Induction of a DNA Nickase in the Presence of Its Target Site Stimulates Adaptive Mutation in *Escherichia coli*

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Adaptive mutation to Lac\(^+\) in *Escherichia coli* strain FC40 depends on recombination functions and is enhanced by the expression of conjugal functions. To test the hypothesis that the conjugal function that is important for adaptive mutation is the production of a single-strand nick at the conjugal origin, we supplied an exogenous nicking enzyme, the gene II protein (gIIp) of bacteriophage f1, and placed its target sequence near the lac allele. When both gIIp and its target site were present, adaptive mutation was stimulated three- to fourfold. Like normal adaptive mutations, gIIp-induced mutations were recA\(^+\) and ruvC\(^+\) dependent and were mainly single-base deletions in runs of iterated bases. In addition, gIIp with its target site could substitute for conjugal functions in adaptive mutation. These results support the hypothesis that nicking at the conjugal origin initiates the recombination that produces adaptive mutations in this strain of *E. coli*, and they suggest that nicking may be the only conjugal function required for adaptive mutation.

When populations of microorganisms are exposed to nonlethal selection, mutations that relieve the selective pressure can appear, a phenomenon called adaptive mutation (4, 5, 15). The mutational process is not directed to specific targets, because nonselected mutations also occur (14). But the process does require the presence of the selective agent; e.g., simply starving nonselected mutations also occur (14). But the process does require the presence of the selective agent; e.g., simply starving cells for a carbon source does not result in mutations (4, 5).

Adaptive mutation has been extensively studied in *Escherichia coli* strain FC40. This strain cannot utilize lactose (i.e., it is Lac\(^-)\) because of a +1-bp frameshift mutation that affects the lacZ and lacY genes (6, 40). Although the Lac\(^-)\) allele, \(\Phi(lacI33-lacZ)\), is slightly leaky, the amount of \(\beta\)-galactosidase produced is not sufficient to allow the cells to grow on medium containing lactose as the only carbon source (4, 13). For ease of genetic manipulation, the lac and proAB operons are carried on an episome, F\(_{\text{proAB}}\), and the corresponding region is deleted from the chromosome.

During nonselective growth, Lac\(^+)\) revertants of FC40 appear at a rate of about \(10^{-7}\) per cell per generation; when incubated on lactose minimal medium, Lac\(^-)\) revertants arise for about a week at a constant rate of about \(10^{-7}\) per cell per day (4, 14). The Lac\(^+)\) mutations arising during lacose selection are distinguished from the Lac\(^-)\) mutations that arise during nonselected growth in several ways: (i) whereas mutations arising during nonselected growth consist of a variety of deletions and duplications that can revert the \(\Phi(lacI33-lacZ)\) allele, adaptive Lac\(^+)\) mutations are almost exclusively –1-bp frameshifts in runs of iterated bases (20, 50); (ii) unlike mutations occurring during nonselective growth, adaptive mutations require recombination functions, specifically *E. coli*’s RecA-RecBCD pathway for double-strand break (DSB) repair (4, 32); (iii) to achieve the high level of recombination-dependent mutation in FC40, the Lac\(^-)\) allele must be on the episome and one or more conjugal functions must be expressed (21, 27, 47). Although we have argued that actual conjugation is not required (21, 22), others maintain that conjugal transfer of the DNA is essential to the mutagenic process (27, 30, 47).

During bacterial conjugation, transfer of the episomal DNA is initiated by a site-specific nick in the DNA at the conjugal origin, oriT. However, even in stationary-phase cells that are not conjugating, the nick persists (25). Several years ago it was proposed that this nicking was the initiating event for adaptive mutation in FC40 (21). This hypothesis was inspired by the fact that recombination between homologous alleles residing on the F\(^+\) and on the chromosome is 20- to 50-fold higher than recombination between the same alleles when both are on the chromosome (43). This enhancement requires the presence of oriT in cis to one of the alleles, the expression of the conjugal nicking functions, and the RecA-RecBCD pathway for recombination (7, 8). These requirements are the same as those for adaptive mutation in FC40.

To test whether a DNA nick stimulates adaptive mutation, we utilized the nicking function of the filamentous bacteriophage, f1. Rolling circle replication of filamentous coliphage genomes is initiated by the gene II protein (gIIp), which recognizes a 140-bp sequence at the phage origin of replication and nicks the plus-strand DNA at a specific site within this sequence. gIIp also unwinds and displaces the plus strand and then cleaves and religates this strand for packaging (58). A 37-bp sequence containing the nick site is sufficient to allow nicking but not to allow the other activities of gIIp (11, 53). When gIIp is expressed in yeast carrying this minimum target sequence, recombination and gene conversion are stimulated in both directions from the nick site (52). Recent evidence suggests that the nick must be converted into a DSB by replication in order to stimulate recombination (29).

