

Mutations caused by the Insertion of Genetic Material into the Galactose Operon of *Escherichia coli*

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(Received 1 August 1968, and in revised form 25 October 1968)

Density-gradient analysis shows that λ transducing phages which carry spontaneous extreme polar mutations of the galactose operon have higher buoyant densities than otherwise identical phage which carry a wild-type galactose operon. Control experiments show that the density increases are not artifacts of the techniques used to isolate mutant transducing phages and that removal of the spontaneous extreme polar mutations by reversion or recombination leads to a loss of buoyant density. The simplest explanation for these results is that the mutations analysed are the consequences of the linear insertion of foreign DNA into the *E. coli* galactose operon. The insertion hypothesis provides an explanation for the strong polarity and unusual genetic properties of spontaneous extreme polar mutations.

1. Introduction

We have identified a class of spontaneous extreme polar mutations of the galactose operon which do not appear to be the results of base substitutions, frameshifts, or extended deletions (Adhya & Shapiro, 1969; Shapiro & Adhya, 1969). These mutations arose from a forward selection for *gal*⁻ mutations based on the fact that certain mutants of *Escherichia coli* cannot grow in the presence of galactose (Adhya & Shapiro, 1969). Similar mutations of the lactose and galactose operons have been isolated by others in analogous selection experiments (Malamy, 1966; Saedler & Starlinger, 1967).

To explain the genetic peculiarities and strong polarity of these spontaneous mutations, we proposed that they are the consequences of the random insertion of large pieces of foreign DNA into a structural gene (Shapiro & Adhya, 1969). The reasoning behind this hypothesis is as follows.

Polarity. Because we postulate random insertion, the probability of the inserted fragment's being out of the proper reading frame or even inverted from its normal orientation is high. Thus, a ribosome travelling along a messenger RNA molecule transcribed from an operon containing a fragment of foreign DNA will almost certainly encounter a nonsense codon soon after passing the site of insertion. If the inserted fragment is sufficiently large and contains no chain initiation signals in the proper reading frame, a strong polar effect will result. On the simplest model, the strength of the polarity will increase with the size of the inserted fragment (cf. Newton, Beckwith, Zipser & Brenner, 1965; Newton, 1966).

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Genetic properties. Clearly insertion mutations will not be suppressible by extragenic or intragenic suppressors. Those spontaneous extreme polar mutations which do revert can do so in one of two ways: (1) by deletion of the inserted fragment; or (2) by recombination ("selfing") with other regions of the bacterial chromosome homologous to the mutated locus (cf. Clark, 1964). Neither of these mechanisms should be greatly influenced by base-analogue or frameshift mutagens. Hence our negative results on mutagen-induced reversion (Adhya & Shapiro, 1969).

The fact that many presumed insertion mutations do not revert at all is to be expected if the insertion process resembles the integration of known episomes into structural genes: integration of a temperature-sensitive *F-lac* into various sites on the bacterial chromosome results in many non-reverting mutations (Beckwith, Signer & Epstein, 1966). Providing that the insertion process does not destroy large regions of the mutated gene, insertion mutations should behave as point mutations in genetic crosses (possibly displaying strong marker effects on quantitative results).

The insertion hypothesis predicts that mutant operons will have a higher DNA content than the parent wild-type operon. The observation that each λdg transducing phage has a characteristic buoyant density in CsCl solution depending on its DNA content made it possible to test this prediction (Weigle, Meselson & Paigen, 1959; Kayajanian & Campbell, 1966). λdg^- phages carrying various spontaneous extreme polar mutations were isolated from a wild-type λdg^+ phage of known density by homogenote selection (Morse, Lederberg & Lederberg, 1956). The densities of these λdg^- phages were then compared with that of the original λdg^+ phage. If a mutant operon contains added DNA, then the corresponding λdg^- phage should be found in a denser region of the gradient than the λdg^+ phage. Four independent spontaneous extreme polar *kt*⁻ mutations of the *galT* gene were chosen for study: S101, S104, S114 and S188 (Adhya & Shapiro, 1969). The positions of these four mutations in the *galT* gene are shown in italic characters in Figure 1. The experiments presented below

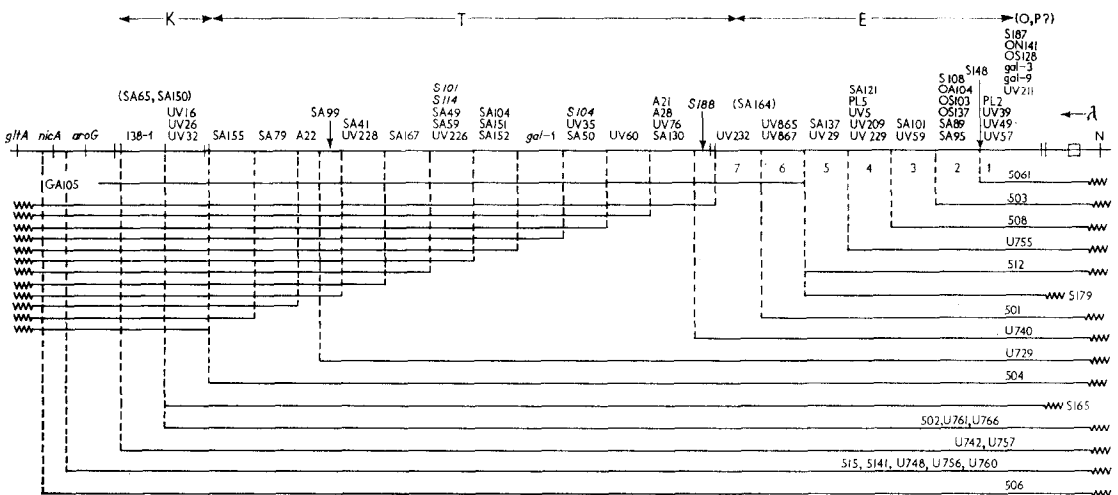


FIG. 1. A deletion map of the *E. coli* galactose operon taken from Shapiro & Adhya (1969).

