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SUPPRESSION OF MUTATIONS IN THE ALKALINE PHOSPHATASE STRUCTURAL CISTRON OF E. COLI*

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A suppressor mutation is defined as one that overcomes the defect caused by another mutation. Of particular interest are the "external" suppressors that are located outside the cistron in which the suppressed mutations occur, and are active only on some of the mutations within the cistron.[1, 2] The present communication describes the properties of an external suppressor for a class of mutations in the structural cistron for alkaline phosphatase of *E. coli*.[3-5] The results suggest that this class of phosphatase-negative mutations produces a nucleotide configuration that is nonsense (unable to specify any amino acid) in the absence of the suppressor but which functions as a sense configuration when the suppressor is present.

Materials and Methods.—Bacterial strains: In all experiments, the *Hfr* was *E. coli* strain *K10* and the F^- was *E. coli* strain *W1*. The characteristics of these strains have been described elsewhere.[3-6] Several of the P^- mutants were previously isolated in collaboration with C. Levinthal and E. Lin. The selective markers employed for the genetic crosses were T^+ and L^+ (ability to grow in a medium lacking threonine and leucine), S^r (ability to grow in the presence of 0.1 mg/ml of streptomycin), and M^+ (ability to grow in a medium lacking methionine).

Media: The composition of the low phosphate medium used to prepare cultures for alkaline phosphatase assays was as follows: 1.2×10^{-1} *M* tris buffer; 0.2% glucose; 6.4×10^{-5} *M* KH_2PO_4; 8×10^{-2} *M* NaCl; 2×10^{-2} *M* KCl; 2×10^{-2} *M* NH_4Cl; 3×10^{-3} *M* Na_2SO_4;

1×10^{-3} *M* $MgCl_2$; 2×10^{-4} *M* $CaCl_2$; 1×10^{-5} *M* $ZnCl_2$; 2×10^{-6} *M* $FeCl_3$; 0.02% Difco casamino acids (phosphate free).

Genetic crossing and blendor experiments: The procedures have been described elsewhere.[6]

Preparation of cell extracts: Cultures were grown to saturation in low phosphate medium at 37°C with aeration. The cells were harvested and resuspended in a solution of 0.05 *M* tris buffer pH 7.5 + 0.02 *M* $MgCl_2$. This suspension was passed once through a French pressure cell to disintegrate the cells and afterwards was centrifuged at 10,000 *g* for 10 min. The supernatant was used for enzymatic activity and CRM assays.

Enzymatic activity assay: A sample of the cell extract was added to a solution containing 4 mg/ml *p*-nitrophenylphosphate in 1 *M* tris buffer pH 8.0 maintained at 32°C, and the rate of change in the optical density at 410 mμ was measured.

Immunological cross-reacting material (CRM) assay: A technique developed by Preer[16] was used. Rabbit antiserum was prepared by inoculations of purified normal enzyme as antigen. A constant concentration of the serum was assayed in microtubes against serial twofold dilutions of cell extract. The relative amount of CRM in an extract was estimated by the position of the precipitation band in the microtubes as compared with the position of the band formed by a standard solution of the normal enzyme. The CRM value is given as per cent of the normal enzyme, in categories of 100, 50, 25, 12, 6, and 3 per cent. Each extract preparation could be unambiguously classified in one of the categories.

Response to 5-fluorouracil:[7] Cultures of phosphatase-negative mutants were grown in low phosphate medium (without casamino acids) to a concentration of about 3×10^8 cells/ml. The cells were centrifuged, washed, and resuspended in an equal volume of low phosphate medium. To one portion of the culture, 20 μg/ml 5-fluorouracil was added; the untreated cells served as the control. After 6 hr of incubation at 37°C, the cultures were shaken with a few drops of toluene and subsequently assayed for enzymatic activity.

