

# Pseudoreversion of Lactose Operator-Constitutive Mutants<sup>1</sup>

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A set of pseudorevertants of lactose operator-constitutive (*lacO<sup>c</sup>*) mutants has been obtained. Analysis of a subset of these pseudorevertants indicates that, in some cases, the secondary mutation alters the lactose repressor (*lacI* gene product), whereas in others it seems to have occurred in the lactose operator (*lacO*) itself. Of the *lacI* gene mutations, the *lacI<sup>s</sup>* mutation, already known to suppress all *lacO<sup>c</sup>* mutations nonspecifically, was recovered by a selection technique developed for this study. However, two additional *lacI* gene mutants were selected which appear to suppress *lacO<sup>c</sup>* sequences in a more-or-less specific fashion; repressor interaction with some operator sequences is facilitated, whereas the binding with *lacO<sup>+</sup>* and others is attenuated concomitantly.

A logical point of departure for the investigation of protein-deoxyribonucleic acid (DNA) interactions in general and the *lac* repressor-operator interaction specifically is the isolation of mutants in which these interactions have been modified. Given a sufficient number of such mutants, it should be possible in principle to determine the number and genetic locations of the elements in the two species (operator and repressor) which are involved in their mutual binding. Such studies have, in fact, been performed on the repressor (1, 12, 15; J. Miwa and J. Sadler, in preparation) and the operator (7, 18), but these investigations dealt mainly with mutants in which repressor-operator interaction was either weakened or destroyed. Mutants in which the repressor-operator interaction is actually strengthened should be at least as significant for understanding the nature of the binding, and the major concern of this investigation has been to find and characterize such mutants.

Because of certain limitations on the selection techniques available, the appropriate mutant phenotype was sought and found among the revertants of base substitution operator-constitutive (*O<sup>c</sup>*) mutants. These revertants are designated *O<sup>c</sup>* pseudorevertants and are defined as mutants in which repression of the lactose operon is restored to some degree from the parental *LacO<sup>c</sup>* phenotype, but which differ distinctly in this regard from the true *LacO<sup>+</sup>* revertant. Beyond confirming the existence of such pseudorevertants (see reference 18 for ten-

tative identification), the concern of this study was to determine whether such secondary suppressor mutations occur in the operator, the repressor, or elsewhere and, in addition, to explore the range of repression effects obtained.

## MATERIALS AND METHODS

**Bacterial strains.** Mutagenesis of the lactose operon was carried out on *F'lac proA<sup>+</sup>* episomes because of the ease of transferring the episome to multiple *Escherichia coli* K-12 backgrounds, thereby facilitating rapid characterization of lactose operon function to the exclusion of secondary genetic changes outside of *lac*. The origin and properties of the strains used in the initial construction and subsequent characterization of the lactose regulatory gene mutants isolated and studied are given in Table 1.

**Media.** All chemically defined minimal media (liquid or agar-solidified media) were based on either Davis minimal salts and buffer (5) [per liter:  $K_2HPO_4$ , 7 g;  $KH_2PO_4$ , 2 g; sodium citrate  $\cdot 5H_2O$ , 0.5 g;  $MgSO_4 \cdot 7H_2O$ , 0.1 g;  $(NH_4)_2SO_4$ , 1 g] or R buffer (18) [per liter: NaCl, 5.3 g; KCl, 3.0 g;  $NH_4Cl$ , 1.1 g;  $KH_2PO_4$ , 0.095 g;  $Na_2SO_4$ , 0.03 g;  $MgCl_2 \cdot 6H_2O$ , 0.21 g; tris(hydroxymethyl)aminomethane-hydrochloride (Sigma), 13.22 g; and Trizma base (Sigma), 1.94 g; pH 7.1 at 37°C]. Preparation of minimal media employed the addition of salts and nutrients as concentrated solutions to autoclaved water-agar mixes.

