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Use of Gene Fusions to Determine the Orientation of Gene *phoA* on the *Escherichia coli* Chromosome

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We present genetic evidence which demonstrates that the *phoA* gene is transcribed in the clockwise direction on the *Escherichia coli* chromosome, in contrast to an earlier proposal. Our conclusion is based on analysis of various genetic fusions between the *lac* operon and the *phoA* gene.

The *phoA* gene, which codes for the periplasmic enzyme alkaline phosphatase, lies between 8 and 9 min on the *Escherichia coli* linkage map (1). The order of genes in this region of the *E. coli* chromosome, read in a clockwise direction, is *lac phoA proC phoR* (7, 16). Suzuki and Garen (13) analyzed fragments of alkaline phosphatase produced by strains of *E. coli* carrying different nonsense mutations in the *phoA* gene. They found that protein chain-terminating mutations located at one end of the *phoA* gene yielded small protein fragments related to alkaline phosphatase. It was concluded that these were amino-terminal fragments of the enzyme. Subsequently, Nakata et al. (7) oriented the *phoA* gene relative to the nearby *proC* gene by doing four-factor crosses with *phoA* mutations located at opposite ends of the gene. Based on this mapping and the previous report of Suzuki and Garen, they concluded that the *phoA* gene was transcribed in the direction away from the *proC* gene (counterclockwise).

During an analysis of genetic fusions between the *phoA* and *lacZ* genes, we encountered a number of confusing results which led us to question the validity of the previous conclusions regarding the direction of transcription of the *phoA* gene. In this paper, we present evidence which shows that the direction of transcription of the *phoA* gene is indeed clockwise rather than counterclockwise. A more detailed analysis of the data of Suzuki and Garen (13), in conjunction with recent amino acid sequence data on alkaline phosphatase (R. Bradshaw, personal communication), is also consistent with this conclusion.

MATERIALS AND METHODS

Media and chemicals. Media and chemicals used are described in the accompanying paper (10).

Bacterial and phage strains. Bacterial and phage strains used are listed in Table 1 and in the accompanying paper (10). To obtain Hfr strains carrying *phoA* mutations, the Hfr strain CA7087 was transduced to *pro*$^+$ with P1 grown on various *phoA* mutants. *proC* and *phoA* are 95% cotransducible. *pro*$^+$ transductants were selected on Tris-minimal glucose-XP (5-bromo-4-chloro-3-indolylphosphate) agar. The *phoA* transductants of the Hfr strain were identified as white colonies.

Isolation of fusions. The *phoA-lacZ* fusions characterized in this paper have been isolated in a derivative of XPh26 that is deleted for the *lac* region. This strain is constitutive for the production of alkaline phosphatase. The technique used for the isolation of fusions has been described previously (9).

Preparation of phage lysates carrying the *phoA-lacZ* fusions. The Casadaban technique (4) allows for isolation of λ transducing phage particles carrying gene fusions. These phages are easily obtained by UV induction of the strain containing the fusion.

A portion of a UV-induced phage lysate from a fusion strain was spread on a lawn of MC4100 in F top agar (2.5 ml) on Tris-minimal glucose-XG (5-bromo-4-chloro-3-indolyl-β-D-galactoside) agar containing 10^{-4} M inorganic phosphate. Lambda transducing phage carrying the fusion yielded dark blue plaques on these plates. After repurification of the plaques on the same medium, several well-isolated independent plaques were obtained. Plate lysates were made from each plaque.

Mapping the amount of the *phoA* gene present in *phoA-lacZ* fusions. Fusion-containing strains XPh93, XPh94, and XPh95 were mated with Hfr CA7087 derivatives containing genetically characterized *phoA* mutations. Matings were performed for 60 min in liquid at 30°C if the Hfr contained a Mu(*cts*) insertion, or at 37°C otherwise. Dilutions of the mating mixture were spread on Tris-glucose-XP-streptomycin plates containing no inorganic phosphate (10). After 4 days of incubation, *phoA*$^+$ recombinants appeared as large blue colonies against a background of small white colonies. This technique is sensitive enough to detect a recombination frequency of 10^{-8}.

When fusions were carried on a λ transducing phage, the amount of *phoA* material present was determined by spotting a drop of the phage lysate onto these same plates (without streptomycin) which had been spread

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with MC4100 derivatives containing different *phoA* mutations. After a 4-day incubation period, *phoA*$^+$ recombinants appear as dark blue colonies.