Results.—Effect of the suppressor on enzyme synthesis: The first indication of the existence of a suppressor in the phosphatase system came from the anomalous behavior of certain phosphatase-negative mutants in genetic crosses. Starting with a phosphatase-negative mutant of the *Hfr* strain, it was observed that enzymatic activity appeared when the P^- marker was transferred to an F^- strain (not isogenic with the *Hfr*).[3, 4] Of 220 independently isolated mutants examined, 15 responded in this way. The enhanced enzymatic activity in the genetic environment of the F^- strain suggested that the F^- harbored a suppressor for these P^- mutations. A comparison of the amounts of enzyme produced in nonsuppressed (*Hfr*) and suppressed (F^-) strains of the mutants is shown in Table 1. The main points to be noted are that essentially no enzyme is produced by any of the nonsuppressed strains, while in the suppressed strains the levels of both enzymatic activity and CRM range from 3 to 100 per cent of the values for the standard P^+ strain.

Genetic mapping of the suppressor: The map position of a suppressor for one of the P^- mutants (*U8*) was established by means of a Blendor experiment[8] which measured the time of transfer of a suppressor marker from an *Hfr* to an F^-. Two strains were required for this experiment, an F^- that did not contain a suppressor for *U8* (su^-) and an *Hfr* with the suppressor (su^+). The first strain was prepared by the following cross: $Hfr\ U8\ su^-\ M^+\ S^s \times F^-\ U8\ su^+\ M^-\ S^r$. The purpose of this cross was to transfer the su^- marker to the F^-. A selection was made for $F^- M^+ S^r$ recombinants, and it was found that about 0.5 per cent were su^-. This result furnished preliminary evidence that the *su* marker was transferred later than *M*, which enters 35 min after the start of mating. The second strain was prepared by isolating enzymatically active revertants from the $Hfr\ U8\ su^-$ strain. These revertants were of two types, one in which the reverse mutation was in the *P* cistron (P^+ revertant) and another in which the reverse mutation was a suppressor ($U8\ su^+$ re-

vertant). The latter was used as the *Hfr U8* su^+ strain for the Blendor experiment. The amount of enzyme produced by the *Hfr U8* su^+ was nine per cent of the amount in the standard P^+ strain. This is approximately the same value previously obtained with the F^- strain of this mutant (see Table 1). It is possible that the same suppressor for *U8* is involved in the *Hfr* and the F^-.

TABLE 1

ALKALINE PHOSPHATASE LEVELS IN NONSUPPRESSED AND SUPPRESSED MUTANT STRAINS

Suppressible P^- mutants	Nonsuppressed Strains: Enzymatic activity	Nonsuppressed Strains: CRM	Suppressed Strains: Enzymatic activity	Suppressed Strains: CRM
U2	0.02	<1	13.	12
U5	0.02	<1	7.	6
U8	0.04	<1	12.	12
U20	0.03	<1	3.	3
U46	0.04	<1	24.	25
U56	0.07	<1	15.	12
E35	0.04	<1	3.	3
G5	0.03	<1	10.	12
G55	0.05	<1	18.	25
A7	0.02	<1	5.	6
H7	0.03	<1	5.	6
S16	0.04	<1	18.	25
S26	0.02	<1	100.	100
S42	0.05	<1	18.	25
S45	0.02	<1	18.	25

The P^- mutants were isolated after treatment of the standard P^+ strain with one of the following mutagenic agents: ultraviolet light (U), X ray (E), N-methyl-N'-nitro-N-nitrosoguanidine (G), 2-aminopurine (A), hydroxylamine (H), or ethylmethanesulfonate (S).

The nonsuppressed strains are the mutants as originally isolated in the *Hfr*. The suppressed strains were produced by crossing the *Hfr* mutants with an F^- strain that carried a nonsuppressible P^- mutation. The selective markers used in the crosses were T^+L^+ in the *Hfr* and S^r in the F^-. The recombinant class $T^+L^+S^r$ was tested for alkaline phosphatase activity, and it was found that about half the recombinants were enzymatically active. These were used as the suppressed strains.