show unambiguously that the presence of each of these four mutations on the DNA of a λdg phage results in an increase of that phage's buoyant density.

2. Materials and Methods

(a) *Bacteria and bacteriophages*

All of the *E. coli* K12 strains used are gal^- . The origins of all but one of the gal^- mutations are described in Adhya & Shapiro (1969). The map position of all the mutations except UV12 are shown in Fig. 1.

Strains MS71, MS72, MS73 and MS80 carry, respectively, the S101, S104, S114 and S188 kt^- spontaneous extreme polar mutations. Strains H57 and MS91 carry, respectively, the polar UV57 e^- amber mutation and the UV16 k^- point mutation. These six strains were used to prepare various λdg^- phages by homogenote selection as described below.

Strain MS2 carries the PL5 non-polar e^- mutation of Buttin (1963), strain MS8 the UV12 non-polar t^- mutation, strain MS132 the UV16 k^- mutation and the su^+_{III} amber suppressor, and strain S165 the S165 gal^- deletion which covers all the other mutations. These strains were used as transduction recipients for the differential assays of various λdg phages.

Strain MS2 was used as indicator strain for active λ^{857} phage in plaque assays.

λ^{857} (Sussman & Jacob, 1962), λc_{Tsu34} , and λvir were obtained from the collection of F. Jacob. A λ^{857} lysogen was made from a derivative of Hfr H carrying a wild-type galactose operon. This lysogen was induced to provide an LFT \dagger lysate. λ^{857} has a mutant, temperature-sensitive repressor; hence, strains lysogenic for λ^{857} can be induced by a simple temperature shift from 30°C to 42°C.

(b) *Media*

These have already been described (Adhya & Shapiro, 1969).

(c) *Isolation of $\lambda^{857}dg^+XV$ by the "orgy" technique*

An LFT lysate of λ^{857} was adsorbed to strain MS91 at high multiplicity (m.o.i. ≥ 10), and the infected bacteria plated on M9-galactose agar. After 72 hr at 30°C, approximately 500 gal^+ transductant clones appeared on each plate. These would virtually all be double lysogens for active λ^{857} and various $\lambda^{857}dg^+$ phages. Approximately 1500 transductant clones were resuspended *en masse* in L-broth and induced to yield an HFT orgy lysate containing several hundred particles per ml. of many different λdg^+ phages. This HFT lysate was centrifuged to equilibrium in CsCl. Fractions of this gradient were assayed for transducing activity on strain MS91. A gal^+ transductant clone arising from the assay of a light fraction was purified and induced to yield an HFT lysate of $\lambda^{857}dg^+XV$, hereafter referred to as λdg^+ .

(d) *Selection of λdg homogenotes*

Cultures of various gal^- strains were infected at low multiplicity (m.o.i. $< 10^{-2}$) with an HFT lysate of λdg^+ and plated on M9-galactose agar. Transduction at low multiplicity ensured that all transductants would carry only λdg^+ as prophage. After 72 hr at 30°C, transductant clones were repurified on galactose-tetrazolium-agar, and from each transduction a gal^+ clone which segregated red gal^- colonies was grown to saturation in L-broth at 25°C. These cultures were streaked on galactose-tetrazolium-agar previously spread with 10^{10} particles of λc_{Tsu34} to counter-select non-immune segregants. Red gal^- segregants were repurified and tested for λ immunity (by cross-streaking against λc_{Tsu34} and λvir) and for production of active phage. Those gal^- strains which proved to be defective lysogens were homogenotes carrying λdg^- prophages containing the appropriate gal^- mutation. The nature of the gal^- mutation on each prophage was verified in all cases by testing HFT lysates obtained after superinfection for recombination and complementation with suitable gal^- strains. We call a derivative of λdg^+ carrying the S101 mutation λdg^-_{S101} , and so forth.

\dagger Abbreviations used: LFT, low-frequency transducing; HFT, high-frequency transducing; m.o.i., multiplicity of infection.

(e) *Production of HFT lysates by superinfection of defective lysogens*

Defective lysogens for various λdg phages were grown at 30°C to approximately 10^9 cells/ml. in M9-maltose medium supplemented with 0.4% Casamino acids. The bacteria were resuspended in 0.01 M-MgSO₄ and a lysate of λ^{857} was adsorbed at a multiplicity of 5 for 20 min at 30°C. The adsorption mixture was diluted into twice the volume of L-broth, incubated 20 min at 42°C, and then incubated at 37°C with vigorous aeration until lysis. The HFT lysates obtained in this way never contained more than 10% transducing phages.