β-Galactosidase assays. β-Galactosidase assays were done according to Miller (6).

RESULTS

Isolation of fusions between the *lac* operon and the *phoA* gene. We have used the Casadaban technique (4) to isolate strains in which the genes of the *lac* operon are put under the control of the regulatory elements for alkaline phosphatase. Two types of fusions are possible with this technique. Operon fusions are those in which an intact *lacZ* gene is placed under the control of the *phoA* promoter. Gene fusions result in the formation of a hybrid *phoA-lacZ* gene which has the portion of the *lacZ* gene corresponding to the amino terminus of β-galactosidase replaced by a portion of the *phoA* gene corresponding to the amino terminus of alkaline phosphatase. The steps used to construct operon and gene fusions are summarized in Fig. 1.

TABLE 1. *Bacterial strains and phages used*[a]

Bacterium/phage	Genotype or bacterial genes carried	Origin
Bacterium		
CA7087	Hfr *lac*$^+$ *proC* YA221 *thi*	F. Jacob
MZ10	F$^-$ Δ*lac*(*X74*) *phoA* *phoR*$^+$ *trp rpsL*	J. Beckwith
XPh93	F$^-$ Δ*lac-169 phoB*$^+$ *phoR glpD rpsL* Φ(*phoA-lacZ*)$_{hyb1611}$	
XPh94	F$^-$ Δ*lac-169 phoB*$^+$ *phoR glpD rpsL* Φ(*phoA-lacZ*)$_{hyb242}$	
XPh95	F$^-$ Δ*lac-169 phoB*$^+$ *phoR glpD rpsL* Φ(*phoA-lacZ*$^+$)$_{821}$	
Phage		
λp1.209		(4)
φ80 *supC*		(3)

[a] XPh93 and XPh94 carry gene fusions derived from Mu insertions *M16* and *M2*, whereas XPh95 carries a gene fusion derived from *M8*.

phoA-lacZ fusions were obtained from strains carrying each of three different Mu insertions in the *phoA* gene. The order of these insertions in the *phoA* gene is *M2, M16, M8, proC* (10). An operon fusion (*821*) was isolated from *M8* and gene fusions (*1611* and *242*) from *M16* and *M2*. The isolation and partial characterization of the latter two fusions have been described previously (9). That the fusions have, in fact, put *lacZ* under the control of the *phoA* regulatory elements is shown by the strong repression of β-galactosidase synthesis in the presence of inorganic phosphate (9; Table 2). Alkaline phosphatase itself is normally controlled in this way (14).

Which portion of the *phoA* gene is deleted in *phoA-lac* fusions? Deletions which are re-

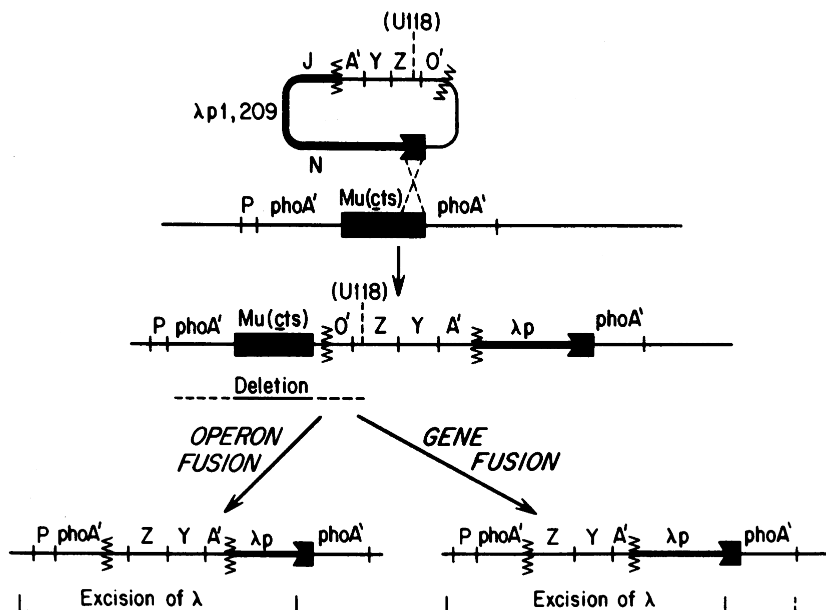

FIG. 1. *Isolation of gene and operon fusions by the Casadaban technique (4). "P" indicates the promoter for the phoA gene. lacZ(U118) is an early ochre mutation in lacZ which is incorporated into the λP1.209 phage when gene fusions are being sought.*