We placed the minimum gIIp nick sequence close to the reversion site of the \(\Phi(lacI33-lacZ)\) allele. We then supplied,
from a plasmid, gene II under control of an exogenous promoter. With this system we were able to demonstrate that adaptive mutation is stimulated when both gIIp and its nick site are present in the cell and, further, that gIIp activity can substitute for conjugal functions in adaptive mutation. These results suggest that nicking may be the only conjugal function that is required for adaptive mutation.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** Bacterial strains and plasmids are listed in Table 1. All these strains were originally derived from P90C (CSH142 [41]; P90C and the two F strains used were obtained from J. H. Miller). FC40, the revertible strain, and FC29, the nonrevertible scavenger strain, have been described elsewhere (4). All genetic constructions used standard protocols (41). The traD411::Km allele was introduced onto episomes as described previously (21, 36) (the allele was obtained from K. Ippen-Ihler [deceased]). The recA938::lacI33-lacZ (gpt-lac):Km allele (obtained from R. Lloyd) were introduced by transduction with bacteriophage P1; transductants were selected for drug resistance and then screened for sensitivity to UV light. In some cases the alleles were transduced into the F− strain and in other cases they were transduced into its F− parent FC36 (4), and then the appropriate episome was introduced by conjugation. Even when the product strains were the same, these different constructions have different numbers as shown in Table 1.

**Strains carrying the gene II plasmid, pCRS4**

<table>
<thead>
<tr>
<th>Name</th>
<th>Derivation</th>
<th>Relevant properties</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>CRS10</td>
<td>CRS18/pCRS4</td>
<td>FC40 + nick site/pPBAD-gene II</td>
<td>This study</td>
</tr>
<tr>
<td>CRS117</td>
<td>CRS174/pCRS4</td>
<td>traD reversible Lac− strain + nick site/pPBAD−gene II</td>
<td>This study</td>
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<tr>
<td>CRS179, CRS248</td>
<td>CRS183/pCRS4</td>
<td>FC40/pPBAD−gene II</td>
<td>This study</td>
</tr>
<tr>
<td>CRS184</td>
<td>CRS186/pCRS4</td>
<td>recA FC40 + nick site/pPBAD−gene II</td>
<td>This study</td>
</tr>
<tr>
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<td>CRS189/pCRS4</td>
<td>recD recA reversible Lac− strain + nick site/pPBAD−gene II</td>
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</tr>
<tr>
<td>CRS190</td>
<td>CRS215/pCRS4</td>
<td>traD411::Km allele (obtained from R. Lloyd)</td>
<td>This study</td>
</tr>
<tr>
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<td>SCavenger/pPBAD−gene II</td>
<td>This study</td>
</tr>
<tr>
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<td>This study</td>
</tr>
<tr>
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<td>traD scavenger/pPBAD−gene II</td>
<td>This study</td>
</tr>
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<td>traD FC40/pPBAD−gene II</td>
<td>This study</td>
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**Plasmids**

<table>
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<td>Control vector</td>
<td>This study</td>
</tr>
<tr>
<td>pCRS4</td>
<td>pCRS3 carrying gene II; Cbr Te′</td>
<td>Gene II under control of PBAD</td>
<td>This study</td>
</tr>
</tbody>
</table>
this plasmid was called pCRS2. and P. L. Foster, unpublished data). pFC530 was digested with \( \text{Eco} \)I and \( \text{Bam} \)HI, obtaining \( \text{Sce} \)I fragment of plasmid pCRS1, and \( \text{HI} / \text{H9021} \)Posfai). The new plasmid was called pCRS8. The protocol for large-scale adaptive mutation experiments was as described elsewhere (4, 13). Cells were grown to saturation in M9-glycerol medium plus antibiotics, then diluted 105-fold into fresh medium, divided into five independent cultures, and allowed to again reach saturation. Scavenger strains were FC29 or FC364 carrying the same plasmids as the revertible strains; these cells were also grown in M9-glycerol medium plus antibiotics. At saturation, culture titers were determined on LB medium and on LB medium plus antibiotics; the experiment was continued only if the two titers were comparable, meaning that nearly all the cells maintained their plasmids. An appropriate number of cells from each of the independent cultures was mixed with 104 cells of the scavenger and spread onto three M9-lactose plates, which were incubated at 37°C. Lac+ colonies on one set of plates were counted daily starting 2 days after the bacteria were plated. The other sets were used to determine the number of Lac− cells, as previously described (19). Plugs were taken daily from the plates, and the cell numbers were determined on LB plus rifampin plates. These plates were then replicated onto MacConkey-lactose plates to determine if any of the colonies were Lac−; if Lac− colonies were present, their numbers were subtracted from the total.