The broth media utilized were SLB (per liter: tryptone, 37.5 g; yeast extract, 22.5 g; NaCl, 3 g; 1 M potassium phosphate buffer, 100 ml; pH 7.4), Penassay broth (per liter: antibiotic medium 3 [Difco], 17.5 g), and tryptone broth (per liter: tryptone [Difco], 8 g; yeast extract [Difco], 1 g; NaCl, 5 g).

Purified agar (Difco) was used at a concentration of 11 g/liter for all minimal plates. All other plates (nutrient broth) were made with 15 g of agar (Difco) per liter.

**Selection strategy.** The basic selection strategy

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TABLE 1. Bacterial strains

Strain designation	Genotype	Use
D431	F <sup>-</sup> lacI2524 <sup>-</sup> O <sup>+</sup> Z <sup>-</sup> Y <sup>+</sup> proA recA	Selector strain (lacZ <sup>-</sup> mutation is non-polar)
P91	F <sup>-</sup> (lac-proA)Δ recA strA	lac deletion strain in which episomes underwent mutagenesis
P92	F <sup>-</sup> (lac-proA)Δ recA Spc <sup>r</sup>	lac deletion selector strain
D531 <sup>a</sup>	(lac-proA)Δ recA strA/F'lacI <sup>+</sup> O3p5 <sup>+</sup> Z <sup>+</sup> Y <sup>+</sup> proA <sup>+</sup>	P91 containing episome with class IIa lacO <sup>c</sup>
D532	(lac-proA)Δ recA strA/F'lacI <sup>+</sup> O116 <sup>+</sup> Z <sup>+</sup> Y <sup>+</sup> proA <sup>+</sup>	P91 containing episome with class IIIa lacO <sup>c</sup>
D533	(lac-proA)Δ recA strA/F'lacI <sup>+</sup> O103 <sup>+</sup> Z <sup>+</sup> Y <sup>+</sup> proA <sup>+</sup>	P91 containing episome with class IIIb lacO <sup>c</sup>
D536	(lac-proA)Δ recA strA/F'lacI <sup>+</sup> O12 <sup>+</sup> Z <sup>+</sup> Y <sup>+</sup> proA <sup>+</sup>	P91 containing episome with class Vb lacO <sup>c</sup>
RV/80 <sup>b</sup>	F <sup>-</sup> (lacX74)Δ λh80cI857St68 λh80cI857St68 dIacO <sup>+</sup>	Source of lacO <sup>+</sup> DNA
D1030	F <sup>-</sup> lacI <sup>-</sup> O <sup>+</sup> recA proA strA	Made lysogenic with lac pseudorevertant φ80 phage as source for altered repressor
W2, W5, W6, W7, W10, W23		lac pseudorevertant candidate episome in strain D431
W33, W40		lac pseudorevertant episome candidate in strain P92
W41, W44		lacO <sup>+</sup> true revertant episome in strain P92
φ33, φ41	D1030 lysogenic with λh80cI857St68 λh80cI857St68dIac	Phage lac contains lac pseudorevertant from W33 and W41, respectively, as source for altered repressor

<sup>a</sup> The characteristics of the lacO<sup>c</sup> strains are described in reference 17.

<sup>b</sup> Details of phage genetics described in reference 11.

used for the isolation of lac regulatory mutants, in which the repressor-operator interaction is strengthened, is dependent on the growth inhibitory effects of o-nitrophenyl-β-D-thiogalactoside (NPTG) (Cyclo or Calbiochem). This galactoside is neither an inducer of the Lac function nor an effective carbon source. It is, however, a substrate for permease and therefore selects against the induced or constitutive lacY gene function, presumably on the basis of a metabolic energy wastage mechanism (9). Mutagenesis of *E. coli* lac deletion strains (P91 or P92) carrying F'lacI<sup>+</sup>O<sup>+</sup>Z<sup>+</sup>Y<sup>+</sup> proA<sup>+</sup> episomes was carried out with N-methyl-N'-nitro-N-nitrosoguanidine (NMNG) (Aldrich Chemical Co.). The mutagenized episomes were then transferred into strain D431 or P92 recA for selection on minimal media plates containing 0.1% succinate as a carbon source, isopropyl-β-D-thiogalactoside (10<sup>-3</sup> M), 40 μg of NPTG per ml, and 20 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (BCIG; Sigma) per ml. The latter compound is not an inducer of the lac operon but is a substrate for β-galactosidase, and its hydrolysis yields a blue indigo dye that provides a good qualitative estimate of β-galactosidase concentrations within colonies on BCIG-containing plates.