For the vegetative phage crosses between λdg^{-}_{S114} and the λdg^{-}_{UV16} and λdg^{-}_{UV57} phages, an HFT lysate of λdg^{-}_{S114} and λ^{857} was used to infect defective lysogens for the other λdg^{-} phages. In these crosses, adsorption was carried out at 42°C.

(f) *Isolation of λdg^{+} revertants of λdg^{-}_{S104} and λdg^{-}_{S188}*

The λdg^{+} revertant phage in HFT lysates of λdg^{-}_{S104} and λdg^{-}_{S188} were isolated by transduction of strain S165 (see Table 1). Transduction was performed at m.o.i. ~ 5 to ensure that practically every transductant obtained was lysogenic for both λ^{857} and a revertant λdg^{+} . The *gal*⁺ transductant clones from each experiment (32 from the transduction with λdg^{-}_{S104} , 136 from the transduction with λdg^{-}_{S188}) were then mixed together and induced to provide two HFT lysates containing several thousand copies of each of the revertant λdg^{+} phages isolated. This amplification procedure should not have altered the densities of the λdg^{+} revertant phages (Weigle, 1961).

(g) *Preparative density-gradient centrifugation*

In general, preparative centrifugation was performed as described by Weigle *et al.* (1959). 3-ml. gradients were centrifuged in the SW39 or SW50L swinging-bucket rotor at 22,000 rev./min for 48 hr at 10°C and collected in 1-drop fractions (except for the experiment presented in Fig. 2, in which 2-drop fractions were collected). 10-ml. gradients were run in the SW40 fixed-angle rotor at 25,000 rev./min for 48 hr at 10°C and collected in 3-drop fractions. Phage suspensions with a density of approximately 1.50 g cm⁻³ were prepared either by mixing phage lysates with an equal volume of a saturated solution of CsCl in 0.01 M-Tris buffer (pH 7) or by dissolving the appropriate mass of CsCl in a given volume of phage lysate. L-broth was used as the buffer for phage lysates. Various Spinco model L centrifuges were used.

(h) *Assay of transducing phages*

Each fraction to be assayed was diluted appropriately in M9 buffer. A 0.1-ml. sample of the dilution was then added to approximately 0.2 ml. of a lysate of λ^{857} helper phage grown lytically on strain MS2. (The helper phage lysate contained more than 10^{10} active phage/ml. and no transducing phage.) To this mixture was added approximately 0.2 ml. of the appropriate *gal*⁻ indicator strain resuspended in 0.01 M-MgSO₄ at approximately 2×10^9 cells/ml. Adsorption was carried out for 30 min at 30°C. The adsorption mixture was stored overnight at 4°C, and then all of it was plated on M9-galactose-agar. Plates were scored for transductant clones after 72 hr at 30°C. Because the entire mixture was plated, the volumes of helper phage and indicator strain used could vary within a factor of 2 without affecting the final result.

Differential assays of more than one λdg phage in a single gradient were based on the transduction specificities summarized in Table 1. λdg^{+} could be assayed uniquely on the *gal* deletion strain S165 because none of the λdg^{-} phages can transduce this strain to *gal*⁺. λdg^{+} transduces S165 by complementation (i.e. by lysogenization). Because λdg^{+} phages were never in excess, λdg^{-} phages carrying the UV16 *k*⁻ marker and the four *kt*⁻ mutations could be assayed on the *e*⁻ strain MS2 which they all transduce by complementation. In the experiment presented in Fig. 6(a), λdg^{-}_{UV16} could be distinguished from λdg^{-}_{S114} by assaying on the *t*⁻ strain MS8: λdg^{-}_{UV16} transduces MS8 efficiently by complementation, while λdg^{-}_{S114} transduces MS8 at a negligible frequency by recombination. In the experiment presented in Fig. 6(b), λdg^{-}_{UV57} could be distinguished from λdg^{-}_{S114} by assaying on the *k*⁻*su*⁺ strain MS132 because the efficiency of transduction by λdg^{-}_{UV57}

is at least tenfold higher than by λdg^-_{S114} . λdg^-_{S114} transduces MS132 only by recombination; λdg^-_{UV57} transduces MS132 by weak complementation and recombination because (i) the su^+_{III} suppressor will suppress the polarity of the UV57 amber mutation and partially restore kinase gene activity to the transducing phage and (ii) the distance between the UV57 and UV16 sites is sufficiently large to permit frequent recombination (Fig. 1). (Note that the su^+_{III} suppressor does not suppress the UV57 mutation to gal^+ : Adhya & Shapiro, 1969.)

TABLE I

Transduction specificities of the different λdg phages for various recipient strains

Recipient strain:	S165 (Δgal)	MS2 (e^-)	MS8 (t^-)	MS132 ($k^- su^+$)
λdg :				
λdg^+	+	+	+	+
λdg^-_{UV16}	—	+	+	
λdg^-_{UV57}	—	—	—	+
λdg^-_{S101}	}	+		
λdg^-_{S104}				
λdg^-_{S188}				
λdg^-_{S114}				

The symbols indicate the probability that infection of a given strain by a λdg particle (in the presence of excess helper phage) will yield a gal^+ transductant. (+): $\geq 10^{-1}$; (—): $< 10^{-3}$; (weak +): $\sim 10^{-2}$.