For β-galactosidase assays, cells were grown to saturation in M9-glycerol medium plus appropriate drugs. Assays were performed as described previously (19, 41).

**DNA manipulations.** All DNA manipulations were carried out using standard protocols (2). Plasmid DNA was extracted with Wizard kits (Promega Corp.). Restriction enzymes, bacterial alkaline phosphatase, and T4 polynucleotide ligase were used according to the manufacturer’s instructions (New England Biolabs).

**Construction of a plasmid with a regulated gene II.** Plasmid pTTL, carrying gene II from bacteriophage \( \text{f1} \) (26), was digested with \( \text{Bam} \)HI and \( \text{EcoR} \)I, yielding a fragment containing gene II from its ribosome binding site to 3 bp downstream of its terminator. To facilitate further subcloning, the polylinker-containing vector, pCR2.1 (Invitrogen, Co.), was digested with \( \text{EcoR} \)I, removing a 15-fragment, and religated. This plasmid was named pCRS1. The \( \text{Bam} \)HI EcoRI fragment containing gene II was cloned in pCR2.1 EcoRI-cut pCRS1, and this plasmid was called pCRS2.

We wished to put gene II under the control of the \( \text{araC} \)-\( \text{P}_{\text{ARAD}} \) regulatory system, but the \( \text{pBAD} \) series of vectors designed for this purpose contains the M13 intergenic region that includes the gIIp target site (31). To eliminate this problem, the araC\( \text{P}_{\text{ARAD}} \) control region was cut out of \( \text{pBAD}18-	ext{Cm} \) (31) with Clal and PstI and ligated into the same restriction sites of plasmid pBR322 (3), generating a plasmid pCRS3. pCRS3 was digested with \( \text{Kpn} \)I and MboI, obtaining gene II with its ribosomal binding site, and ligated in the same restriction sites of plasmid pCRS3, maintaining the proper orientation to the araC\( \text{P}_{\text{ARAD}} \) regulatory system. This plasmid was called pCRS4. Plasmid pCRS4 and the empty vector, pCR3, were transformed into the derivatives of \( \text{E. coli} \) FC40 generated for the purpose of this study (Table 1).

We confirmed gIIp activity by showing that pCRS4 increased the ability of \( \beta \) bacteriophage R12, which has a nonsense mutation in gene II, to form plaques on a nonsuppressing host, \( \text{E. coli} \) strain K38 (26) (bacteriophage and bacteria were obtained from P. Model). The number of plaques was additionally increased when arabinose was added, confirming that gene II was under control of \( \text{P}_{\text{ARAD}} \) (data not shown).

**Construction of a plasmid with the gIIp target site in \( \Philac333-lacZ \).** The target for gIIp was a 45-base sequence (Table 2, oligonucleotides 3 and 4) from the origin of bacteriophage \( \text{f1} \) that contains the minimum recognition site necessary for nicking but not the sites required for plus-strand synthesis or ligation by gIIp (11, 52). These oligonucleotides also have \( \text{Sac} \)I sites on their ends. Oligonucleotides 3 and 4 were used for PCR, and the product was ligated into the TA cloning vector pCR2.1 (Invitrogen Co.). The analog was cloned by PCR with oligonucleotide pair 1 and 4 and pair 3 and 2 (Table 2) and then sequenced using oligonucleotides 1 and 2 (Table 2) as primers. This plasmid was named pCRS5. Plasmid pHCS50 carrying \( \Philac333-lacZ \) was created by replacing the \( \text{Bam} \)HI-\( \text{Sac} \)I fragment of plasmid pMC1403 (51) with a 3-kb \( \text{Bam} \)HI-SacI fragment of \( \Philac333-lacZ \) generated by long, accurate PCR from strain FC40 (P.-E. Yeh and P. L. Foster, unpublished data). pHCS50 was digested with \( \text{Bam} \)HI and Clal, yielding a 1,962-bp fragment carrying \( \Philac333-lacZ \) and the now-unique restriction site \( \text{Bcl} \)I (compatible with \( \text{Sac} \)I). This fragment was cloned into \( \text{Bam} \)HI-\( \text{Clal} \)-cut plasmid pBR322 (3), creating plasmid pCRS6. Because \( \text{Bcl} \)I is methylated by \( \text{Sac} \)I, pCRS6 was transformed into the \( \text{dam} \) strain GM2163 (obtained from M. Marinus).