Only the bacteria with low β-galactosidase specific activities (Z/B values, as described in reference 18) of less than 0.01 or with altered permeation of NPTG form sizable colonies after 60 h of incubation at 32°C. On this medium, both true (lacO<sup>+</sup>) revertants and lacZ<sup>-</sup> polar mutants appear white, but these two colony types can be distinguished by replication onto TTC-Lac agar (50 μg of triphenyltetrazolium chloride per ml, 1% lactose, tryptone broth), where the former are Lac<sup>+</sup> and the latter are Lac<sup>-</sup>. Likewise, both LacY<sup>-</sup> mutants and the desired pseudorevertants, which form light blue colonies, can be distinguished by replication onto TTC-Lac agar.

Mutagenesis was carried out in cultures grown in Davis minimal media with 0.1% lactose-thiamine to about 5 × 10<sup>8</sup> cells per ml. Cells were washed twice by centrifugation with 0.1 M potassium phosphate buffer (pH 6.2) and incubated in a shaker bath for 20 min at 37°C. NMNG dissolved in dimethyl formamide (10 mg/ml) was added to a final concentration of 100 μg/ml, and the mixture was shaken at 37°C for 20 min. Suspensions were then brought to 4°C, washed by centrifugation with 2 volumes of Penassay broth, and incubated at 37°C for 90 min prior to mating.

## RESULTS

Three hundred one mutant candidates that appeared light blue on NPTG-BCIG plates and were Lac<sup>+</sup> on TTC-Lac replica plates were isolated. Based on induced and uninduced  $Z/B$  values ( $Z/B_{max}$  and  $Z/B$ , respectively), all isolated mutants could be categorized into several functional groups. True revertants were characterized by basal and maximal  $Z/B$  values indistinguishable from those of the wild type. Some mutants were found to have no alteration in repression, in that the induced and uninduced  $Z/B$  values were reduced to the same degree; i.e., the ratios of uninduced to induced  $Z/B$  values (the  $P$  value) of the mutants were the same as the parental  $lacO^c$  strains. These promoter or promoter-like mutants are not discussed further here.

As Fig. 1 amply demonstrates, many "revertants" were obtained which lie either above or below the *iso-P* line (18) drawn through the parental  $lacO^c$   $Z/B$ / $Z/B_{max}$  point. Such "revertants" cannot represent simple promoter-like mutations (which by definition lie on the *iso-P* line), but must be alterations that change one of the variables,  $Z/B$  or  $Z/B_{max}$ , more than the other. The revertants lying above the diagonal must represent alterations restoring a measure