For the enzymatic activity measurements, extracts of cultures grown in limiting phosphate medium were prepared as described under *Methods*. The CRM (immunological cross-reacting material) assays were done on the same extracts. All results in the table have been normalized to a standard cell concentration and are expressed as the per cent of the value obtained with the P^+ strain. Each experiment was done in duplicate on separate cultures. The enzymatic activities were reproducible to ±25%, and the CRM results to within the limits indicated in the section on *Materials and Methods*.

The blendor experiment was done by crossing *Hfr U8* su^+ S^s with F^- *U8* su^- S^r. At different times, samples were blended to stop mating and were then plated on a medium selective for *U8* su^+ S^r recombinants, to score for the entry of the su^+ marker into the F^-. The time of entry of su^+ was at 90 min, as compared to 9 min for the *P* marker. Thus, the suppressor for *U8* is well separated from the *P* cistron, by a distance equivalent to about 75 per cent of the total length of the bacterial chromosome.[8]

The map position of a suppressor for another mutant, *S26*, was established by an analogous blendor experiment. The cross was between an *Hfr S26* su^+S^s and an F^- *S26* su^- S^r, each strain being produced by the procedures described for the preceding cross. The *S26* suppressor was transferred at about the same time as was the *U8* suppressor, suggesting that the same suppressor may be involved with both mutants.

The two F^- strains prepared for the preceding blendor experiments, one containing an su^- marker for the *U8* mutation and the other an su^- marker for the *S26* mutation, were each crossed with all of the suppressible *Hfr* mutants to determine whether these su^- markers prevented suppression of the entire group of P^- mutations. None of the P^- mutations were suppressed when crossed into either F^-

strain. This is additional evidence that a single suppressor is responsible for suppression of all the P^- mutations listed in Table 1.

Genetic mapping of suppressible P^- mutations: The results in Table 1 show that the suppressible mutations have a wide range of responses to the suppressor. To determine whether they are located at different genetic sites, the following crosses were performed. (In all of these crosses, both the *Hfr* and F^- strains were su^-.) Each suppressible *Hfr* mutant was crossed against a P^- "deletion" mutant in which the genetic defect spans about two thirds of the *P* cistron. Five of the mutants (*U2, U5, U20, U56,* and *S45*) produced P^+ recombinants and therefore are located outside the span of the deletion. One of these (*U5*) and four of the mutants within the span (*U8, S26, E35,* and *U46*) were each crossed against the other suppressible mutants and against each other. In every case, P^+ recombinants were found. Thus, the number of suppressible sites must total at least seven, five within the span and two outside.

Properties of the enzyme produced by the suppressed mutants: A comparison of the enzymatic activity and CRM values for the suppressed mutants (Table 1) shows that the specific enzymatic activities per unit of CRM are approximately normal for all mutants. Two other properties, heat stability and electrophoretic mobility, were also examined to test for possible structural alterations in the enzymes. The standard P^+ enzyme is exceptionally heat-stable,[9] capable of surviving exposure of up to 90°C for 30 min without loss of activity. The enzymes from eight suppressed mutants (*U5, U8, U46, U56, S16, S26, S42, S45*) were as heat-stable at the standard enzyme. With regard to electrophoretic mobility, it is known that electrophoretically altered enzymes can result from mutations in the *P* cistron.[3, 4, 10] The electrophoretic behavior of the enzymes from six suppressed mutants (*U5, U8, U46, S45,* and *G5*) was found to be identical to that of the standard P^+ enzyme.

Complementation tests with suppressible mutants: Complementation has been observed between pairs of different P^- mutants.[11] The mechanism for complementation appears to be the formation of hybrid enzyme molecules containing one protein chain derived from each P^- cistron (the standard P^+ enzyme molecule is a dimer composed of two identical chains).[12] Of particular importance for the present work is the fact that complementation can occur with P^- mutants that by themselves produce no detectable enzymatic activity or CRM. Such mutants presumably are capable of producing the protein chains of the enzyme in a defective form. We have carried out complementation experiments with the suppressible mutants as a measure of their capacity to synthesize the protein chains. Five of the suppressible mutants (*U5, U8, U46, S26,* and *E35*) were tested for complementation against each of five nonsuppressible P^- mutants that are known to be capable of complementing.[11] There was no indication of complementation in any test involving a suppressible mutant.