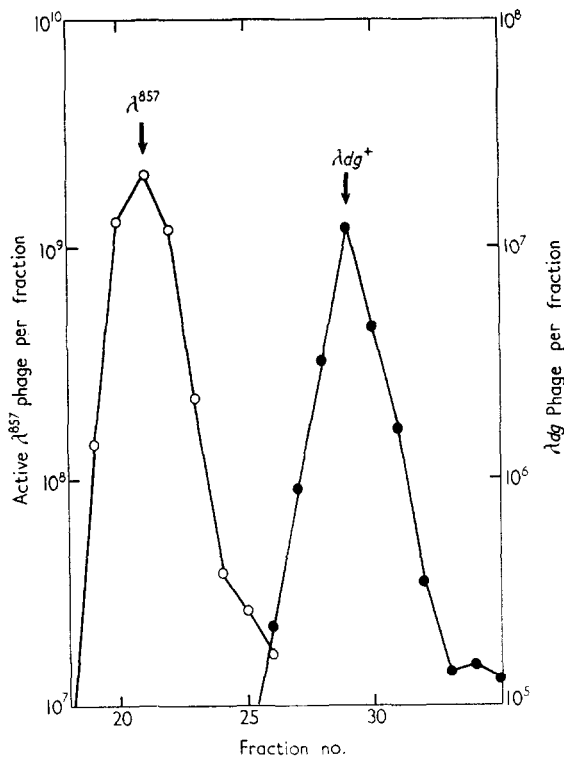


FIG. 2. CsCl density-gradient profile of an HFT lysate of λdg^+ . 22,000 rev./min, SW50L rotor, miniscus at fraction 54.

—○—○—, Plaque-forming units; —●—●—, transducing phage assayed on strain S165.

3. Results

(a) Isolation of λdg^+

An HFT lysate of a λdg^+ phage with a low buoyant density derived from λ^{857} was obtained as described in Materials and Methods. The equilibrium CsCl density-gradient profile of this lysate is shown in Figure 2. Both the active λ^{857} and λdg^+ phages form single bands. The distance between the two peaks is approximately 15% of the total gradient. Knowing the geometry and speed of the rotor and the mean density of the gradient, the density difference ($\Delta\rho$) between λ^{857} and λdg^+ can be calculated. This has been done for several gradients, and the results vary between $\Delta\rho = 0.012$ and $\Delta\rho = 0.014 \text{ g cm}^{-3}$. This corresponds to a DNA difference between λ^{857} and λdg^+ of between 0.12 and 0.14 λ equivalents (Weigle *et al.*, 1959) or at least 6,000 base pairs (Caro, 1965).

(b) Homogenote formation using a gal^- point mutation

A λdg^- phage carrying the UV16 k^- point mutation was isolated by homogenote selection as described in Materials and Methods. An HFT lysate of this λdg^-_{UV16} phage was prepared by superinfection and mixed with an HFT lysate of the parental λdg^+ phage so that the λdg^-_{UV16} particles were in excess. The mixture of lysates was centrifuged in CsCl to equilibrium and differentially assayed for active λ^{857} , λdg^+ and λdg^-_{UV16} phages. The results are shown in Figure 3. There is no significant difference

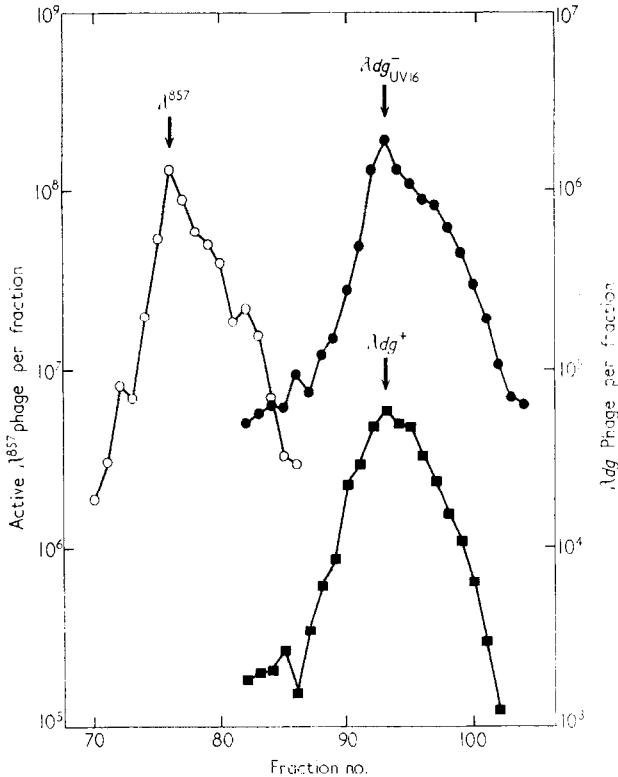


FIG. 3. A mixture of HFT lysates of λdg^-_{UV16} and λdg^+ . 22,000 rev./min, SW39 rotor, miniscus at fraction 109.

—○—○—, Plaque-forming units; —●—●—, transducing phage assayed on strain MS2; —■—■—, transducing phage assayed on strain S165.

between the buoyant densities of the two transducing phages. Thus, the process of homogenote formation does not *per se* cause any change in the density of a transducing phage. This observation was previously made by Weigle *et al.* (1959).