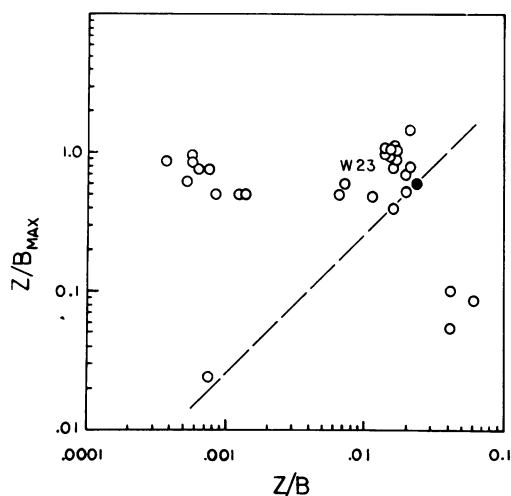


FIG. 1.  $\beta$ -Galactosidase activities of some mutants isolated from D533 ( $lacO^c$  class IIIb) on NPTG selector plates. The mutant episomes were assayed as merodiploids with P92. The assay was performed as defined in Fig. 2, except that the cultures were grown in tryptone broth rather than glycerol minimal medium. The solid circle indicates the locus of the  $Z/B$  and  $(Z/B)_{max}$  values of the parental  $lacO^c$ . The location of the one pseudorevertant, W23, from this set, which was investigated further, is also indicated. The dashed line is the *iso-P* line for the class III  $lacO^c$ .

of repression without effecting a proportionate reduction in the fully induced  $Z/B_{max}$  value, and the converse must be true for the "revertants" lying below the diagonal. Among the latter type, one would expect to recover classical  $lacI^s$  mutants in which the repressor affinity for inducers is reduced with little or no alteration in operator affinity (10). Pseudorevertants lying above the line could be explained a priori as alterations in either the repressor or the  $lacO^c$  operator. Such pseudorevertants were recovered in numbers approximately equal to those found for true revertants as well as promoter-like mutants (Table 2).

Twenty-one of the 43 pseudorevertants isolated from six different  $lacO^c$  strains were felt to be independent by  $\beta$ -galactosidase assay. Although the revertant frequency is not precisely quantifiable because of selection techniques, the relative frequencies of mutant types are consistent with the proposition that these pseudorevertants represent mainly single-step mutations from the parent  $lacI^+O^c$  strain. One caution is that NMNG can cause linked secondary mutations. However, pseudorevertants were also obtained at a similar frequency with Treffers mutator.

A small group of true revertants and pseudorevertants was selected for further characterization. Preliminary to this, the pseudorevertant-containing episomes were transferred into P92 *recA* (a *lac* deletion strain) and re-assayed for induced and uninduced  $\beta$ -galactosidase levels after growth in 0.1% glycerol minimal medium. (Fig. 2).

In interpreting this figure, an important longstanding observation is pertinent: namely, that a fully induced  $lacI^+O^+$  or  $lacI^+O^c$  strain is fully derepressed; i.e., it exhibits a  $Z/B_{max}$  value indistinguishable from that of an otherwise isogenic  $lacI^-$  strain (viz.  $lacI^-O^c$ ). This

TABLE 2. Distribution of 310 regulatory gene mutant candidates

Parental $lacO^c$	"Pseudorevertants" <sup>a</sup>	Promoter mutants	True revertants	Parental $lacO^c$	$lacI^s$
IIa(D531)	8	10	14	11	0
IIIa(D532)	13	8	3	19	0
IIIb(D533)	12	2	10	7	3
IVa(D534)	2	9	5	21	2
IVb(D535)	1	25	3	22	0
Va(D537)	0	2	0	25	0
Va(D536)	7	9	6	20	6

<sup>a</sup> The pseudorevertant candidates characterized after selection by induced and uninduced  $Z/B$  values are discussed in Results. Fifteen of the 310 candidates proved to be *lacZ* gene mutants.