Effect of 5-fluorouracil on suppressible P^- mutants: A physiologial method for reversing the phenotype of certain *rII* mutants of T4 phage (and also of phosphatase-negative mutants of *E. coli*) by the action of 5-fluorouracil (FU) has recently been reported.[7] FU appears to reverse specifically those mutational defects that result from the transition of a G-C to an A-T base pair in DNA. This finding makes it possible to test for such a base pair change in a P^- mutant: a positive response to

FU would be an indication that this change had occurred, although failure to respond would not necessarily mean the contrary. The 15 suppressible P^- mutants in Table 1 were tested (in an su^- strain) for their response to FU, the criterion being an increase in amount of enzymatic activity in cultures treated with FU. Positive results were obtained with all except two of the mutants (Table 2). The magnitude of the response to FU varied, the maximum value being 0.3 per cent of the activity of the standard P^+ strain. This is considerably below the level of suppression achieved with the su^+ strains (see Table 1) but nevertheless is significant.

TABLE 2

RESPONSE OF PHOSPHATASE-NEGATIVE MUTANTS TO 5-FLUOROURACIL

Suppressible P^- mutants	Enzymatic Activity		Response
	Without FU	With FU	
U2	0.03	0.2	+
U5	<0.03	<0.03	–
U8	0.03	0.13	+
U20	0.03	0.2	+
U46	0.04	0.14	+
U56	0.06	0.34	+
S16	0.03	0.16	+
S26	<0.03	0.48	+
S42	0.08	0.25	+
S45	<0.03	0.08	+
E35	<0.03	0.14	+
G5	<0.03	0.25	+
G55	0.03	0.4	+
A7	<0.03	0.04	(+)
H6	0.03	0.2	+
Nonsuppressible P^- mutants			
U3	<0.05	<0.05	–
U7	<0.05	<0.05	–
U18	<0.05	<0.05	–
U24	<0.05	<0.05	–
U38	0.13	0.13	–
U58	<0.05	<0.05	–
S10	<0.05	0.16	+
S36	<0.05	<0.05	–
S41	<0.05	<0.05	–

All of the mutants were *Hfr* strains that contained an su^- marker. The procedures involved in the FU tests are described in the section on *Materials and Methods*. The enzymatic activities are expressed as per cent of the value obtained with the P^+ strain without FU.

As a control, a group of P^- mutants not suppressible in the su^+ strain was also tested with FU. Only one of these responded to the FU treatment. Thus, the general responsiveness of the mutants in Table 1 to FU implies that they may have in common a G-C to A-T alteration in the P cistron.

Discussion.—All 15 of the suppressible P^- mutants are incapable of synthesizing any enzyme protein when the suppressor is not functioning, as indicated by the absence of CRM and enzymatic activity and by the failure to complement other P^- mutants. This behavior distinguishes these mutants from other P^- mutants that do not respond to this particular suppressor, since a majority of the latter do have the capacity to synthesize enzyme protein. These suppressible mutants may, therefore, all originate from nonsense mutations that block the formation of the protein molecule.

It is striking that suppression of one of the P^- mutants enables the fully normal amount of enzyme to be synthesized, thus completely restoring the function of the

P cistron despite the continued presence of a P^- mutation. This mutation would not have been detectable if the screening for P^- mutants had been carried out with a strain that contained the suppressor. Here, we have an extreme example of suppression, an absolute negative mutant being transformed into an apparently normal cell.

