*RECESSIVE LETHALS: A NEW CLASS OF NONSENSE SUPPRESSORS IN ESCHERICHIA COLI**

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Abstract.—Two new nonsense suppressors in *Escherichia coli* were found in partial diploids carrying F'14 and were shown to be on the episome. These suppressors can exist only in cells which also contain the su^- allele, i.e., su^+/su^- heterozygotes. Presumably the mutations cause an alteration of an essential cellular component, the complete loss of which is lethal. *Su7*, an amber suppressor, has an efficiency of 76 per cent and *su8*, an ochre suppressor, an efficiency of 4 per cent.

Extensive searches by several workers have revealed only three amber suppressors in *Escherichia coli*.[1–3] Such suppressors are thought to result from changes in the anticodon of a transfer RNA (tRNA) allowing the altered tRNA to pair with UAG, the amber triplet, at the ribosome.[4–6] Seven amino acids have codons related to UAG by a single base change: therefore, it should be possible to generate at least one suppressor for each of these amino acids by a mutation affecting the anticodon. The fact that no suppressors have been found which insert some of these amino acids (tryptophan, lysine, and glutamic acid) suggests a fundamental restriction on the generation of suppressors.[7] Assuming that anticodon changes in certain tRNA's do not prevent aminoacylation, the most likely restriction is that the tRNA which might become the suppressor is indispensable, and its loss is lethal to the cell. If this were true, such potentially lethal mutations affecting tRNA's should be recoverable in cells which are diploid for these genes. Such strains can retain one copy of a duplicated indispensable tRNA gene to perform its normal function, allowing the second to mutate to yield a suppressor. Other potential mutations leading to suppression but not involving tRNA's might also be lethal to the haploid cell but could be revealed in partial diploids. This report presents a general approach for the isolation of such recessive lethal suppressors and describes a new amber and a new ochre suppressor of this type.

Materials and Methods.—Bacterial strains: All strains, except where noted, were derived from W3110 $R^-_{uvr}trpA9605$ obtained from Dr. Charles Yanofsky. Strains harboring *su4* (H12R7a), *su5* (U11R1d), and *su6* (AB1206/FH12-8-RZ) were provided by Dr. Alan Garen. Additional amino acid requirements were introduced by UV mutagenesis (1800 ergs mm^{-1} to 10 ml of cells at $OD_{590} = 1$ in a 9-cm Petri dish) followed by penicillin selection.[8] Spontaneous Thy mutants were isolated after growth in L-broth containing 40 μg/ml trimethoprim[9] (trimethoprim was kindly provided by Dr. G. Hitchings, Burrows Wellcome Co., White Plains, N.Y.). *RecA56* strains were prepared as described by Hill *et al.*[10]

Bacteriophage: Bacteriophage P1Kc (P1) were obtained from Dr. Charles Yanofsky. Phage f2 was prepared by Dr. John Foulds. A T4 ochre mutant, 427, originally from Dr. Stan Person, was obtained from Dr. Charles Yanofsky.

Media: Minimal medium was that of Vogel and Bonner[11] supplemented with 0.2% glucose. Unless otherwise noted, L-amino acids were present when needed at a concentration of 20 μg/ml. L-Broth contained 1% Difco Bacto-tryptone, 0.5% Difco yeast extract, 0.5% NaCl, and 0.1% glucose. Nutrient broth contained 0.8% Difco nutrient broth and was adjusted to pH 7.6 with 1 *N* NaOH for acridine curing experiments.

Transductions: Transductions were performed according to Lennox.[12] Transductants were tested for P1 lysogeny by restriction of bacteriophage λ replication,[13] and only nonlysogenic strains were retained.

Matings: Bacterial conjugation was performed according to Adelberg and Burns[14] using L-broth. Mating was interrupted by vigorous agitation with a vortex mixer for 60 sec or, where the F^- parent was $T6^r$, by the addition of bacteriophage T6.

Acridine curing: Curing experiments were performed by diluting overnight cultures grown in selective media 1000-fold into nutrient broth pH 7.6 containing 20 μg/ml acridine orange. After growth to saturation (36–48 hr) had been allowed, appropriate dilutions were plated and single colonies tested for loss of markers.