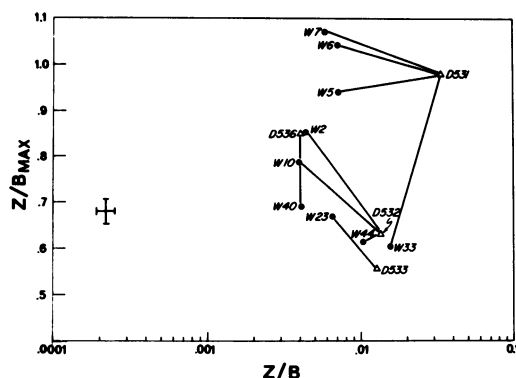


FIG. 2.  $\beta$ -Galactosidase specific activities of pseudorevertant and parent  $lacO^c$  episomes as merodiploids in the P92  $lac$  deletion strain. Colonies were picked from BCIG indicator agar and grown in  $D$  buffer plus 0.1% (vol/vol) glycerol with and without isopropyl- $\beta$ -D-thiogalactopyranoside ( $5 \times 10^{-4}$  M) for  $\beta$ -galactosidase activities and bacterial concentration determinations (17). Units of  $\beta$ -galactosidase specific activity ( $Z/B$ ) are as defined previously (17). Tie lines link the parental  $lacO^c$  to derived pseudorevertants. The crossed error bars to the left of the figure give the location of six true ( $lacI^+O^+Z^+$ ) revertants also assayed.

observation was recently systematically confirmed by comparison of the  $Z/B_{max}$  values obtained for a series of  $lacI^+O^cZ^+$  strains and the derived  $lacI^+O^cZ^+/F'lacI^+Z^-$  strains (J. Betz, personal communication). Together with the observation that many  $lacO^c$  mutations have "up-promoter" effects (18), this forces the conclusion that an increase seen in the  $Z/B_{max}$  for a pseudorevertant implies a mutational change in the operator rather than in the repressor. On this basis, pseudorevertants W2, W6, W7, W10, and W23 were initially considered altered in the operator. However, the pseudorevertants with reduced  $Z/B_{max}$  values cannot be treated so conclusively; pseudorevertants W5, W33, W40, and W44 could represent either a repressor or operator alteration.

To further test these preliminary conclusions, the effects of pseudorevertant-containing episomes on various operators located *trans* were assessed. In such a test, pseudorevertant episomes specifying tight-binding repressors with an altered affinity for certain  $lacO^c$  operators are expected to repress *trans*  $lacO^c$  operators in a manner different than that of the wild-type repressor. Conversely, episomes carrying secondary mutations in the operator will provide wild-type repressor. For this test, the pseudorevertant episomes were transferred into a set of  $F'lacI^+O^cZ^+ recA proA$  strains, and the  $\beta$ -galactosidase levels of the resulting merodip-

loids were compared with those found for the  $F'lacI^+O^+Z^+ proA^+B^+$  episomes in the same  $F'lacI^+O^c recA proA$  strains. In computing  $\beta$ -galactosidase synthesis from a chromosomal  $lacI^+O^cZ^+$  segment, it was assumed that the enzyme production by an episomal lactose operon in the merodiploid was proportional to that in the  $lac$  deletion strain P92  $recA$ . This assumption was found to be correct for the parental  $lacI^+O^cZ^+$  episomes. From the results obtained (Table 3) with the various pseudorevertants, it may be concluded that: (i) W2, W7, and W10 are best understood as having operator alterations; viz., the chromosomal contributions to the  $\beta$ -galactosidase activities are the same as those obtained with the  $lacI^+O^+$  episome. (ii) W33 and W44 are examples of strains having altered repressors; viz., the chromosomal contributions to  $\beta$ -galactosidase activities differ greatly from those found with the  $lacI^+O^+Z^-$  episome in several cases. In particular, W33 represses two of the highest  $lacO^c$  classes very well, whereas W44 represses the two lowest  $lacO^c$  types very poorly. (iii) W5, W6, and W23 cannot be categorized conclusively from these data. Although, in general, they show much the same behavior in the  $lacI^+O^c$  set as do W2, W7, and W10, there are anomalies (Table 3). As noted earlier, W6 and W23 probably contain operator alterations because of their increased  $Z/B_{max}$  values. (iv) W40 has a  $lacI^F$  repressor mutation that depresses all  $Z/B_{max}$  levels in a constant fashion (data not shown). This effect is seen in Fig. 2.

To confirm the notion that W33 is an altered repressor pseudorevertant and to better appreciate some of its altered operator-binding properties, *in vitro* repressor-operator binding studies (lifetime kinetics) were performed for  $\phi 33$  (W33 on the  $\lambda h80dlac$  prophage) and  $\phi 41$  (wild-type) repressor extracts against  $^{32}P$ -labeled  $\lambda h80dlacO^+$  DNA by the nitrocellulose filter assay method of Bourgeois and collaborators (2, 3).

