Topological repression of gene activity by a transposable element

*Escherichia coli* ebgA gene/plasmid pBR322/transposon/gamma-delta/position effect)

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**ABSTRACT** The ebgA (evolved β-galactosidase) gene of *Escherichia coli* was isolated as part of a 9.6-kilobase (kb) sequence cloned into plasmid pBR322. The position of the ebgA gene within that 9.6-kilobase sequence was identified by insertional inactivation by means of the transposon gamma-delta. In addition to the gamma-delta insertions that inactivate ebgA by disrupting the coding sequence, seven additional gamma-delta insertions reduce expression of the gene by a factor of >200 by insertions elsewhere into the replicon. One of these insertions is into the pBR322 sequence itself. This action at a distance to reduce expression requires that gamma-delta is cis with respect to the ebgA gene. The effect is independent of the orientation or position of gamma-delta within the replicon, but it does depend both upon the orientation of the ebgA-bearing sequence within the replicon and upon the total size of the replicon. Transcription readthrough (promoter occlusion) does not explain this phenomenon, and we suggest that the presence of gamma-delta may alter the local supercoiling in the region of the ebgA promoter in such a way as to inhibit transcription. This repression by a transposable element appears to represent a novel mechanism for altering gene expression.

The evolved β-galactosidase gene, ebg, of *Escherichia coli* has been used extensively as a model for the study of acquisitive evolution (1). The ebg system consists of a repressor encoded by ebgR and at least two structural genes, ebgA and ebgB (2, 3). The ebgA gene specifies a β-galactosidase, while the ebgB gene product has no known function. In the unevolved (wild-type) operon, the repressor responds poorly to lactose as an inducer (4), and ebg β-galactosidase is a poor lactase (5). Mutations have been isolated in both genes that enhance the properties of the operon for growth on β-galactosides (6-9). The product of the wild-type allele, ebgA, is a poor β-galactosidase (10) and is designated ebg° enzyme. Several classes of mutations have been isolated that considerably enhance the activity of this enzyme toward lactose, and one particular class (class IV) permits the efficient hydrolysis of several β-galactoside sugars. The molecular details of the ebg system are not well understood; thus, the cloning and DNA sequencing of ebgA and ebgR is of considerable interest in understanding the mutational events that occur to change the properties of the respective proteins.

We show here that the expression of the cloned ebgA gene can be drastically altered by a transposon through a mechanism that involves neither insertional inactivation nor promoter occlusion (11).

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions.** Strains and plasmids are shown in Table 1. Cells were grown in L-broth (12) or in minimal medium (5) at 37°C with aeration.

**Isolation and Manipulation of DNA.** Chromosomal DNA was prepared by the method of Stauffer (13). Large-scale preparations of plasmid were prepared from 1-liter overnight cultures in L-broth supplemented with 100 µg of ampicillin per ml by standard methods (14). Small-scale preparations were prepared from 10-ml cultures. After the ethanol precipitation step, DNA was digested with 40 units of T1 RNase (Bethesda Research Laboratories), and the mixture was loaded onto a Sepharose CL-6B column preequilibrated with 1.0 M NaCl in TE buffer (12). DNA-containing fractions were dialyzed overnight against TE buffer, precipitated with ethanol, and dissolved in TE buffer. Plasmid DNA was transformed into *E. coli* cells rendered competent by the method described by Maniatis et al. (12) except that 100 mM CaCl₂ was used. Restriction endonucleases were obtained commercially and used according to the instructions of the manufacturer. Liguations were performed in a total volume of 40 µl at a 1:3 molar ratio of vector to insert by adding 2.5 units of T4 DNA ligase to the ligation mix and incubating for 2 hr at room temperature and then overnight at 4°C.