Assays: Extracts for measuring enzyme levels were prepared according to Yanofsky and Ito[15] from cells grown under conditions that repressed the production of tryptophan biosynthetic enzymes. Tryptophan synthetase α and β_2 proteins were assayed as described by Smith and Yanofsky.[16] Protein was determined by the method of Lowry *et al.*[17]

Results.—Selection of episomal suppressors: The search for recessive lethal suppressors first entailed isolating suppressors in a strain carrying an episome, then showing that the suppressor was located within the duplicated region and, finally, demonstrating the recessive lethality of the mutation. The construction of the parental partial diploid is shown in Figure 1. Both *trpA9605* and *his 29* were determined to be amber mutants; their auxotrophic phenotype could be reversed by the introduction of *su*1, *su*2, or *su*3.[18] *Ilva 1* and *pro 2* were not suppressible by any known amber or ochre suppressor. F'14 was introduced by mating with AB1206 (obtained from Dr. E. A. Adelberg) for 60 minutes, followed by addition of T6 to kill the F'14 donor cells. The resulting strain LS302 was sensitive to the male specific bacteriophage f2 and could donate $Ilva^+$ in matings. Furthermore, growth in acridine orange, which is known to cause

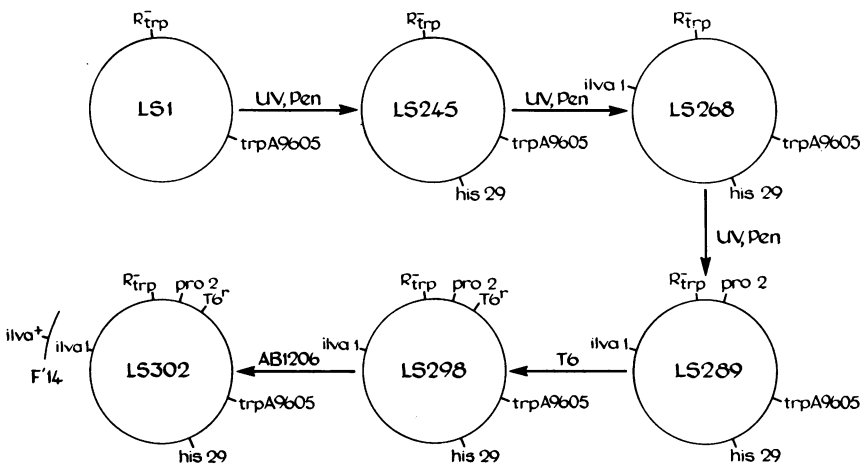

FIG. 1.

elimination of the episome,[19] eliminated the f2-phage sensitivity as well as the Ilva$^+$ phenotype.

LS302 was mutagenized by placing a filter disc saturated with ethyl methane sulfonate on a minimal proline plate spread with 0.1 ml of a late log-phase culture. Alternatively, cells were exposed to UV and plated on minimal proline plates immediately after exposure. Suppressor selection was accomplished by demanding simultaneous tryptophan and histidine prototrophy. It was found that greater than 95 per cent of the colonies selected contained suppressors, indicating that a suppressor mutation is far more frequent than simultaneous reversions in the two structural genes.

Independently arising Su$^+$ colonies were picked and transferred to grids on minimal proline plates and allowed to grow 16–18 hours. They were then replicated to minimal plates previously spread with 0.1 ml of a culture of a recA56 derivative of LS268 (W3110 R^-_{trp} *trpA9605 his 2 9ilva 1 recA56*). It has been shown that a *recA* allele in the recipient cell prevents integration of chromosomal markers in Hfr $\times$ F$^-$ matings, but does not appreciably interfere with the transfer of episomes.[20] Growth on the replica plate can occur only if both the suppressor of the *trp* and *his* amber mutations and the *ilva*$^+$ marker are transferred on the episome. Twelve per cent of the UV-induced and 35 per cent of the ethyl methanesulfonate-induced suppressors were transferred with F'14. Spot tests with a set of λsus mutants and a T4 ochre phage revealed that all of the 150 isolates tested with the suppressor on the episome fell into two classes, which have been designated *su*7 and *su*8. The patterns obtained are shown in Table 1, along with those exhibited by other amber and ochre suppressors.