(c) *Homogenote formation using spontaneous extreme polar mutations*

λdg^- phages carrying the four spontaneous extreme polar kt^- mutations were also prepared by homogenote selection, and the experiment described in Figure 3 was repeated for each of the four phages. The results are shown in Figure 4. In each case the peak of λdg^- phage is closer to the λ^{857} peak than is the peak of λdg^+ phage. In those gradients where the ratio of λdg^- to λdg^+ particles was relatively low (Fig. 4(b), (c) and (e)), assay of transducing phage on strain MS2 gave a bimodal distribution with one of the peaks corresponding to the λdg^+ phage. The slight shoulder on the λdg^-_{S101} peak in Figure 4(a) is also due to λdg^+ phages. Comparison of Figure 4(e) with Figure 4(c) shows that essentially the same results are obtained in the fixed-angle and swinging-bucket rotors. In contrast with the previous experiment, these results show that formation of λdg^- phages carrying these four spontaneous kt^- mutations results in an increase of buoyant density.

(d) *λdg^+ revertants of λdg^- phages carrying the S104 and S188 mutations*

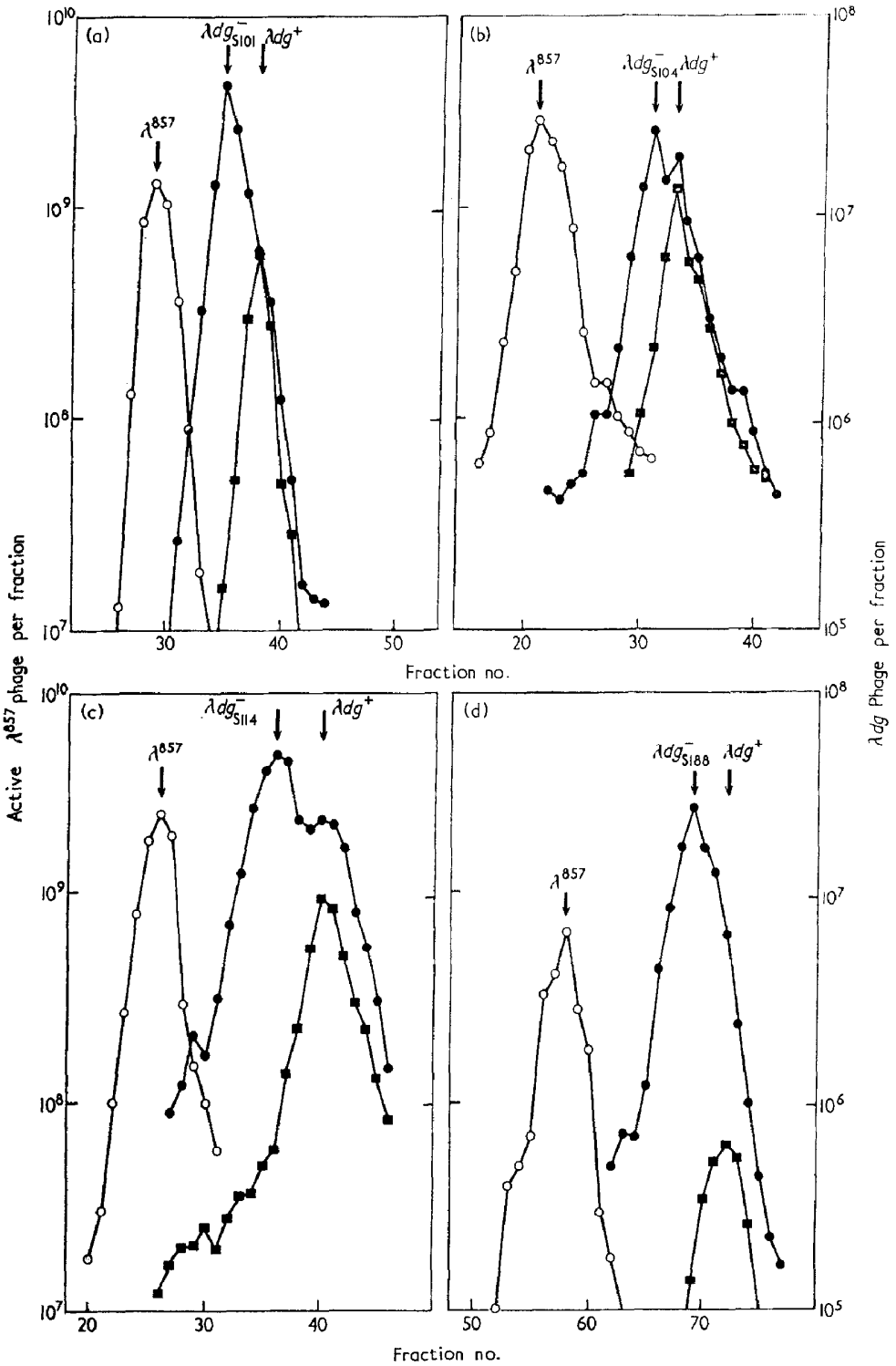
Despite the control experiment presented in Figure 3, the possibility remained that the density increases associated with isolation of λdg^- phages carrying the spontaneous kt^- mutations were somehow artifacts of the homogenote selection procedure. Hence it was necessary to demonstrate that the density differences were specifically associated with the presence of the gal^- mutation on the λdg phage. One way to do this was to isolate λdg^+ revertants of the λdg^- phages and show that the λdg^+ phages have a lower density than the parental λdg^- phages.