According to a recently proposed mechanism of suppression,[1, 2, 13] a suppressor strain might contain a new kind of transfer RNA-amino acid complex that generates a specific kind of mistake in proteins by substituting one particular amino acid for another. This could result in the restoration of a function in a mutant if the mistake happened to compensate for the amino acid defect produced by the mutation. Since such a mistake mechanism would affect all proteins, only a limited number of mistakes are likely to be tolerated by the cell.

In the special case of suppression of a nonsense mutation, this difficulty would not arise. While the same type of suppressor mechanism could operate, its effect would be limited to the nonsense site which would not recur elsewhere. Instead of producing mistakes, the suppressor would transform nonsense into sense. Since the only protein affected would be the one under the control of the cistron containing the nonsense mutation, there need not be any limitation to the efficiency of the process. The high efficiency of the alkaline phosphatase suppressor could be accounted for in this way.

Other evidence in support of the conclusion that the alkaline phosphatase suppressor acts on nonsense mutations is presented in an accompanying paper by Benzer and Champe,[14] in which they describe an ingenious test for nonsense mutations in the *rII* region of phage T4. They have found that the alkaline phosphatase suppressor can also suppress certain *rII* mutations[15] and that all of these concomitantly suppressible *rII* mutations appear to be of the nonsense type.

Summary.—A group of 15 phosphatase-negative mutants, involving at least seven separate sites in the structural cistron, are suppressible by an external suppressor. All of the mutants are incapable of synthesizing any enzyme protein when the suppressor is not functioning. In the presence of the functional suppressor, the amount of enzyme synthesized ranges from 3 to 100 per cent of normal for different mutants. The enzyme formed by the suppressed mutants is not detectably different from the normal enzyme in electrophoretic mobility, heat stability, or specific activity. It is proposed that the suppressor acts by converting nonsense mutations into sense.

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ON GAUSSIAN SUMS

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1. Let χ denote a nonprincipal character (mod p), where p is an odd prime. Denote by χ_0 the principal character. I made the following

Conjecture: It is known that

$$\tau(\chi) = \sum_{1}^{p-1} \chi(n)\, e^{2n\pi i/p} = \sqrt{p}\,\epsilon(\chi)$$

where $|\epsilon(\chi)| = 1$; $\epsilon(\chi) =$ *is a root of unity only when* $\chi^2 = \chi_0$.

In this paper, I prove the conjecture. In the special case when $(p - 1)/2$ is also a prime a proof was recently given by Straus, Peck, and me, by a method whose power in other directions we hope to investigate later.

For a recent study of Jacobi and Gaussian sums, I would like to refer to a paper of A. Weil "Jacobi sums as Grössencharactere" in *Trans. Amer. Math. Soc.*, 1952. My thanks are due to A. Selberg for a stimulating conversation on the subject of this paper.

2. Let k be the least positive integer such that $\chi^k = \chi_0$. Then we have $p - 1 = qk$, where q is an integer. Write $\tau(\chi)$ in the form

$$T_1(\omega, \zeta) = \sum_{m=0}^{k-1} \omega^m S_m,$$

where
$$\omega = e^{\frac{2\pi i b}{k}}, \quad (b, k) = 1, \quad S_m = \sum_{t=1}^{q} \zeta^{g^{tk+m}}$$

and g denotes a primitive root (mod p); $\zeta = e^{2\pi i/p}$. We operate in the field $R(e^{2\pi i/w})$ where $w = 4\,pk$. We write $\theta = e^{2\pi i/w}$ and note that the automorphisms of $R(\theta)$ are given by $\theta \rightarrow \theta^h$ where $0 < h < w$, $(h, w) = 1$.

3. Suppose that $\epsilon(\chi)$ is a root of unity. Since $\tau^k(\chi)$ lies in $R(\omega)$ (Hasse, *Vorlesungen über Zahlentheorie*, Springer Verlag, pp. 440–450), it is easy to see that our theorem is true for k odd > 1. Hence suppose k even. Since $\tau^k(\chi)$ lies in $R(\omega)$, we easily see that if $\epsilon(\chi)$ is a root of unity, we must have