sponsible for operon and gene fusions may extend into the *phoA* gene, thus removing *phoA* genetic material (Fig. 1). Any genetic material removed must correspond to the portion of the *phoA* gene which is between the *phoA* promoter and Mu. Genetic sites beyond the point of the Mu insertion should be unaffected by the events which lead to fusions. Thus, determination of what, if any, genetic material has been removed as a result of a fusion event should give an indication of the end of the *phoA* gene at which the promoter lies.

To determine if any *phoA* material was deleted in the three fusion strains, crosses were carried out with Hfr strains carrying a number of different *phoA* mutations (Table 3). As expected (2), in the case of the *phoA-lacZ* operon fusion *821*, no detectable *phoA* DNA was deleted. Similarly, with the gene fusion *242*, *phoA*$^+$ recombinants were recovered with all *phoA* mutants tested except for the original Mu insertion.

In contrast to the previous two cases, the formation of the gene fusion *1611* resulted in the deletion of *phoA* genetic material. Specifically, the site corresponding to *M2*, which is on the *proC*-distal side, was deleted, whereas no material in the *proC*-proximal portion of the gene was missing. This result is consistent with the suggestion that the *phoA* promoter is at the end of the gene farthest from the *proC* gene.

Proper regulation of a *phoA-lacZ* fusion: requirement for the *proC*-distal portion of the *phoA* gene and nonrequirement for the *proC*-proximal portion. An important feature of the Casadaban technique is that, starting with fusion strains, specialized λ transducing phages can be isolated which contain the fusion. *phoA-lacZ* fusions carried by λ, if properly regulated, should carry the entire promoter-proximal region of the *phoA* gene which is fused to the *lac* genes. This region can be identified by crossing the λ transducing phage with various mutations in *phoA*. This technique has been used previously to characterize fusions of the *malB* operon to the *lac* genes (12).

Lambda transducing phages for the three *lac-phoA* fusions were obtained and verified as such by showing inorganic phosphate control (14) of β-galactosidase synthesis in lysogens (Table 2). These phages were then crossed with a series of *phoA* mutations. When three independently isolated phages carrying the *phoA-lacZ* operon fusion *821*, which was obtained from *M8*, were crossed with *phoA* mutations that map in the *proC*-distal portion of the *phoA* gene, *phoA*$^+$ recombinants were obtained at a frequency of 10^{-6}. However, no *phoA*$^+$ recombinants were

TABLE 2. *β-Galactosidase activity in MC4100 lysogenized with λ phage carrying phoA-lacZ fusions*[a]

MC4100 lysogenized with λ transducing phage containing *phoA-lacZ* fusion from strain:	β-Galactosidase activity (U/mg of protein) in:		Repression ratio
	Low phosphate	High phosphate	
XPh93 (*1611*)	3,962	1.5	2,641
XPh94 (*242*)	1,092	1.2	910
XPh95 (*821*)	1,923	2.0	962

[a] Units of β-galactosidase activity are defined in reference 6. The synthesis of alkaline phosphatase itself is normally increased several hundredfold in wild-type strains by phosphate starvation.

TABLE 3. *Recombination frequency obtained from crosses between phoA mutants and strains containing phoA::Mu(cts) insertions or phoA-lacZ fusions*[a]

Strains containing Mu(cts) in *phoA* gene or carrying *phoA-lacZ* fusion	*phoA* mutations contained in Hfr CA7087						
	M2	*M20*	*U12*	*U5*	*S19*	*E35*	*S10*
XPh95 (*821*)	NT[a]	NT	$>2 \times 10^{-5}$	$>2 \times 10^{-5}$	NT	9×10^{-6}	2×10^{-7}
XPh26 containing *M8*	NT	NT	$>2 \times 10^{-5}$	$>2 \times 10^{-5}$	NT	8×10^{-6}	2×10^{-7}
XPh93 (*161*)	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	3.5×10^{-6}	6.9×10^{-6}	3.5×10^{-6}
XPh96 containing *M16*	NT	NT	8×10^{-7}	1.6×10^{-5}	1×10^{-6}	5.6×10^{-6}	1.8×10^{-6}
XPh94 (*242*)	$<1 \times 10^{-8}$	2×10^{-6}	1.6×10^{-6}	2×10^{-5}	6×10^{-6}	9.6×10^{-6}	2.2×10^{-6}
XPh26 containing *M2*	NT	NT	2×10^{-7}	$>2 \times 10^{-5}$	4×10^{-6}	3×10^{-6}	1.2×10^{-6}
MC4100 containing *M31*	1.5×10^{-6}	NT	NT	NT	NT	NT	NT