To generate a nonpolar insertion of the gIIp target site in \( \Philac333-lacZ \), the 45-bp \( \text{Sac} \)I fragment from plasmid pCRS5 was ligated into the \( \text{Bcl} \)I site of plasmid pCRS6. This insertion site corresponds to nucleotide 443 of the Ecolac sequence in GenBank (J01636.1), 593 bases to the 5′ side of the \( \text{lac} \) mutation, a +1 frameshift at nucleotide 1036 (6, 20). To identify the correct orientation of the insert, clones were analyzed by PCR using the primer pairs 5 and 4 and 5 and 3 (Table 2). A plasmid with the correct orientation was named pCRS7. Finally, to generate a suicide plasmid with the construction, the \( \text{Bam} \)HI-\( \text{Clal} \) fragment of plasmid pCRS7 was cloned into the same restriction sites of plasmid pSG76-K, which requires the \( \text{pin} \) gene for replication and carries a recognition site for the endonuclease I-SceI (45) (pSG76-K and the host strain were obtained from G. Posfai). The new plasmid was called pCRS8.

**Construction of \( E. coli \) strains carrying lacI333-lacZ with the gIIp nick site.** \( \Philac333-lacZ \) with the gIIp nick site was introduced into the episome of \( E. coli \) FC40 by in vivo gene replacement stimulated by a DNA DSB (44). First, the \( \text{pin} \)-dependent suicide plasmid, pCRS8, was transformed into FC40, and recombination between the plasmid and the episome was selected on LB-Km. Then, plasmid pST98-\( \text{ASceP} \) was introduced by transformation. pST98-\( \text{ASceP} \) is a temperature-sensitive suicide plasmid (it cannot replicate at 42°C) that constitutively expresses the I-SceI nuclease (plasmid obtained from G. Posfai). I-SceI will cleave a DNA DSB at its recognition site, which was recombined onto the episome in the previous step. The DSB stimulates intramolecular recombination between the introduced and the resident alleles. Recombinants that had eliminated the plasmid sequences were identified by loss of kanamycin resistance at the nonpermissive temperature. To verify that the desired gene replacement had taken place, clones were analyzed by PCR using oligonucleotides 5 and 4 (Table 2). This primer pair yields a PCR product only if the nick site is in the correct orientation. The strain carrying the gIIp nick site was called CRS18. The sequence from CRS18 was mated into an F′ Met− Nal+ strain and then back into C60, to create strain CRS241.

**PCR amplification and nucleotide sequencing.** PCR amplifications were carried out using the Platinum PCR Supermix (Gibco-BRL Corp.) for 30 cycles consisting of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, except for amplification with primer pair 5 and 4, which was at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min. For sequencing reactions an ABI Prism Big Dye terminator (Applied Biosystems Inc.) was used for 25 cycles consisting of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min. Products were purified with DyeEx Spin columns (Qiagen, Inc.) and sequenced using ABI 377 or 3700 DNA sequencers.

**Statistical analyses.** In most cases, standard statistical methods were used. Because the results presented below in Fig. 4 and 5 are ratios of numbers, both of which have associated errors, somewhat more complicated statistics were needed. The ratios and the standard errors for the ratios were calculated using the formulas described by Rice (49). The results were confirmed by simulating the experiments with the program Mathematica (Wolfram Research, Inc.).
results

induction of the gIIp nickase in the presence of its target site increases the rate of adaptive mutation to Lac+. Adaptive mutation in FC40 can be assayed in large-scale (4) or in small-scale (19) experiments. For small-scale assays, cultures are inoculated from single colonies and grown to saturation in M9-glycerol medium. Then, 10-μl aliquots (approximately 2 x 10^7 cells) are spread on each quadrant of a minimal lactose plate, which is incubated at 37°C. Most of the colonies that appear 2 days later are due to mutations that occurred during the prior growth of the culture; the colonies that appear on days 3 to 5 are due to adaptive mutations (4). The mean number of Lac+ colonies appearing on days 3, 4, and 5 gives a reasonable estimate of the rate of adaptive mutation (19).