The W33 repressor displayed kinetics of release from the  $lacO^+$  operator distinctly different from those of the wild-type repressor (Fig. 3). Such biphasic kinetics suggest that the W33 repressor exists in two states with distinctly different binding characteristics and that these forms (when operator bound) are not interconvertible or are sluggishly interconverted. More recently, another altered repressor has been discovered which shows similar biphasic release kinetics (J. Betz and J. Sadler, submitted for publication). In this case, the mutant was isolated from the over-producing, or  $lacI^q$ , allele, and hence it should be possible to purify the repressor in sufficient amounts to

TABLE 3. *Trans-dominant tests on pseudorevertants*

<i>lacI</i> <sup>-</sup> <i>O</i> <sup>c</sup> <i>Z</i> <sup>+</sup> strain <sup>a</sup>	Chromosomal <i>Z/B</i> contributions <sup>b</sup> of <i>lacI</i> <sup>-</sup> <i>O</i> <sup>c</sup> and pseudorevertant episome								
	<i>lacI</i> <sup>-</sup> <i>O</i> <sup>c</sup> <i>Z</i> <sup>-</sup>	W2	W5	W6	W7	W10	W23	W33	W44
69 77	0.0017	0.0018	0.0025	0.00082	0.0017	0.0021	0.0046	0.0022	0.0040
63 81	0.0041	0.0052	0.0045	0.0038	0.0042	0.0045	0.0082	0.0056	0.0083
96 99 71	0.0057	0.0067	0.0062	0.0054	0.0060	0.0061	0.013	0.0067	0.0074
42 94	0.016	0.016	0.015	0.014	0.014	0.014	0.015	0.0085	0.014
95	0.015	0.019	0.0040	0.015	0.012	0.015	0.023	0.019	0.018
67 104 108	0.044	0.047	0.046	0.039	0.038	0.043	0.045	0.027	0.46

<sup>a</sup> The isolation and classification of *F*<sup>-</sup>*lacI*<sup>-</sup>*O*<sup>c</sup>*Z*<sup>+</sup> mutants will be described elsewhere (Betz and Sadler, in preparation); *lacO*<sup>c</sup> strains are grouped according to similar *Z/B* and *Z/B*<sub>max</sub> values of *lacI*<sup>-</sup>*O*<sup>c</sup>*Z*<sup>+</sup>/*F*<sup>-</sup>*lacI*<sup>-</sup>*O*<sup>c</sup>*Z*<sup>-</sup>.

<sup>b</sup> Chromosomal *Z/B* contributions were computed as *Z/B* of the merodiploid (*lacI*<sup>-</sup>*O*<sup>c</sup>*Z*<sup>+</sup>/*F*<sup>-</sup> *lac* pseudorevertant) minus the *Z/B* of the merodiploids (P92/*F*<sup>-</sup> *lac* pseudorevertant), where P92 is a *lac* deletion strain. The average standard deviation of duplicate assays was  $\pm 7\%$  of the mean *Z/B* value.

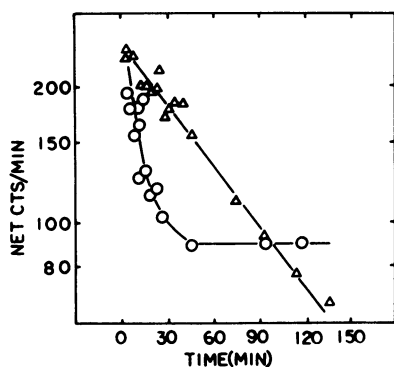


FIG. 3. Dissociation kinetics of  $\phi 41$  ( $\Delta$ ) and  $\phi 33$  ( $\circ$ ) repressor-*lacO*<sup>+</sup> operator complexes. The *lacO*<sup>+</sup> DNA was derived from strain RV/80. The experiments were performed as described in the footnotes of Table 3, except that partially purified preparations of the repressors were employed (2). Net counts per minute bound were obtained by subtracting the counts filter bound in the presence of isopropyl- $\beta$ -D-thiogalactopyranoside ( $10^{-3}$  M) on parallel reaction mixtures.

permit extensive in vitro characterization.