**Enzyme Assays and Protein Determinations.** Cultures for ebg enzyme assays were grown in succinate minimal medium unless otherwise indicated, and extracts were prepared as described (15). ebg enzyme activity was determined from O-nitrophenyl-β-D-galactopranoside hydrolysis, and protein concentration was determined from absorbance at 225 nm, both as described (2). β-Lactamase activity was assayed in the supernatants of exponentially growing cultures in L-broth by measuring the hydrolysis of 0.1 mM Nitrocefin (Gallaxo Research Group, Greenford, Middlesex, England) in 50 mM potassium phosphate buffer (pH 7.0). Culture supernatant (25 µl) was added to 0.5 ml of substrate, and the increase in absorbance at 482 nm was monitored at 25°C. One unit is defined as an increase of 1.0 absorbance unit per min. Specific activities are reported as units per 10⁶ cells in the culture.

**Construction of Gamma-Delta-Containing Plasmids.** Plasmids containing gamma-delta were constructed as described by Sancar and Rupp (16). The donor strain was MG1063 harboring a plasmid carrying ebgA, and the recipient was strain SJOR. The ebg (Lac) phenotype was determined on MacConkey lactose indicator plates supplemented with 0.2 mM isopropyl β-D-thiogalactoside, streptomycin (300 µg/ml), and ampicillin (100 µg/ml).

**Test for Hydrolysis of 5-Bromo-4-chloro-3-indolyl-β-D-galactopranoside (X-gal).** Cultures of strain SJO48R harboring plasmids were streaked onto minimal medium supplemented with 0.2 mM isopropyl β-D-thiogalactoside, and 40 µg of X-gal per ml. Hydrolysis was indicated by the production of blue pigment.

Abbreviations: kb, kilobase(s); X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ebg, evolved β-galactosidase; IS, insertion sequence.

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Table 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/phenotype</th>
<th>Reference or comment</th>
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<tbody>
<tr>
<td>SJOR</td>
<td>argB metC ΔlacZ(W4680) rpsL ebgA0 ebgR+ recA56 srl05::Tn10</td>
<td>2 (recA transduction of SJOR)</td>
</tr>
<tr>
<td>SJ60</td>
<td>argB metC ΔlacZ(W4680) rpsL ebgA205 ebgR25</td>
<td>10</td>
</tr>
<tr>
<td>SJ84R</td>
<td>argB metC ΔlacZ(W4680) rpsE ΔebgRAB(1011) recA1</td>
<td>30</td>
</tr>
<tr>
<td>MG1063</td>
<td>F' recA56</td>
<td>16</td>
</tr>
</tbody>
</table>

RESULTS

Construction of the ebgA Clone and Isolation of Gamma-Delta-Containing Derivatives. Genomic DNA of strain SJ60, carrying the class IV allele ebgA205 (7), was partially digested with Sau3A enzyme to give about 10-kb fragments and was ligated into BambHI-cut pBR322. ebg' transformants of strain SJOR were selected by plating onto lactose minimal medium. One Lac- transformant harbored a plasmid carrying a 14-kilobase (kb) insert. Preliminary restriction mapping of this plasmid, designated pUF1, permitted the subcloning of a single 9.6-kb SalI fragment carrying ebgA into pBR322. The restriction map of this plasmid, designated pUF2, is shown in Fig. 1.

Several lines of evidence show that the lactase activity encoded by pUF2 is due to the ebgA gene. First, when pUF2 was transformed into the ΔlacZ-ΔebgAB strain SJ84R, all transformants were Lac-. Second, crude extracts of strain SJ84R/pUF2 synthesized a protein that cross-reacted with anti-ebg antibody. Neither lacZ-β-galactosidase nor any other β-galactosidase cross-reacts with anti-ebg antibody (18, 19). Third, strains carrying plasmid pUF2 exhibited exactly the pattern of β-galactosidase sugar utilization expected of class IV ebgA strains (9). Finally, several additional ebgA alleles have been cloned as 9.6-kb SalI fragments. In each case the pattern of β-galactosidase sugar utilization was exactly that expected for its ebgA class (9), and in each case the restriction pattern was either identical to that of pUF2 or to that expected when the fragment is inserted into pBR322 in the opposite orientation.

