It is conceivable that the first evolutionary use of a Toll–IL-1 pathway involved a primitive immunity. Perhaps this pathway was independently coopted by the Drosophila embryo and the mammalian immune system. Thus, the Toll–IL-1 signaling pathway is not dedicated to specific batteries of target genes, but instead controls completely divergent genes involved in distinct processes. We propose that enhancers, like exons, are fundamental units of evolutionary change that can couple conserved pathways to divergent gene functions. According to this view, both genes and their promoters are modular and contain discrete, separable elements permitting, at least in principle, rapid evolutionary changes in genetic networks.

Fig. 3. The dl binding sites and E boxes are required for neuroectoderm stripes. Mutagenized κ-lacZ fusion genes were examined in P-transformed embryos. Expression patterns were visualized by in situ hybridization. (**A** and **B**) Mutagenized κ enhancer without the κB site. When this site is disrupted, there is a loss of staining in lateral stripes within the presumptive neuroectoderm (B). However, during precellular stages the initial pattern appears normal, with ubiquitous staining throughout the embryo [(A); compare with Fig. 1A)]. The enhancer is completely inactive after cellularization (22). (**C** and **D**) Mutations in the κE2 site and neighboring sna site. There is weak, transient staining in ventral regions of precellular embryos. Similar results were observed when the κE1 and κE3 sites were disrupted.
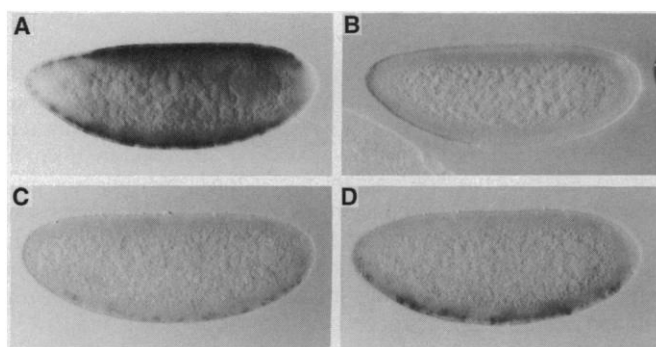

Fig. 4. The κ enhancer is repressed by sna and kni. The wild-type κ-lacZ fusion gene was crossed into sna⁻ (A to C) or kni⁻ (D to F) embryos. (**A**) Lateral view of a cellularized embryo. Intense staining is observed along the ventral surface, except for a gap in the pattern in abdominal regions. (**B**) Ventrolateral view of a cellularized embryo. Staining encompasses the ventral mesoderm and ventrolateral neuroectoderm and spans a total of about 26 to 28 cells in the ventral half of the embryo. Note the abdominal gap and the rather sharp dorsal limit of the staining pattern. (**C**) Ventral view of a gastrulation-stage embryo. In sna⁻ mutants there is no ventral furrow, and ventral cells fail to invaginate. Expression of the κ-lacZ fusion gene includes the lateral neuroectoderm and ventral regions that normally form mesoderm. There is normal anteroposterior modulation of the pattern, which foreshadows the formation of proneural clusters. The posterior region in which staining is reduced corresponds to the original gaps seen during earlier stages. (**D**) A cellularizing kni⁻ embryo. The lateral stripes extend uniformly through posterior regions that normally contain reduced expression. (**E**) Ventral view of a gastrulating embryo. There is no gap in the pattern in the presumptive abdomen. (**F**) Lateral view of a mutant embryo undergoing germ-band elongation. Again, there is no gap in the presumptive neuroectoderm.
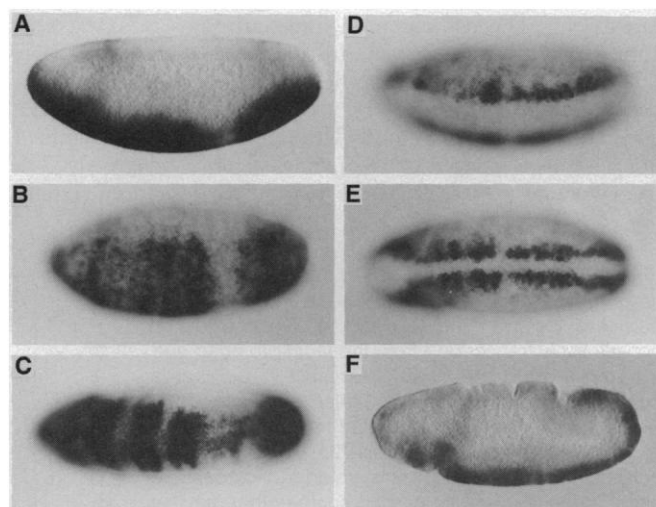
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13. The mouse κ light chain immunoglobulin enhancer was cloned by polymerase chain reaction with the use of appropriate oligonucleotides and mouse genomic DNA. The identity of the resulting genomic DNA fragment was confirmed by DNA sequencing. Two and four copies of a 200–base pair (bp) Dde I–Dde I genomic DNA fragment (4) were cloned into the P-element vector pWHL (3).
14. Plasmids containing κ-lacZ fusion genes were injected into w⁶⁷ embryos, along with the Δ2,3 transposase helper plasmid (3). At least three independent germline transformants were analyzed for each construct. Expression patterns were visualized in whole mount preparations of embryos by in situ hybridization with the use of lacZ antisense RNA probes labeled with digoxygenin–uridine 5′-triphosphate and antibody to

digoxygenin conjugated to alkaline phosphatase (3). The stained embryos were photographed with Nomarski optics.

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18. GST-dl and GST-sna plasmids were expressed in the Escherichia coli strain HB101 as described (3). The GST-dl fusion protein contains the NH$_2$-terminal 378–amino acid residues of dl, which spans the entire rel domain (1). The GST-sna fusion protein contains the 213 COOH-terminal amino acid residues of sna, which includes the five zinc fingers comprising the DNA binding domain (3). Deoxyribonuclease I protection as-

says were performed as described in (3).
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31. We thank H. Cai, K. Tatei, D. Arnosti, and the other members of the lab for help and encouragement. We also thank C. Murre, J. Posakony, and E. Bier for helpful discussions and encouragement. Funded by a grant from NIH (GM 46638). S.G.-C. is a recipient of a postdoctoral fellowship from the Spanish Ministerio de Educación y Ciencia.

16 December 1993; accepted 18 February 1994

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.
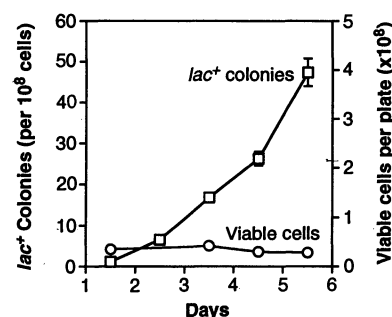
The system used to monitor adaptive mutation is that described in (2, 3). Sam-

ples 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

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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 Δlac 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).