The fact that *su*8 and not *su*7 suppressed the T4 ochre mutation indicates that the former is an ochre suppressor, while the latter may be an amber suppressor. In order to confirm these assignments, the *trpA9605* allele of LS302 was replaced[21] by a known ochre lesion, *trpB9676*. Because this strain contains one amber and one ochre nonsense mutation, the presence of an ochre suppressor

TABLE 1. *Phage spot tests for various suppressors.*

Phage	*su*1 (Ser) amber	*su*2 (Glu-NH_2) amber	*su*3 (Tyr) amber	*su*4 (Tyr) ochre	*su*5 (Lys?) ochre	*su*6 (Leu) amber	*su*7 — amber	*su*8 — ochre
λ^{++}	+	+	+	+	+	+	+	+
$\lambda SusR_{60}$	+	+	+	+	+	+	+	+
$\lambda SusR_{216}$	0	+	0	0	+	0	+	+
$\lambda SusR_{221}$	0	+	+	+	+	0	+	+
$\lambda SusP_3$	+	+	0	0	0	0	+	0
$\lambda SusQ_{157}$	0	0	+	+	0	0	0	0
$\lambda SusN_7$	+	+	+	+	0	+	+	0
$\lambda SusB_{10}$	+	+	+	+	0	+	+	0
$\lambda SusE_{13}$	+	+	0	0	0	+	+	0
$\lambda SusJ_{27}$	±	0	0	0	0	+	+	0
T4 ochre-427	0	0	0	+	+	0	0	+

All suppressors were tested in an LS302 background except *su*4, *su*5, and *su*6, which were introduced into LS268. Lawns of each bacterial strain were prepared by mixing 0.2 ml of overnight cultures with 2 ml of soft agar and pouring onto tryptone-agar plates. Approximately 0.01 ml of each phage suspension at 10^7 PFU/ml was spotted on this lawn. Plates were scored after 18 hr incubation at 42°C, with complete clearing of the spot considered a positive response.

should produce a Trp$^+$His$^+$ phenotype, while an amber suppressor should relieve only the histidine requirement. P1 grown on LS302 *su7* and LS302 *su8* were used to transduce this strain. According to this criterion *su7* was found to be an amber suppressor, whereas *su8* is of the classical ochre type.

Additional proof that the isolated suppressors were located on the episome was obtained from acridine curing experiments: it was found (Table 2) that both suppressors were eliminated concomitantly with the *ilva*$^+$ marker during growth in the presence of acridine orange. These results show that *su1*, *su2*, and *su3* are not sensitive to acridine curing when they are present in this background.

Evidence for recessive lethality of su7 and su8: If the *su7* and *su8* suppressors are indeed recessive lethal mutations, two predictions can be made: (1) the generation of these suppressors should not be possible in haploid bacteria and (2) transduction of the suppressor mutation into diploid recipients (i.e., α F'14 merodiploid) should give viable prototrophs, while transduction of the same mutations into a haploid recipient should not. Experiments designed to test both of these predictions have been performed.

TABLE 2. *Acridine curing of suppressor strains.*

LS302 containing	Total colonies tested	SU$^+$Ilva$^+$	SU$^+$Ilva$^-$	SU$^-$Ilva$^+$	SU$^-$Ilva$^-$
su1	52	2	50	0	0
su2	52	1	51	0	0
su3	52	0	52	0	0
su6	52	1	0	0	51
su7	52	3	0	0	49

Overnight cultures grown in minimal + proline media were diluted 1:10^3 into nutrient broth pH 7.6 containing 20 μg/ml acridine orange. The cultures were grown to saturation at 37°C. Appropriate dilutions were plated on tryptone media and scored for loss of Su$^+$ or Ilva$^+$ by replicating onto plates lacking tryptophan and histidine or isoleucine-valine, respectively.

In the course of transducing the *su7* and *su8* suppressors, it was found that both were linked to the *ilva* marker (60–65% cotransduction frequency). This fact enabled us to search for the occurrence of these suppressors in a haploid strain. Approximately 500 independent Su$^+$ colonies were generated by EMS mutagenesis in LS245 (W3110R^-_{trp}*trpA9605 his 29*), a haploid strain, and in LS302 (W3110R^-_{trp}*trpA9605 his 29 ilva 1 pro2/F'14 ilva*$^+$), the F' 14 merodiploid derived from LS245. P1 lysates prepared on each of these mixed Su$^+$ populations were used to transduce LS268 (W3110 R$^-_{trp}$*trpA9605 his 29 ilva 1*). If *su7* or *su8* had arisen in the haploid strain, transduction of the Su$^+$ alleles would have yielded Su$^+$Ilva$^+$ transductants: the failure to detect *ilva*-linked suppressor mutations in haploid cells and their presence in the suppressed diploid strains (Table 3) suggest that *su7* and *su8* cannot arise and exist in haploid cells.