Gamma-Delta Can Generate an ebgA- Phenotype by a Mechanism Other Than Insertional Inactivation. To locate the ebgA coding sequence, the transposable element gamma-delta was inserted into random sites within pUF2. The positions of 12 Lac- gamma-delta insertions were determined by restriction mapping (Fig. 1). All of the insertions were mapped by digestion with single and double restriction enzyme combinations and were placed unambiguously. The molecular weight of the ebgA-encoded protein is estimated as 120 kilodaltons (15), a size that requires approximately 3 kb of DNA coding sequence. Since the insertions were distributed over 9 kb, it seemed unlikely that the loss of lactase activity could be due solely to insertion within the ebgA gene itself. One plasmid, designated pUF211, consisted of gamma-delta inserted into the vector DNA (Fig. 1). The restriction pattern for pUF211 is shown in Fig. 2.

Because gamma-delta in pUF211 is within the pBR322 sequence, it was not possible that it had inactivated the ebgA gene by physically disrupting the coding sequence. To demonstrate the physical integrity of the ebgA sequence, the 9.6-kb SalI fragment was excised from pUF211 and cloned into pBR322. The resulting recombinant plasmids conferred a Lac- phenotype on strain SJ84R, and all gave restriction patterns identical to that of pUF2 or consistent with insertion of the 9.6-kb fragment in the opposite orientation. The restriction pattern of one such recombinant, pUF12, is shown in Fig. 2.

Because pUF211 contains gamma-delta inserted into the pBR322 sequence, gamma-delta must in that case act at a distance to prevent expression of the ebgA gene. To determine whether ebgA in pUF211 was expressed at all, strain SJ84R/pUF211 was assayed for ebg enzyme activity. The crude extract contained 8.7 units mg⁻¹ of activity toward o-nitrophenyl-β-D-galactopyranoside, or the equivalent of 3.0 units mg⁻¹ of in vivo lactase activity (10), a level of lactase activity insufficient to support growth on lactose or to generate a Lac+ phenotype on MacConkey lactose agar (9). The low level of activity indicated that the ebgA gene had not been inactivated but that its expression had been reduced by the presence of gamma-delta within the replicon. The extent of the repression was estimated by comparison with SJ84R/pUF2 grown in lactose (Table 2).

The observed reduction in ebg enzyme synthesis to 1/200 is probably an underestimate. To maximize expression of ebgA, SJ84R/pUF211 was grown on the non-catabolite-repressing carbon source, succinate. Because of extreme hyperexpression of the ebgA gene from pUF2, strain SJ84R/pUF2 cannot be grown in succinate. (The plating efficiency of SJ84R/pUF2 on succinate is 5 × 10⁻⁶ relative to plating on L-broth, whereas the SJ84R/pUF211 plates equally well on both media.) To select for retention of the plasmid, SJ84R/pUF2 was grown in lactose minimal medium containing ampicillin. Lactose catabolite represses expression of the ebgA gene to one-third, which markedly reduces hyperexpression toxicity. Correcting for catabolite repression, we estimate that the presence of gamma-delta in pUF211 reduces the expression of ebgA to 1/1000. To provide a direct comparison of SJ84R/pUF211 with SJ84R/pUF2, extracts of these strains grown in L-broth containing ampicillin were assayed for ebg enzyme activity. SJ84R/pUF2 synthesized 100 units mg⁻¹, whereas SJ84R/pUF211 synthesized <0.4 units mg⁻¹. Thus, we confirm that under identical conditions gamma-delta represses expression of ebgA by a factor of 100.
To determine whether plasmid copy number contributed to the magnitude of the repression observed here, we assayed β-lactamase activity in the supernatants of exponentially growing cultures of SJ84R/pUF2 and SJ84R/pUF211. Plasmid pUF2 yielded 19 units per 10^9 cells, while plasmid pUF211 yielded 6.3 units per 10^9 cells. Thus, there is at most a reduction in plasmid copy number to one-third, and gamma-delta must repress the level of ebgA gene expression by a factor of 100.

To determine whether the repression exerted by gamma-delta involved a diffusible gene product, a derivative of pBR322 was constructed harboring an insertion of gamma-delta. This plasmid, designated pUF6, was introduced into strain SJ60. Because of the high copy number of pBR322, a trans-acting gene product encoded by gamma-delta would be expected to repress a chromosomal gene more efficiently than a plasmid-borne gene. All of the transformants remained Lac^−, indicating that the observed repression was not mediated by a classical trans-acting effector such as is found in negative control systems.