To determine if induction of a DNA nick increases adaptive mutation, we did small-scale assays with cells grown for 24 h in M9-glycerol medium supplemented with nothing, fucose (a nonmetabolizable repressor of the pBAD promoter [42]), or arabinose (an inducer of the pBAD promoter [31]). With strains FC40 and CRS179 (FC40/pCRS4; pCRS4 is the pBAD plasmid carrying gene II) grown under the different conditions, the rates at which Lac+ colonies appeared were similar, indicating that, in these assays, the presence and expression of gIIp from plasmid pCRS4 did not affect adaptive reversion to Lac+ (Fig. 1A, bars 1 to 6). When strain CRS18, which carries the gIIp nick site near the lacI33 mutation, was grown in the different media, again a similar rate of mutation to Lac+ was obtained, indicating that the nick site alone did not affect reversion to Lac+ (Fig. 1A, bars 7 to 9). But when strain CRS10 (CRS18/pCRS4), which carries both the nick site and gene II, was fully induced for gIIp expression, the rate at which Lac+ colonies appeared increased threefold relative to FC40 (Fig. 1A, bars 10 to 12). These results indicate that induction of a DNA nick increases adaptive mutation to Lac+.

For large-scale experiments, cells are grown from very small inocula and take up to 48 h to reach saturation (4, 13). We found that such long periods of continuous induction resulted in a decrease in adaptive Lac+ mutation in strain CRS10, which has the nick site and gene II. Figure 1B shows a small-scale assay done with the cultures described above but diluted 10^5-fold into fresh media and grown for an additional 36 h. While the number of Lac+ revertants was similar among strains FC40, CRS179, and CRS18 (Fig. 1B, bars 1 to 9), the reversion rate was significantly reduced in strain CRS10 grown with arabinose (Fig. 1B, bars 10 to 12). Large-scale experiments gave similar results (data not shown). One explanation is that prolonged continuous nicking of the episomal DNA resulted in loss of all or part of the episome, including the reversion site. Microscopic examination of CRS10 cells grown in the presence of arabinose revealed many filamentous cells, which is symptomatic of induction of the SOS response (24).

induction of the gIIp nickase in the presence of its target site compensates for the loss of conjugal functions in adaptive mutation. If conjugal functions are not expressed in strain FC40, the rate of adaptive mutation to Lac+ falls about 10-fold (21). Using the small-scale assay described above, we tested whether gIIp plus its target site could restore adaptive mutation to a traD mutant, which is defective for conjugal functions (see Discussion). As shown in Fig. 2A, CRS174, the traD strain with just the gIIp target site, had the expected low rate of adaptive mutation (Fig. 2A, bars 1 to 3). But strain CRS177, which has both gIIp and its target site, had an increased rate of adaptive mutation (Fig. 2A, bars 4 to 6). Indeed, when gIIp was fully induced, the reversion rate of CRS177 was 10-fold higher than that of strain CRS174 and nearly 70% that of the wild-
type FC40 strain. These results indicate that induction of a DNA nick compensates for loss of conjugal functions.

As observed for the wild-type strain, when the traD mutant strain CRS177, which has both the nick site and gene II, was grown under inducing conditions for about 60 h, the adaptive reversion rate to Lac$^+$ was decreased (Fig. 2B, bars 4 to 6). That is, the continuous production of DNA nicks was detrimental to traD mutant cells, as it was to wild-type cells.

**Most nickase-induced Lac$^+$ adaptive mutations are recA$^+$ dependent.** Adaptive mutations in *E. coli* FC40 are reduced 100-fold when RecA is defective (4, 32). In small-scale assays of saturated cultures grown for only 24 h, adaptive mutation was likewise severely reduced in recA derivatives of strains carrying gIIp and its target site (Fig. 3A.). Thus, nickase-induced adaptive mutations are recA$^+$ dependent. In contrast to recA$^+$ strains, when gIIp was fully induced by growing the recA mutant strains with the nick site and the gene II plasmid, CRS187 (tra$^+$) and CRS190 (traD), for 36 to 60 h in the presence of arabinose, adaptive mutation to Lac$^+$ was increased (Fig. 3B). However, these mutation rates were still less than those of rec$^-$ strains (see Discussion).