[a] Recombination frequency is defined as the number of *phoA*$^+$ recombinants obtained divided by the total number of input Hfr. In the control experiment *M31* × *M2*, Hfr containing *M2* transferred the *phoA*::Mu(cts) insertion. *M2* and *M20* are Mu(cts) insertions in the *phoA* gene. *U12, U5, S19, E35,* and *S10* are *phoA* mutations. NT, Not tested.

obtained when the three phages were crossed with *phoA* mutation *S10*, which maps at the *proC*-proximal end of *phoA*. From results in the previous section we know that in strain XPh95, containing the *phoA-lacZ* operon fusion *821*, the site corresponding to the *phoA* mutation *S10* was not deleted. Thus, in order to incorporate a functional *phoA-lacZ* operon fusion *821*, the phage must include *proC*-distal regions but no *proC*-proximal region of the *phoA* gene.

Four independently isolated λ phages containing the *phoA-lacZ* gene fusion *1611*, which was derived from *M16*, were crossed with strains containing *phoA* mutations that are present in the *phoA* gene on either side of *M16*. No $phoA^+$ recombinants were obtained in any of the crosses. From the previous section we know that the deletion that generated the *phoA-lacZ* gene fusion *1611* had removed the region of *phoA* that corresponds to *M2*, which lies at the *proC*-distal end of the *phoA* gene. However, no *proC*-proximal sites in *phoA* were deleted in the original fusion. These results are similar to those with fusion *821*.

However, two phages carrying *phoA-lacZ* fusion *1611* did recombine with *phoA* mutations which lie in the *proC*-proximal portion of the *phoA* gene. We suspect that in the formation of these two phages the λ phage excised in such a way as to include chromosomal material at both ends (Fig. 1). Thus, in addition to carrying the fusion *1611*, they also carry *phoA* DNA which is not part of the fusion.

The same analysis was done with two independently isolated phages containing *phoA-lacZ* gene fusion *242* obtained from *M2*. No $phoA^+$ recombinants were recovered when the fusion was crossed with *phoA* mutations which lie in the *proC*-proximal portion of the *phoA* gene.

All of the phages analyzed in this study contain fusions of the *lacZ* gene to the *phoA* gene and hence must carry the promoter of *phoA* and regions of *phoA* DNA between the *phoA* promoter and the *lacZ* gene. Therefore, the mapping studies of the *phoA* gene contained in *phoA-lacZ* fusion indicate that the promoter of the *phoA* gene is located at the end of *phoA* distal to the *proC* gene.

Use of a nonsense mutation in the *phoA* gene to establish the direction of transcription of *phoA*. The evidence presented so far indicates that the promoter of the *phoA* gene is at the end of the *phoA* gene farthest from *proC*. If this is the case, in *phoA-lacZ* fusions, the *proC*-distal portion of the *phoA* gene will be the *phoA* segment which is properly transcribed from its promoter. It is possible to interrupt the transcription of distal genes of operons by introducing chain-terminating mutations in an early gene (8). If our conclusions so far are correct, the introduction of a *phoA* chain-terminating mutation into the *proC*-distal portion of *phoA* in a *phoA-lacZ* operon fusion should reduce the expression of the *lac* genes.

We have tested this prediction in the following way. In the previous section, the isolation of a λ transducing phage which carries the *phoA-lacZ* operon fusion *821* was described. The λ phage carries the end of the *phoA* gene which is distal to the *proC* gene. Strain MC4100 containing a chain-terminating mutation, *phoA*(*U24*) (15), which is located at the *proC*-distal end of the *phoA* gene, was infected with the λ transducing phage containing the operon fusion *821*. Since this phage carries *phoA* DNA corresponding to the *phoA*(*U24*) site, λ lysogens which gave rise to $phoA^+$ recombinants could be selected. These $phoA^+$ recombinants can arise by a single reciprocal crossover event at homologous regions of *phoA* DNA contained in the operon fusion and present in the chromosome (Fig. 2). This recombination event also allows for integration of λ together with the fusion directly into the chromosome. However the nonsense mutation *phoA*(*U24*), which was originally present in the *phoA* gene on the chromosome, would now be crossed by the reciprocal recombination event onto that part of the *phoA* gene which is fused to the *lacZ* gene in the *phoA-lacZ* operon fusion *821*. If this mutation is located in the region of the *phoA* gene which is present between the *phoA* promoter and the fused *lacZ* gene, then expression of the *lacZ* gene should be reduced.