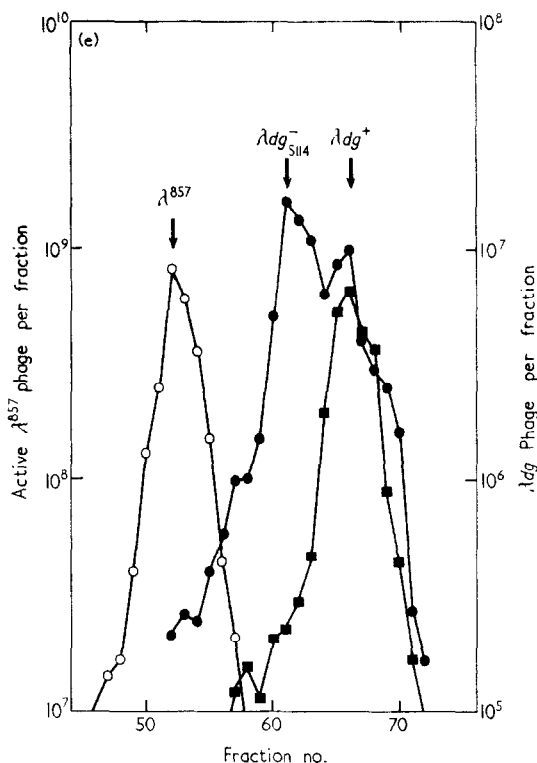
Accordingly, the revertant λdg^+ phages in HFT lysates of λdg^-_{S104} and λdg^-_{S188} were isolated as described in Materials and Methods. † HFT lysates of these revertant λdg^+ phages were centrifuged to equilibrium together with lysates of the parental λdg^- phages and assayed differentially. The results are presented in Figure 5. It can be seen that reversion of both λdg^-_{S104} and λdg^-_{S188} to λdg^+ entails a loss of buoyant density. The positions of the revertant λdg^+ peaks suggest that these phages are the same as the original λdg^+ phage.

(e) *Segregation of the density difference associated with the S114 mutation in vegetative crosses*

A second method of demonstrating the specific association between the increased density and gal^- character of λdg^- phages carrying the spontaneous kt^- mutations was to show that both properties segregate together. For this purpose, vegetative crosses between λdg^-_{S114} and λdg^- phages carrying the UV16 k^- and UV57e e^- amber markers were performed as described in Materials and Methods. Because these two gal^- markers are located on either side of the S114 mutation (Fig. 1), a density marker outside the galactose operon should be detectable among the λdg^+ recombinants in at least one of the two crosses. The density-gradient analyses of the lysates

† This experiment could not be done with the λdg^-_{S101} or λdg^-_{S114} phages because neither the S101 or S114 mutations ever reverts (Adhya & Shapiro, 1969).





arising from these crosses are shown in Figure 6. A schematic representation of each cross is given in the upper left-hand corner. In both crosses the λdg^+ recombinant phages have a lower buoyant density than the λdg^-_{S114} phage. Moreover, the coincidence of the λdg^+ peak with the λdg^-_{UV16} peak in Figure 6(a) and with the λdg^-_{UV57} peak in Figure 6(b) demonstrates that the recombinant λdg^+ phages are identical to the original λdg^+ . The shoulder on the λdg^-_{UV57} peak in Figure 6(b) is due to recombinational transduction of strain MS132 by λdg^-_{S114} . Thus, the higher density of the λdg^-_{S114} phage segregates rigorously with the S114 mutation and is not due to a change in either the right- or left-hand end of the transducing phage genome.

Comparable results were obtained in marker-rescue experiments in which an HFT lysate of λdg^-_{S114} was used to infect non-lysogenic strains carrying *gal*⁻ markers on either side of the S114 mutation.

FIG. 4. Density-gradient analyses of spontaneous *kt*⁻ mutations.

(a) A mixture of HFT lysates of λdg^-_{S101} and λdg^+ . 22,000 rev./min, SW50L rotor, miniscus at fraction 67.

(b) A mixture of HFT lysates of λdg^-_{S104} and λdg^+ . 22,000 rev./min, SW50L rotor, miniscus at fraction 90. Phage titres multiplied by 10.

(c) A mixture of HFT lysates of λdg^-_{S114} and λdg^+ . 22,000 rev./min, SW50L rotor, miniscus at fraction 103. Active phage titre multiplied by 10, transducing phage titre multiplied by 100.

(d) A mixture of HFT lysates of λdg^-_{S188} and λdg^+ . 25,000 rev./min, SW40 rotor, miniscus at fraction 88.

(e) A mixture of HFT lysates of λdg^-_{S114} and λdg^+ 25,000 rev./min, SW40 rotor, miniscus at fraction 87.

—○—○—, Plaque-forming units; —●—●—, transducing phage assayed on strain MS2; —■—■—, transducing phage assayed on strain S165.