Since pUF211 produced a small amount of ebg enzyme, SJ84R/pUF211 was tested on X-gal medium because X-gal is an extremely sensitive substrate for class IV ebg enzymes. SJ84R/pUF211 produced the blue pigment indicative of X-gal hydrolysis; consequently, all of the gamma-delta-containing plasmids were tested on X-gal medium. The plasmids fell into two classes (Table 2): those that produced pigment (X-gal⁺) and those that did not (X-gal⁻). More significantly, X-gal⁻ plasmids all contained gamma-delta within a 3.2-kb region of the 9.6-kb SalI insert. This is consistent both with the X-gal⁻ class being Lac⁻ as the result of insertion of gamma-delta within ebgA itself and with the assumed length of the ebgA coding sequence. As expected, the X-gal⁺ plasmid produced no detectable ebg enzyme activity (Table 2). On the other hand, insertions of gamma-delta outside of that 3.2-kb region resulted in levels of ebg enzyme synthesis similar to that observed for pUF211 (Table 2). These data demonstrate that gamma-delta can alter the level of expression of the ebgA gene by two methods: (i) by classical insertional inactivation in which gamma-delta disrupts the coding se-
Table 2. Assay of ebgA clone and gamma-delta-containing derivatives for ebg enzyme activity and determination of their phenotype on X-gal medium

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Enzyme activity, units mg(^{-1})</th>
<th>Position of gamma-delta(^{a}) (Fig. 1)</th>
<th>Phenotype on X-gal(^{b})</th>
</tr>
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<tbody>
<tr>
<td>pUF2(^{2})</td>
<td>1742.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pUF211</td>
<td>8.7</td>
<td>11.9</td>
<td>+</td>
</tr>
<tr>
<td>pUF201</td>
<td>7.8</td>
<td>2.3</td>
<td>+</td>
</tr>
<tr>
<td>pUF202</td>
<td>4.2</td>
<td>8.9</td>
<td>+</td>
</tr>
<tr>
<td>pUF203</td>
<td>NT</td>
<td>2.4</td>
<td>+</td>
</tr>
<tr>
<td>pUF204</td>
<td>5.3</td>
<td>10.2</td>
<td>+</td>
</tr>
<tr>
<td>pUF205</td>
<td>10.0</td>
<td>3.2</td>
<td>+</td>
</tr>
<tr>
<td>pUF207</td>
<td>NT</td>
<td>9.1</td>
<td>+</td>
</tr>
<tr>
<td>pUF206</td>
<td>NT</td>
<td>7.0</td>
<td>-</td>
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<td>pUF209</td>
<td>0</td>
<td>5.0</td>
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<tr>
<td>pUF210</td>
<td>NT</td>
<td>6.7</td>
<td>-</td>
</tr>
<tr>
<td>pUF212</td>
<td>NT</td>
<td>4.6</td>
<td>-</td>
</tr>
<tr>
<td>pUF215</td>
<td>NT</td>
<td>3.8</td>
<td>-</td>
</tr>
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</table>

All plasmids were tested in an SJ84R (ebg deletion) background to ensure that there was no contribution from a chromosomal ebgA gene. NT, not tested.  
\(^{a}\) gamma-delta insertions within pUF2 (Fig. 1A). Positions are in kb from the EcoRI site of pBR322.  
\(^{b}\) +, Pigmented; –, nonpigmented.

sequence and no active enzyme is produced, and (ii) by insertion of gamma-delta into the replicon but outside of the ebgA gene itself, repressing enzyme synthesis by a factor of >100. This second method, involving action at a distance, does not depend upon either the orientation or position of gamma-delta within the replicon (Fig. 1).