To test the second prediction, a P1 lysate prepared with a diploid strain carrying *su7* was used to transduce the suppressor to LS302 (the unsuppressed parental diploid), to LS289 (the haploid progenitor of LS302), and to an F$^+$ derivative of LS289. When the transduction frequency is normalized for the transduction of a chromosomal marker, there is only a two- to threefold preference for transduction into the F'14 merodiploid compared to the haploid recipients (Table 4). This

TABLE 3. *Absence of suppressor mutations linked to Ilva in haploid cells.*

Source of P1 lysate	Su^+ transductants tested	Su^+Ilva^+ cotransductants
LS245 Su^+ (haploid)	2170	0
LS302 Su^+ (diploid)	168	41

The recipient strain was LS268 (W3110$R^-_{trp}trpA_{9509}$*his 29 ilva 1*). Transductants were plated on minimal + isoleucine-valine media to score for Su^+ transductants and on minimal media to determine Su^+Ilva^+ cotransduction.

TABLE 4. *Transduction of suppressors into haploid and diploid strains.*

Recipient strain	Su^+ transductants/ml $\times 10^{-3}$ P1 (*su1*)	P1 (*su7*)	*su7*/*su1*
LS289	5.7	2.1	0.37
LS289F$^+$	10.4	4.2	0.40
LS289F'14 (LS302)	3.1	2.9	0.94

finding is clearly unexpected if the suppressor mutations are recessive lethals; therefore, the transductants found in the haploid were examined in more detail.

Twenty of the *su7 ilva*$^+$ cotransductants produced in the haploid strain were purified by four single-colony isolations. Each of these was grown in broth for ten generations and checked for retention of the *su7* and *ilva*$^+$ alleles by replicating colonies arising on nonselective plates to plates lacking either isoleucine-valine or tryptophan and histidine. With each of the 20 transductants, approximately 10 per cent (7–13%) of the population were Su^-. Since all Su^- segregants were also $Ilva^-$, we conclude that segregation was the result not of selective enrichment of Su^- revertants but of a loss of a DNA segment. This and the relatively high frequency of segregants suggest that the Su^+ transductants arising in the haploid bacteria contain a duplication of the suppressor locus yielding a *su7*/*su*$^-$ partial heterozygote. Evidently the duplication may also include *ilva*. The mechanism by which this duplication is created and its nature are presently under study; but it should be pointed out that duplications arising during transduction have already been observed and a theory for their origin has been suggested.[22] The instability of *su7* and of *su8*, for which similar data have been obtained, resembles that shown by a suppressor described by Schwartz[23, 24] which is also cotransducible with *ilva*. This latter suppressor has been shown to reverse mutations associated with the UGA triplet[25] and thus is not identical to either *su7* or *su8*. It is possible, however, that the same gene could give rise to the Schwarz suppressor and either *su7* or *su8*.

Efficiency of suppression: The tryptophan synthetase protein provides an elegant system for measuring the efficiency of nonsense suppression *in vivo*. The two types of subunits of the protein, the β_2 and α proteins, are coded for by the B and A cistrons, respectively, of the tryptophan operon. Nonsense mutations in the A cistron (the most distal to the operator) eliminate the production of a complete α subunit; extracts of such mutants have only the activity characteristic of the β_2 subunit and none of the activity of the $\alpha\beta_2$ complex. Suppression of the nonsense mutation permits the production of a complete α-polypeptide (either normal α-chains or α-CRM, depending upon the amino acid inserted by the suppressor). The complex of α-CRM and β_2 exhibits one enzymic activity of

normal $\alpha\beta_2$, and in this reaction wild-type α subunits and all α-CRM's examined display the same specific activity. Furthermore, it has been shown that under the growth conditions employed, the α and β subunits are produced in equimolar amounts in a Trp$^+$ cell. The ratio of α-CRM/β_2, therefore, directly measures the efficiency of suppression; the amount of β_2 measures the total number of times translation of the A and B cistrons has been attempted, and the amount of α-CRM measures the number of times suppression of the nonsense codon has occurred.

Table 5 summarizes the measurements made for the same *trpA* gene amber lesion with the known amber supressors *su*1, *su*2, and *su*3 and the new suppressors *su*7 and *su*8. Clearly, *su*7 is an efficient suppressor while *su*8, in common with other ochre suppressors which have been studied, is relatively inefficient.