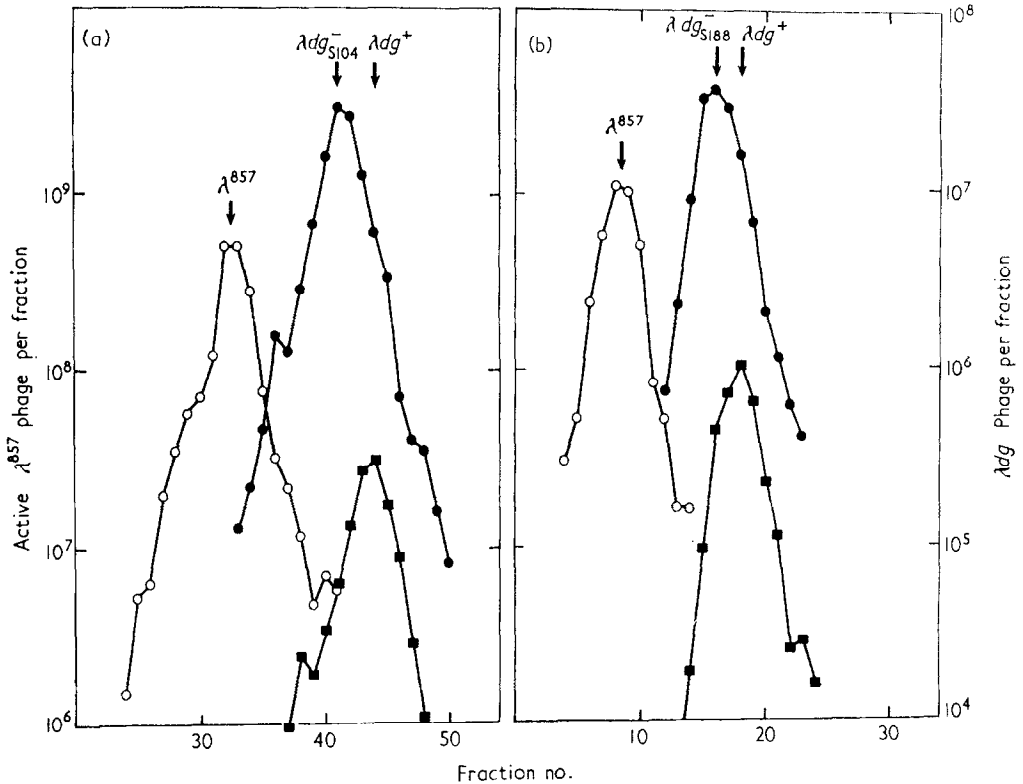


FIG. 5. Density-gradient analyses of revertant galactose operons.

(a) A mixture of HFT lysates of λdg^-_{S104} and λdg^+ revertant phages isolated by transduction of strain S165. 25,000 rev./min, SW40 rotor, miniscus at fraction 70.

(b) A mixture of HFT lysates of λdg^-_{S188} and λdg^+ revertant phages isolated by transduction of strain S165. 22,000 rev./min, SW39 rotor, miniscus at fraction 59.

—○—○—, Plaque-forming units; —●—●—, transducing phage assayed on strain MS2; —■—■—, transducing phage assayed on strain S165.

(f) Sizes of the various mutations

From the data presented above, estimates can be made of the amount of inserted material in each mutation. Assuming a difference of 6×10^3 base pairs between the λ^{857} and λdg^+ genomes (see above), linear interpolations based on the positions of λ^{857} , λdg^- and λdg^+ peaks in different experiments give the values in Table 2. At least for the S114 mutation, the observed density difference is reproducible ($\pm 25\%$) from experiment to experiment.

4. Discussion

The density-gradient experiments presented in Figure 4 show that λdg^- phages carrying any one of the four spontaneous extreme polar mutations studied have a higher buoyant density than the λdg^+ phage from which they are derived. Control experiments show that the increased density is not due to the process of homogenote formation and that removal of the spontaneous mutations by reversion or recombination leads to a loss of buoyant density. Although other possibilities are not excluded, the simplest explanation for these results is that the spontaneous extreme polar