**The pBAD plasmid increases the apparent “leakiness” of**
the lac allele. The lac allele used in these experiments, \( \Phi(lacI33-lacZ) \), is slightly leaky, producing about 2 Miller units of \( \beta \)-galactosidase, an amount insufficient to allow these cells to grow on lactose (4, 13). However, in certain mutant backgrounds (e.g., recD mutants) and in Salmonella enterica serovar Typhimurium, this allele is actually or effectively leakier, allowing those cells to grow on lactose plates (19, 27). Roth and colleagues have argued that the process of adaptive mutation occurs even when cells are growing slowly under selective conditions (27, 35). In the small-scale experiments described above, the Lac\(^{-}\) cells of wild-type strains carrying pCRS4 made visible lawns on lactose plates. Although such growth cannot account for large differences in mutation rates, it can obscure small differences.

As shown in Table 3, cells carrying either pCRS4 or pCRS3 produced more \( \beta \)-galactosidase than cells without a plasmid. This increase required neither gene II nor its target but was a property of the pBAD plasmid. Whether the plasmid caused the lac allele to be expressed at a higher level or caused an increase in the number of episomes or of lac alleles was not investigated. Because of this increased leakiness, we confirmed the key results of the experiments presented above with experiments during which we monitored the growth of Lac\(^{-}\) cells on lactose medium (see below).

The increase in adaptive mutation does not require full induction of the gIIp nickase. The previous experiments indicated that gIIp is induced to some extent even when the cells are grown in glycerol and not exposed to arabinose (e.g., compare CRS174 and CRS177 in Fig. 2A). To minimize the detrimental effects of full induction of gIIp, we grew cells in M9-glycerol medium and determined the rate of adaptive mutation to Lac\(^{-}\) of strains carrying the gIIp nick site and either pCRS3, the empty vector, or pCRS4, the vector with gene II. As mentioned above, these plasmids allow cells to grow slowly on lactose plates. Therefore, we also determined the number of Lac\(^{-}\) cells on the lactose plates and divided the number of new Lac\(^{-}\) colonies appearing each day by the number of Lac\(^{-}\) cells present 2 days earlier (it takes 2 days for a Lac\(^{-}\) revertant to grow into a visible colony [4, 13]). As shown in Fig. 4C, the mutation rate of cells carrying pCRS3 (the empty vector) was about fivefold higher than that previously observed with FC40 (13). We attribute this increase to the extra \( \beta \)-galactosidase being produced (see above), which is roughly correlated with

FIG. 4. The presence of gene II and its nick site increases adaptive reversion to Lac\(^{-}\). The graphs show the results of a large-scale experiment (see Materials and Methods). Approximately \( 10^5 \) (strain CRS243) or \( 10^6 \) (strain CRS244) cells from each of five independent cultures, plus \( 10^9 \) scavenger cells, were spread onto M9-lactose plates. (A) The accumulation of Lac\(^{-}\) colonies divided by the number of cells originally plated. (B) The number of Lac\(^{-}\) cells on the plates relative to the number originally plated. (C) The accumulation of Lac\(^{-}\) colonies divided by the number of Lac\(^{-}\) cells on the plate 2 days earlier. Because it takes 2 days for a Lac\(^{-}\) colony to form, the curves in panels A and C have been shifted back 2 days. Diamonds, CRS243 (FC40 with the gIIp nick site carrying pCRS3, the empty vector); squares, CRS244 (FC40 with the gIIp nick site carrying pCRS4, the vector with gene II). The scavenger cells were CRS234 and CRS235, respectively. Data are the means and standard errors of the means. Some error bars are smaller than the symbols.

**TABLE 3.** \( \beta \)-galactosidase activities produced by various Lac\(^{-}\) strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Properties</th>
<th>Plasmid</th>
<th>( \beta )-galactosidase activity (Miller units)</th>
<th>Fold increase due to plasmid</th>
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<td>FC40</td>
<td>Wild type</td>
<td>None</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>CRS247</td>
<td>Wild type</td>
<td>pCRS3 (vector)</td>
<td>3.7</td>
<td>1.6</td>
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<tr>
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<td>pCRS4 (gene II)</td>
<td>3.0</td>
<td>1.3</td>
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<tr>
<td>CRS241</td>
<td>Wild type + nick site</td>
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<td>2.0</td>
<td></td>
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<td>Wild type + nick site</td>
<td>pCRS3 (vector)</td>
<td>2.7</td>
<td>1.4</td>
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<td>pCRS4 (gene II)</td>
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<td>1.5</td>
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</table>
mutation rates (28). However, even when corrected for cell growth, the presence of gIIp expressed from plasmid pCRS4 increased the rate of adaptive mutation by three- to fourfold (Fig. 4C).