From the cross between the λ phage containing operon fusion *821* and the mutant containing *phoA*(*U24*), eight $phoA^+$ recombinants which were also λ lysogens were isolated and purified.

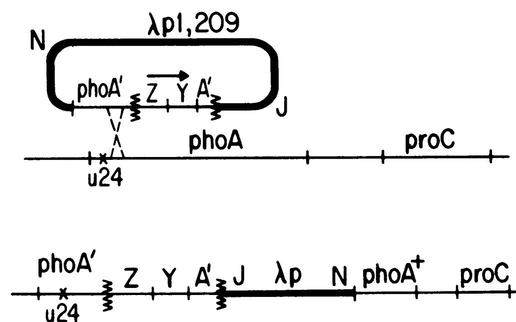

FIG. 2. *Recombination of a λ phage containing phoA-lacZ operon fusion 821 into the phoA gene containing the nonsense mutation phoA(U24). The phoA⁺ recombinant has the phoA(U24) mutation recombined onto the phoA-lacZ operon fusion.*

Ordinarily, strains containing operon fusion *821* when streaked on Tris-minimal glucose-XG agar form blue colonies, indicating high levels of β-galactosidase. To determine the polarity effect of nonsense mutation *phoA(U24)* on expression of the *lacZ* gene in the *phoA-lacZ* operon fusion, each *phoA*$^+$ recombinant was tested on this same medium. Four out of eight *phoA*$^+$ recombinants formed pale blue colonies on this medium, indicating that the nonsense mutation *phoA(U24)* recombined onto the *phoA* DNA fused to the *lacZ* gene and reduced the expression of the *lacZ* gene from the *phoA* promoter. The other four *phoA*$^+$ recombinants formed blue colonies on this medium. However, each of these recombinants when restreaked on the same medium also yielded a fraction of pale blue colonies. These *phoA* recombinants may represent double lysogens of the fusion phage which throw off pale blue single lysogens, including the phage containing the nonsense mutation *phoA(U24)* in the *phoA-lacZ* operon fusion.

If the reduction of *lacZ* expression in the operon fusion is due to the polarity exerted by *phoA(U24)* crossed onto the *phoA* DNA in the *phoA-lacZ* operon fusion, then suppression of *phoA(U24)* by nonsense suppressor mutation *supC* (3) should restore expression of the *lacZ* gene from the *phoA* promoter. Lambda transducing phages containing the operon fusion *821* with the nonsense mutation *phoA(U24)* crossed onto the *phoA* DNA were isolated. These phages appeared as pale blue plaques on a lawn of MC4100 on Tris-minimal glucose-XG agar. The phages were used to test the effect of the suppressor mutation *supC* on expression of the *lacZ* gene. The tester strains were MZ10 and its isogenic *supC* derivative. Since MZ10 is deleted for the *lac* genes, phage containing a functional *lacZ* gene when spotted on a lawn of this strain on Tris-minimal glucose-XG agar under conditions of *lacZ* gene expression will appear as a blue spot. Two independently isolated λ phages containing the operon fusion *821* with *phoA(U24)* were spotted on a lawn of MZ10 ø80 *supC* on Tris-minimal glucose-XG agar. In the presence of suppressor mutation *supC*, a spot of the lysate produced a blue zone in this test, indicating that the nonsense mutation *phoA(U24)* contained in the *phoA-lacZ* operon fusion could be suppressed to restore β-galactosidase activity. A drop of the same lystate, when spotted on MZ10 on Tris-minimal glucose-XG agar, produces a pale blue spot. We then isolated lysogens of these phages in MZ10 and its isogenic *supC*-containing derivative in order to assay β-galactosidase. Assays of these strains showed that suppression of the *phoA(U24)* mutation when present in the *821* operon fusion allowed for a 10-fold increase in synthesis of β-galactosidase.