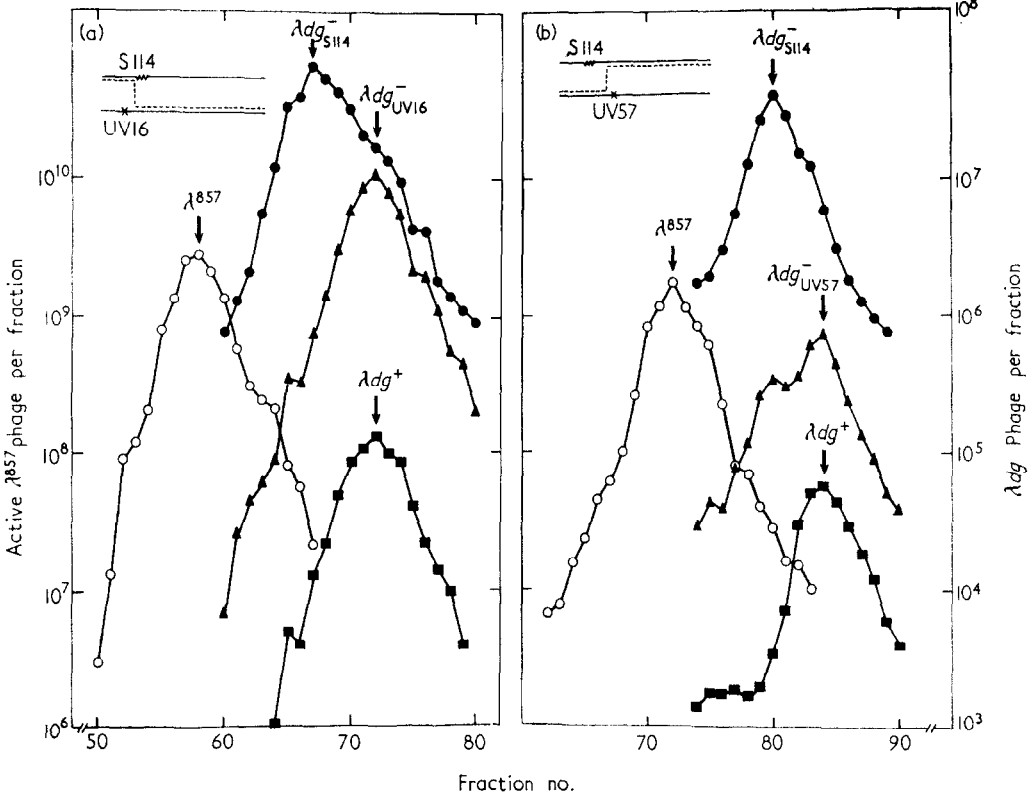


FIG. 6. Segregation of density increase with the S114 mutation in vegetative crosses.
 (a) $\lambda dg^-_{S114} \times \lambda dg^-_{UV16}$: an HFT lysate obtained by superinfection of a defective homogenote strain carrying λdg^-_{UV16} as prophage with an HFT lysate of λdg^-_{S114} . 22,000 rev./min, SW39 rotor, miniscus at fraction 102.
 (b) $\lambda dg^-_{S114} \times \lambda dg^-_{UV57}$: an HFT lysate obtained by superinfection of a defective homogenote strain carrying λdg^-_{UV57} as prophage with an HFT lysate of λdg^-_{S114} . 22,000 rev./min, SW39 rotor, miniscus at fraction 96.
 —○—○—, Plaque-forming units; —●—●—, transducing phage assayed on strain MS2; —■—■—, transducing phage assayed on strain S165; —▲—▲—, transducing phage assayed on strain MS8 (a) or strain MS132 (b).

TABLE 2
 Sizes of the four insertion mutations

Mutation	Approximate mutation size (10 ³ base pairs)	Gradient
S101	2	4(a)
S104	1	4(b)
S114	1.5	5(a)
	1.7	4(c)
	2.1	4(e)
	2.1	6(a)
	2	6(b)
S188	1.3	4(d)
	1.2	5(b)

The approximate mutation size is the number of base pairs of DNA of 50% (G + C) content needed to account for the density difference between each λdg^- phage and the parental λdg^+ phage. For calculations based on Figs 5 and 6, we assume that the revertant or recombinant λdg^+ phage have the same density as the parental λdg^+ phage.

mutations we have examined are the consequences of linear insertion of DNA into the *E. coli* galactose operon. The fact that all four mutations studied fulfill a strong prediction of the insertion hypothesis argues in favour of the validity of the model.

As discussed in the Introduction, the insertion model provides an explanation for the properties of spontaneous extreme polar mutations in the galactose and lactose operons. A possible explanation of why some spontaneous extreme polar mutations appear to be *absolutely* polar (Malamy, 1966; Adhya & Shapiro, 1969) is that they correspond to nucleotide sequences signalling the end of messenger RNA transcription. Although the insertion hypothesis provides a ready explanation for the presence of such transcription signals at many sites within a structural gene, it should be kept in mind that even without special sequences, large insertions should have virtually absolute polar effects (cf. Newton *et al.*, 1965).

The evolutionary importance of insertion mutations depends upon how common they are. Assuming that nearly all spontaneous extreme polar mutations of the galactose operon are the result of insertions, it can be argued that insertion events represent a significant proportion of all spontaneous mutations in *E. coli*. In one experiment, 77 spontaneous mutations of the galactose operon were isolated by selecting for loss of galactokinase activity; of these, at least 14 (18%) have the properties of insertion mutations, and only two are extended deletions (Shapiro, 1967; cf. Adhya & Shapiro, 1969). This high percentage agrees with the results of an analogous but independent experiment of Saedler & Starlinger (1967) in which at least 17 out of 147 spontaneous mutations (12%) have properties ascribable to insertion events. In view of the apparent high frequency of insertion events, it should be pointed out that insertion of DNA fragments containing special sequences can account for the properties of many complex mutations besides polar mutations: for example, *gal*₃ and its constitutive revertants in *E. coli* (Hill & Echols, 1966; Morse, 1967); unstable partial revertants of the *his-203* mutant of *Salmonella* (Ames, Hartman & Jacob, 1963); the *c*₁₇ mutant of phage λ (Pereira da Silva & Jacob, 1968; Packman & Sly, 1968); and some operator-constitutive mutants in various operons.

I would like to thank Alex Fritsch for his patience and tolerance in teaching me how to make preparative CsCl gradients; Harvey Eisen, Ethan Signer, Sydney Brenner, Gerard Buttin, Maurice Hoffnung and Jon Beckwith for lively and helpful discussions; and Hubert Condamine and Jeffrey Miller for reading the manuscript. I am grateful to Professor Francois Jacob for the hospitality of his laboratory. This work was aided by grants from the Délégation Générale à la Recherche Scientifique et Technique, the Centre National de la Recherche Scientifique and the Commissariat à l'Énergie Atomique, and the Jane Coffin Childs Memorial Fund for Medical Research of which I was a Fellow.

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