The fact that roughly 60% of the W33 repressor has a shorter half-life (about 20 min) on the *lacO*<sup>+</sup> operator than the wild-type repressor (about 73 min) is in accord with the in vivo result that the W33 allele represses the *lacO*<sup>+</sup> operator less well than wild-type repressor.

Similarly, lifetime kinetics of W6, W7, W2, and W10 <sup>32</sup>P-labeled  $\lambda h80d\text{lac}$  DNAs against *lacI*<sup>qx86</sup> repressor were measured. The results (Table 4) indicate that these "operator" pseudorevertants are distinctly different from their parental operators by the in vitro test.

## DISCUSSION

In the present study we obtained a set of secondary mutations that offset, or partially compensate for, the constitutive effects of *lacO*<sup>c</sup> operator mutations; some of these mutations clearly result in altered repressors, whereas others seem to represent secondary mutations in the *lacO*<sup>c</sup> operator itself. With regard to the pseudorevertants that act *cis* only, these could represent either secondary mutations in the operator itself or mutations in the second repressor binding site recently discovered in the *lacZ* gene (16). In either case, they should prove useful in the understanding of the repressor-DNA interaction.

If they are in the true operator, the mutations resulting in *cis*-dominant pseudorevertants could represent a base-pair change either at the original *lacO*<sup>c</sup> site or somewhere else in the operator. In their original isolation and mapping of a large number of *lacO*<sup>c</sup> strains, Sadler and Smith (17) found that only one *LacO*<sup>c</sup> phenotype mapped at each *lacO*<sup>c</sup> locus,

TABLE 4. Affinity of QX86 repressor for pseudorevertant and parental lacO<sup>c</sup> operator DNA<sup>a</sup>

Operator	Half-life (min)
RV/1 (class IIa lacO <sup>c</sup> ; parent of W6 and W7)	8.0
W6	14.2
W7	14.0
RV/116 (class IIIa lacO <sup>c</sup> ; parent of W2, W10)	6.1
W2	16.1
W10	21.6

<sup>a</sup> Purified lacI<sup>qx86</sup> repressor at approximately 10<sup>-12</sup> M was allowed to bind to 10<sup>-12</sup> M <sup>32</sup>P-labeled operator DNA in binding buffer (10) (I = 0.05 M) containing 3 μg of chicken blood DNA per ml. The dissociation of the labeled operator and repressor was measured from the time of addition of a 100-fold excess of unlabeled λh80dlacO<sup>+</sup> DNA to the reaction mixture, and the time elapsed until 50% of labeled operator remained associated with repressor was defined as half-life (11). The pseudorevertants were transferred to the λh80cI857St68dlac prophage as described elsewhere (11) for operator DNA purification.

whereas one might have expected three physiologically distinct ones corresponding to the three possible base substitutions at each particular locus. It is possible that some of the *cis*-dominant pseudorevertants represent one of the other possible base substitutions at the original lacO<sup>c</sup> site. If this is the case, however, it is not clear why the *cis*-dominant pseudorevertants isolated here were conspicuously absent in the original lacO<sup>c</sup> search, since these pseudorevertants are not excluded by the selection techniques employed by Smith and Sadler (18). In addition, the original mutations of the class IIa and class IIIa O<sup>c</sup> operators (D531 and D532) are now known to be base pair transitions of guanine·cytosine to adenine·thymine (7). Since mutagenesis to obtain pseudorevertants was by nitrosoguanidine, which causes mutations almost exclusively at guanine·cytosine base pairs (13), it is particularly unlikely that the *cis*-dominant pseudorevertants derived from strains D531 and D532 (W6 and W7, W2, and W10) represent secondary mutations at the original lacO<sup>c</sup> site.