The same experiment with traD mutant strains is shown in Fig. 5. The traD mutant cells grew much less well on the lactose plates than did traD+ cells (Fig. 5B), and the mutation rate of the traD mutant with the nick site carrying the empty vector (strain CRS245) (Fig. 4C) was comparable to that which we previously observed with traD mutants (21). As observed in small-scale experiments, the presence of gIIp (strain CRS246) increased the adaptive mutation rate 10-fold (Fig. 5C).

Nickase-induced adaptive Lac+ mutations are ruvC dependent. Many of the genes encoding proteins that are involved in recombination are repressed by LexA, the repressor of the genes that constitute E. coli's SOS response to DNA damage (24). Because RecA is required to induce these genes (via its coprotease activity against LexA), loss of adaptive mutations in a recA mutant does not show definitively that the mutations are dependent on recombination. ruvC, which encodes an enzyme that resolves recombination intermediates (Holliday junctions), is not under LexA control (24). In otherwise wild-type cells, adaptive mutation to Lac+ is ruvC as well as recA dependent (23, 33). Therefore, we used a ruvC mutant strain to determine whether nickase-induced Lac+ mutations are truly recombination dependent or are simply a consequence of induction of the SOS response. As shown in Fig. 6, all nickase-induced Lac+ mutations were dependent on ruvC+ as well as on recA+ and thus require the resolution of a Holliday junction to be produced or retained.

Nickase-induced Lac+ mutations are stable. Most of the Lac+ colonies that appear on lactose plates during an adaptive mutation experiment are composed of cells that have reverted the Lac– allele. However, a few Lac+ colonies appear that are composed of cells that have amplified the unreverted Lac– allele. These make up only a small percentage of the Lac+ colonies during a normal 5-day experiment (13, 18, 19) but can represent nearly 50% of the Lac+ colonies if plates are incubated for 7 to 10 days (34, 46). The hallmark of amplification is that the Lac+ phenotype is unstable (54). Instability can be detected by allowing individual Lac+ cells to grow on medium containing X-Gal and seeing if white (Lac–) sectors form within blue (Lac+) colonies.

We tested approximately 32 Lac+ colonies each from strains CRS243 (carrying the empty vector) and CRS244 (carrying the plasmid with gene II) appearing on days 4 to 6 during the experiment shown in Fig. 4, and an additional 30 colonies of each strain from a repeat of this experiment. One out of 62 colonies of CRS243 and 4 out of 62 colonies of CRS244 were unstable, a difference that is not significant (χ² = 0.83; P = 0.36). Similar results were obtained with the traD strains: none of 21 Lac+ colonies of CRS245 (carrying the empty vector) and 2 of 32 Lac+ colonies of CRS246 (carrying the plasmid with gene II) were unstable, which was again a nonsignificant difference (χ² = 0.19; P = 0.67). We conclude that induction of a DNA nick does not increase the number of cells that amply the Lac– allele, at least not to the extent that the cells become phenotypically Lac–.

The spectrum of nickase-induced Lac+ mutations is the
same as that of adaptive mutations in wild-type cells. Table 4 gives the sequence of 35 Lac<sup>+</sup> mutations of CRS244 from the experiment shown in Fig. 4. As found in the wild-type strain (20, 50), the spectrum of mutations in CRS244 was dominated by −1-bp deletions in runs of iterated bases. In particular, −1-bp frameshifts at the hotspot at bp 1036, which normally account for about 50% of the adaptive mutations (18), were 53% of the nickase-induced mutations.

**DISCUSSION**

Expressing the bacteriophage f1 gIIp in a strain carrying the gIIp nick site near the revertible lac allele stimulated Lac<sup>+</sup> adaptive mutation. In addition, induction of the nickase was able to restore mutation to a conjugal-defective traD mutant. These results support the hypothesis that the recombination events required for adaptive mutation are normally initiated by a DNA single-strand nick produced by conjugal proteins at oriT, the conjugal origin of the F<sup>+</sup> episome (23, 35). Further, nicking appears to be the most important, and perhaps the only, conjugal function required for adaptive mutation in strain FC40.

oriT is about 80 kb away from the lacI33 site (P. L. Foster, unpublished data), whereas we placed the gIIp nick site about 600 bp away from lacI33. Although the oriT and gIIp nicks are on the same DNA strand, they are on opposite sides of the lacI33 site (dividing the episome at its vegetative origins). It will be interesting to see if the adaptive mutation rate varies as a function of distance and/or orientation relative to the site of mutation.