**Action at a Distance Is Dependent on Replicon Size and Gene Orientation.** To determine whether action at a distance is due to a specific nucleotide sequence within gamma-delta or is simply a property of the physical presence of additional DNA, insertions into three other plasmids were examined. Plasmid pUF25 is identical with pUF2 except that the 9.6-kb SalI fragment is inserted in the opposite orientation (orientation II). Plasmid pUF16 encodes the class II (9) allele ebgA143, with the 9.6-kb SalI fragment inserted in orientation II. The restriction map of pUF25 is identical to that of pUF16, shown in Fig. 1. When gamma-delta was inserted into either of these plasmids, ~80% of the exconjugants were Lac\(^{-}\). In both cases, all of the Lac\(^{-}\) plasmids failed to produce pigment on X-gal. This suggested that, when the 9.6-kb ebgA clone is in orientation II, gamma-delta does not repress gene expression by action at a distance. To confirm this, we mapped several of the gamma-delta insertions in pUF16 (Fig. 1). The insertion generating the Lac\(^{-}\) phenotype mapped, as expected, within the ebgA gene. The remaining insertions were distributed approximately at random, on both sides of ebgA and in both orientations, but they failed to render pUF16 Lac\(^{-}\). Since gamma-delta insertions in approximately the same positions and orientations had strikingly different consequences when inserted into pUF2 and into pUF16, the repression effect is dependent upon gene orientation.

Plasmid pUF161, consisting of pUF16 with the 0.85-kb HindIII fragment removed (Fig. 1), confers a Lac\(^{-}\) phenotype indistinguishable from that of pUF16. When gamma-delta was inserted into pUF161, all of the exconjugants were Lac\(^{-}\). When tested on X-gal, ~80% of those exconjugants formed blue colonies. The positions of several gamma-delta insertions were mapped (Fig. 1), and all insertions that were blue on X-gal were outside the ebgA gene, whereas the insertion that was white on X-gal was within ebgA. Thus, the effect of gamma-delta insertions depends upon both the orientation of the ebgA gene within the replicon and upon the total size of the replicon. Taken together, these observations also show that action at a distance is not solely due to some specific nucleotide sequence contained within gamma-delta.

**DISCUSSION**

The ebgA gene was located within a 9.6-kb SalI fragment into pBR322 by insertional inactivation by the transposon gamma-delta. Seven additional gamma-delta insertions, on both sides of ebgA, in both orientations, and as far as 5 kb away from ebgA, resulted in a repression of the expression of the ebgA gene by a factor of 200. It is noteworthy that the majority of the gamma-delta insertions into pUF2 resulted in repression of ebgA expression. Over 80% of the exconjugants from the original experiment were Lac\(^{-}\). Others (16, 17) have reported that in similar experiments target genes were inactivated in only 5–15% of the exconjugants. On the basis of target size alone, about 20% of the exconjugants were expected to be Lac\(^{-}\). Attempts were made to locate the sites of gamma-delta within the few Lac\(^{-}\) exconjugants, but in each case the restriction patterns were inconsistent with a simple insertion of gamma-delta, suggesting that deletions or rearrangements had occurred. It appears that all "clean" insertions of gamma-delta into pUF2 are capable of repressing the ebgA gene. The roughly random distribution of gamma-delta insertions indicates that the repression effect can be exerted from any region of the plasmid. The failure of gamma-delta inserted into pBR322 to repress a chromosomally located ebgA gene shows that gamma-delta acts only in cis.

Although the effect does not depend upon the location of gamma-delta within the replicon, it does depend upon the orientation of the affected gene within the replicon. When either the original allele, ebgA202, or a different allele, ebgA143, was cloned as a 9.6-kb insert in orientation II, gamma-delta did not repress gene expression. This indicates that the action at a distance is mediated by some mechanism that is independent of gamma-delta nucleotide sequence per se.

Finally, the effect is also dependent upon the overall size of the replicon. When a 0.85-kb fragment was excised from pUF16, carrying ebgA143 in orientation II, the slightly smaller plasmid was once again subject to action at a distance by gamma-delta. This experiment also demonstrates that the effect is not limited to a single ebgA allele. Additional experiments (data not shown) have shown that additional ebgA alleles cloned on 9.6-kb fragments in orientation I are subject to repression by gamma-delta, while those in orientation II are not.