The intriguing possibility that compensating mutations occur at a second site in the operator raises the issue of how such second-site compensation could operate. The lac operator contains a sequence of 21 base pairs which has twofold rotational symmetry (8). This symmetry, however, is imperfect, and an analysis of binding characteristics of lacO<sup>c</sup> operators suggests that repressor-operator interaction is stronger in the

promoter-proximal half of the operator than it is in the distal half (11). If operator interaction with native tetrameric repressor is polymodal (i.e., more than one repressor subunit binds the nearly symmetrical region), mutations in the promoter-distal half of the operator that result in improved symmetry could create a super operator (lacO<sup>s</sup>). A search for such a mutation has been made by Pfahl and Bourgeois, starting with a class IIa lacO<sup>c</sup> (14). They report that the most likely candidate for the lacO2<sup>cs</sup> pseudorevertant resembles the wild type phenotypically. Mutants similar to the wild type *in vivo* were recovered in our selection from several classes of lacO<sup>c</sup> strains but were not analyzed *in vitro*, where Pfahl and Bourgeois noted a two- to threefold difference in operator-repressor interaction of the lacO<sup>cs</sup> candidate compared with lacO<sup>+</sup>. Certainly the *cis*-dominant pseudorevertants reported here are distinctly different from both the wild type and the original operator-constitutive parent.

Aside from changes in primary structure of the operator DNA, a secondary change in operator structure could be a determinant of operator-repressor interaction. The twofold symmetry of the operator allows for a possible secondary hairpin structure of the operator region of DNA, originally proposed by Gierer (6), which would be stabilized by intrastrand base-pair matching and base stacking at the turns. Another type of secondary structural change, envisioned by Crick and Klug (4), is sequential kinking of the DNA within the operator region, forming a zig-zag pattern. Indeed, it has been shown that some secondary operator structural change does take place when the repressor binds to the operator site, resulting in unwinding of the DNA helix through a small angle of 40 to 90° (19). Although this degree of unwinding is not that expected for the Gierer-loop formation, any base-pair substitution that stabilizes the secondary structure of the bound form of operator DNA could partially compensate of lacO<sup>c</sup> mutations.

The lac pseudorevertant-containing episomes that are *trans* dominant are expected to be lacI gene mutants. The lacI<sup>s</sup> mutation was recovered by a selection technique developed for this study, and its repressor suppresses Z/B<sub>max</sub> levels of all lacO<sup>c</sup> strains. Some of the repressor pseudorevertants (*trans* dominants) obtained compensate for some lac operator sequences (lacO<sup>c</sup> and lacO<sup>+</sup>) in a more-or-less specific manner. For example, the W33 repressor represses a high lacO<sup>c</sup> class (probably class IIa) operator better than the wild-type repressor does, but is unable to repress the wild-type operator even as

well as it can the class IVa *lacO<sup>c</sup>* operator (data not shown). An analogous pattern of specificity for the W44 repressor is also seen in the data of Table 3.

These results are only partially consistent with the view that repressor-operator binding is composed of a series of specific, point-by-point interactions. The alteration in the W33 repressor that accommodates it to the changed sequence in the class IIa *lacO<sup>c</sup>* operator also defacilitates its binding to the *lacO<sup>+</sup>* operator sequence. This is still consistent with a model of point-by-point interactions (considering the operator symmetry to be imperfect); however, on such a model, it is not at all clear why the W33 repressor also binds a class IV *lacO<sup>c</sup>* operator better than the wild type, since the class IV and class IIa *lacO<sup>c</sup>* sites are at different locations in the operator (7). It will be of interest to map this unusual repressor mutant (W33), since a current model proposes that the specific operator binding site of the repressor is limited to the first 50 or 60 amino acid residues of the repressor protein (13).

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