It may appear surprising that the nicking function of gIIp can, at least partially, substitute for the loss of TraD in adaptive mutation. The traD allele that we used is not polar (K. Ippen-Ihler, personal communication), and TraD is not required for nicking by TraI, the enzyme that nick at oriT (12). However, TraI is stimulated by TraM (37), and TraM interacts with and may be activated by TraD (10). Thus, our finding that the traD defect can be complemented for adaptive mutation by an exogenous nicking system supports the idea that TraD is required for the maximal level of nicking at oriT in vivo.

Adaptive mutations in FC40 are dependent on the recombination pathway that repairs DNA DSBs (19, 32). This requirement, plus the requirement for conjugal functions but not actual conjugation (21), were reconciled by Kuzminov (38), who proposed that when a replication fork encountered the persistent nick at oriT, the fork collapses, producing a DSB (Fig. 7A). Repair of this DSB by recombination with the sister chromosome would reestablish the replication fork (Fig. 7B to D). We have further postulated (16) that the reestablished repulsion initially contains not the normal replicative DNA polymerase, DNA Pol III, but either DNA Pol II or DNA Pol IV. If accurate Pol II gains access, the new DNA synthesis is error free; if error prone Pol IV gains access, a track of inaccurate DNA synthesis is produced, accounting for some (but not all) of the adaptive mutations. Eventually DNA Pol III continues DNA synthesis, and the errors that it makes account for the rest of the mutations (Fig. 7D). The results presented here support the hypothesis that the initiating event is a DNA nick.

Our results also support a different mechanism for adaptive reversion that involves amplification. First proposed in 1992 (17), amplification of the nonreverted Lac− allele has been shown to account for some, but not all, Lac<sup>+</sup> colonies that appear during lactose selection (13, 34, 46). But Roth and colleagues (1, 35) have proposed that amplification is a necessary precondition for all Lac<sup>+</sup> revertants. According to their model, nicking at oriT leads to amplification of the lac allele and cells with amplifications produce slowly growing clones on lactose medium. True Lac<sup>+</sup> mutations then appear among the amplified arrays, and the arrays then disappear (35). Although the data presented here do not distinguish between the Roth model and the model presented in Fig. 7, the fact that exogenous nicking increased adaptive mutation without significantly

### TABLE 4. The spectrum of nickase-induced Lac<sup>+</sup> mutations

<table>
<thead>
<tr>
<th>Mutation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Site</th>
<th>No. of Lac&lt;sup&gt;+&lt;/sup&gt; revertants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 4</td>
</tr>
<tr>
<td>−G at 1020</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>−C at 1036</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>−C at 1064</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>−A at 1056</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>−T at 1077</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>−C at 1085</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Size change</td>
<td>Delete&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>Other</td>
<td>Not in target&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

<sup>a</sup>The numbers refer to the bases in Ecolac (GenBank J01636.1), except that the extra C at 1036 to 1038, which constitutes the lacI33 mutation, is not numbered. Only the base in the coding DNA strand is given.

<sup>b</sup>Detectable by a mobility shift of the PCR product.

<sup>c</sup>These late-arising Lac<sup>+</sup> colonies are usually due to amplification of the Lac<sup>−</sup> allele (18, 46).
supplying all the conjugal functions from an additional plasmid and showing that adaptive mutation on the chromosome did not increase (data not shown). It has been previously suggested that the E. coli chromosome has both hot and cold spots for recombination-dependent mutation (55).

Finally, we found that prolonged induction of gene II was highly detrimental, resulting in filamentous cells and few Lac+ mutations. However, in recA cells, prolonged induction resulted in a small increase (about threefold) in lac reversion (Fig. 3). This was true whether the recA cells were traD+ or traD− deficient. We interpret this result to mean two things. First, the detrimental effects of continuous induction of gIIp depend on RecA function. It is likely that continuous DNA nicking induces the SOS response and consequent lethal filamentation. As mentioned above, RecA is required to induce the SOS response and may also be required to process the nicked DNA into the SOS-inducing signal, single-stranded DNA. Second, when the DNA is nicked there is at least one pathway for adaptive mutation that is independent of RecA. However, this pathway is normally a minor contributor to adaptive mutation.

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