In contrast, a missense mutation can be expected not to exhibit polarity in the operon fusion. Recombination of missense mutation *phoA(S19)* (11) onto the *phoA* region present in the operon fusion *821* by the same approach did not reduce β-galactosidase activity in this fusion. These results also support the conclusion that the promoter for *phoA* is *proC* distal to *phoA(U24)*.

DISCUSSION

We have used the Casadaban technique (4) to isolate fusions of the *phoA* gene to the *lac* gene. During characterization of the *phoA-lacZ* fusions, we have generated several lines of evidence which suggest that the *phoA* gene is transcribed in a clockwise direction: (i) in the construction of a *phoA-lacZ* gene fusion, some *phoA* DNA which is distal to the nearby *proC* gene on the chromosome has been deleted; (ii) λ transducing phages containing *phoA-lacZ* fusions need only carry *phoA* DNA which is distal to the *proC* gene; and (iii) nonsense mutation *phoA(U24)* located at the end of the *phoA* gene distal to *proC*, when crossed onto the *phoA* DNA contained in a *phoA-lacZ* operon fusion, reduces expression of the *lacZ* gene. A similar analysis with *malT-lacZ* gene fusions was used to show the direction of transcription of the *malT* operon (5).

Further support for this conclusion comes from a combined genetic and biochemical analysis of one of the gene fusion strains. The gene fusion *1611* codes for a hybrid alkaline phosphatase–β-galactosidase protein. The Mu(*cts*) insertion in *phoA(M16)* used in the isolation of this gene fusion is located at the end of the *phoA* gene which is distal to *proC*. The molecular weight of the hybrid protein produced by the gene fusion strain as estimated from sodium lauryl sulfate-polyacrylamide gels is approximately 115,000; hence it should contain only a few amino acids from the amino terminus of alkaline phosphatase. (The monomer of β-galactosidase has a molecular weight of 116,000, and in the generation of the fusion only 19 amino acids from the amino terminus of β-galactosidase were removed.) A determination of the amino acid sequence in this segment of the hybrid protein revealed the presence of only five amino acids, presumably from the signal sequence of the precursor of alkaline phosphatase attached to β-galactosidase, and no amino acid residues from wild-type alkaline phosphatase (9). To generate such a fusion from *M16*, most of the *phoA* DNA between the Mu(*cts*) insertion

and the promoter of *phoA* would have to be deleted. A genetic analysis of this region in strain XPh93 containing the gene fusion *1611* which codes for the hybrid protein $(phoA\text{-}lacZ)_{hyb1611}$ shows that only *phoA* DNA at the end of the *phoA* gene distal to *proC* has been deleted (Table 3).

Our conclusion concerning the orientation of the *phoA* gene on the *E. coli* chromosome contradicts a previous report (7). However, our conclusion is also supported by a reevaluation of earlier data on fragments of alkaline phosphatase from strains containing nonsense mutations (amber and ochre) in the *phoA* gene. From nonsense mutations which map at the *proC*-proximal end of the *phoA* gene, peptides of molecular weight ~4,000 (40 to 50 amino acids long) representing portions of alkaline phosphatase were obtained. It was inferred that these particular lesions causing translation termination must be early (i.e., promoter proximal) in the *phoA* gene. Nakata et al. (7) performed four-factor crosses which oriented two *phoA* mutant alleles relative to *proC* and *lac*. One of these mutations mapped in the same region as the amber and ochre mutants which Suzuki and Garen had examined (13). The genetic data taken together with the earlier findings led to the conclusion that the direction of transcription of *phoA* was counterclockwise on the *E. coli* chromosome (7).

We have reevaluated the data of Suzuki and Garen. We have compared the amino acid composition of the tryptic peptides contained within their putative 4,000-molecular-weight amber and ochre fragments with those predicted from the amino acid sequence of mature alkaline phosphatase as determined by Bradshaw and co-workers (personal communication). We find that at least two of the putative nonsense fragments contain amino acids which lie at positions 150 to 190 of mature alkaline phosphatase. This makes them unlikely candidates for true amber or ochre fragments since these would necessarily contain amino acids lying at positions 1 to 50 of the mature alkaline phosphatase amino acid sequence. Thus, we believe that Suzuki and Garen were examining short degradation products of the true amber and ochre fragments.

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