TABLE 5. *Efficiency of suppression.*

Strain	Specific activity TSase β_2	Specific activity TSase α CRM	α/β_2	Efficiency of suppression
$R^-_{trp}trp^+$	14.6	14.4	0.99	(100%)
LS302*su*1	8.4	3.8	0.45	45
LS302*su*2	7.4	0.65	0.088	9
LS302*su*3	10.4	6.9	0.66	67
LS302*su*7	13.5	10.3	0.76	77
LS302*su*8	6.6	0.30	0.045	5

The values for α CRM have been corrected for the 3% activity of the isolated β_2 subunit in the conversion of indole to tryptophan.

Discussion.—Hill *et al.*[10] have described a missense suppressor which, in a haploid cell, causes multiple phenotypic alterations; these include an extended generation time in minimal media and cell death upon exposure to tryptone broth. These effects are completely absent if this suppressor is present in merodiploids which also contain an *su*$^-$ allele. This implies that the absence of the *su*$^-$ allele and not the presence of the *su*$^+$ is responsible for the haploid phenotype. The nonsense suppressors characterized in this paper are very similar except that the absence of an *su*$^-$ gene in this case is lethal, not merely injurious.

If suppression results from a tRNA modification that permits pairing with a nonsense triplet, then the original coding specificity must concomitantly be lost. This has been demonstrated in the case of *su*3. *In vitro* experiments[6] and sequence analysis[26] have shown that *su*3 results from an anticodon alteration GUA → CUA which allows pairing with UAG, the amber triplet, but abolishes interaction with the normal tyrosine codons UA^U_C. The *su*3 mutation is not lethal, however, since an additional $tRNA^{Tyr}$ species can translate the latter triplets. Perhaps for other amino acids such tRNA degeneracy does not exist, and thus the required anticodon change leaves a gap in the cellular decoding dictionary. For example, there is a single codeword for tryptophan, UGG, which requires that the $tRNA^{Trp}$ anticodon be CCA. A mutation in the anticodon CCA → CUA should allow insertion of tryptophan in response to amber triplets. If there is only a single $tRNA^{Trp}$ gene, the mutation to *su*$^+$ would prevent the cell from incorporating tryptophan into its usual positions in proteins.

No amber suppressor tRNA's derived from $tRNA^{Glu}$ or $tRNA^{Lys}$ have been

detected. The failure to observe these predicted suppressors may be explained if the suppressor mutation affects other properties of the tRNA, rendering it nonfunctional (e.g., the aminoacyl synthetase can no longer charge it, or it cannot be properly modified). Another possibility is that each $tRNA^{Glu}$ species translates $GA\overset{A}{G}$ and each $tRNA^{Lys}$ species translates $AA\overset{A}{G}$: this requires the anticodons UUC (for glutamic acid) and UUU (for lysine). Neither of these can be converted to the anticodon needed to translate UAG specifically (CUA) by a single base change. If either explanation is correct, these suppressors would not be detectable in haploid or diploid cells.

However, if there was a $tRNA^{Glu}$ specific for GAG (anticodon CUC) or a $tRNA^{Lys}$ specific for AAG (anticodon CUU), each could give rise to suppressors of UAG (anticodon CUA). If these were the major tRNA species for the respective amino acids, their loss might produce a deficiency of tRNA for translating glutamic acid and lysine codons. In that case, the presence of both the suppressor and normal alleles would still permit synthesis of the wild-type tRNA's for glutamic acid and lysine and thereby ensure survival.

The method described in this paper can be employed to look for suppressors in any region of the genetic map that can be incorporated into an F′. Recessive lethal missense suppressors, as well as nonsense suppressors, can be isolated. In the case of missense suppressors, suppression could result from an aminoacyl synthetase mutation rendering the enzyme capable of charging a heterologous tRNA to give aay-$tRNA^{AA}x$. If the mutation rendered the synthetase incapable of charging one or more of its homologous tRNA species, the cell would die unless a second gene copy supplied normal synthetase. Codon-specific suppressors arising from other components of the protein biosynthetic complex are also not eliminated, but no mechanism can be suggested.

A unique genetic feature of recessive lethal suppressors is that a cell containing such a suppressor and grown under conditions where the suppressor is required must maintain two copies of the suppressor gene; that is, there is no way a recombinational event can result in the production of a viable, truly haploid cell. This requirement may prove useful for the stabilization of large episomes which otherwise are easily lost.

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