The first mechanism that comes to mind to account for this cis-acting repression is promoter occlusion (11)—i.e., transcription from a strong promoter within gamma-delta interfering with normal function of the ebgA promoter. Several lines of evidence rule out this mechanism and any other mechanism that depends upon transcription from inside gamma-delta. First, since gamma-delta insertions into pUF2 and pUF161 act in both orientations, gamma-delta would have to contain strong outward-reading promoters at both ends. There is no evidence for such promoters in gamma-delta; furthermore, gamma-delta insertions into pBR322 do not interfere with expression of either the Ap' or the Tc' gene (20), and gamma-delta insertions into plasmids carrying cloned uvrA and wdr genes interfered with these gene functions only by disrupting the genes themselves (16, 17). Second, insertions into regions that lead to repression in plasmids pUF2 and pUF161 do not lead to repression in pUF16. Any mechanism that depended simply upon tran-
scription from within gamma–delta would not be affected by the orientation of the 9.6-kb insert or by removal of the 0.85-kb HindIII fragment. 

Trans-acting mechanisms are ruled out by the failure of pBR322 containing gamma–delta to repress a chromosomal ebgA gene. Disruption of unknown upstream elements or positive regulatory elements are ruled out by the fact that gamma–delta inserted into the pBR322 portion of pUF211 gives the repression effect.

If cis-acting repression of ebgA is not accounted for by readout, what other cis-acting mechanisms might account for the ability of gamma–delta to repress a gene located as much as 5 kb away? It is clear that the topological state of DNA is important in determining proper transcription (21). Moreover, it is becoming increasingly evident that mutations that alter the level of DNA supercoiling may alter the expression of a gene (for a review, see ref. 22). For example, mutations at gyrA and gyrB reduce the level of supercoiling (23), and mutations in either of these genes can activate the normally cryptic bgl operon of E. coli (24). The level of expression of the gyrase genes themselves is, in fact, regulated by supercoiling (25). Underlining the importance of DNA supercoiling is the fact that mutations in the topoisomerase I gene, which relaxes negatively supercoiled DNA, also affect transcription (26). In addition, compounds that inhibit the activity of DNA gyrase can alter gene expression (27, 28).

Since DNA topology is important in gene expression and since no other known mechanism accounts for the observed effects of gamma–delta on ebgA expression, we speculate that the ebgA gene may be susceptible to local changes in DNA supercoiling. Under this model we would expect the overall supercoiling of the replicon, in terms of superhelical turns per unit length of DNA, to be unchanged by the presence of gamma–delta. However, the insertion of gamma–delta into pUF2 or pUF161 might, by altering replicon size, place the ebgA promoter into such a position with respect to local supercoiling that RNA polymerase could no longer bind efficiently. If this were the case, then gamma–delta need not affect other genes on the plasmid in the same way. This model would also suggest that it is the alteration in replicon size caused by the presence of gamma–delta, rather than any genetic property of gamma–delta per se, that leads to the repression effect. This also would be consistent with the observations that a purely topological change, inversion of the 9.6-kb SalI fragment, drastically changes the effect of gamma–delta and that the change can be reversed by another topological change, the removal of a 0.85-kb fragment from the replicon.

The idea that an insertion element can alter gene expression by altering supercoiling is not without precedent. It has been reported that the cryptic bgl operon can be activated by insertions of either insertion sequence IS1 or IS5 upstream from it; since neither of the IS elements is known to carry a promoter sequence, the authors suggested that the IS elements might alter the local supercoiling state (24). Finally, insertion of any of several insertion elements can prevent expression of the bop gene of Haloferax halobium when the element is inserted as much as 1.4 kb upstream of the bop gene (29). In one case insertion of a second element, distal to the first element restored the bop phenotype. That reversion cannot be accounted for by readthrough or by disruption of upstream elements and may well be the result of the same mechanism that permits gamma–delta to act on ebgA.

The specific model presented here is clearly speculative, but the general proposition that the effect is topologically mediated appears sound. If some promoters are sensitive to local changes in DNA topology, then this "position effect" would have significant evolutionary implications since, for such genes, the location on the chromosome could profoundly influence its level of expression.

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