SUPPRESSION BY GENE SUBSTITUTION FOR THE leuD GENE OF
SALMONELLA TYPHIMURIUM

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A recent review of suppression by GORINI and BECKWITH (1966) classifies suppressors in terms of direct (intragenic or informational) and indirect suppression. In the former case the suppressor functions by causing an alteration in the mutated genes usually at the transcriptional or translational level. In the latter case the primary mutational lesion is circumvented without altering the mutated gene product.

This report describes a case of indirect suppression in Salmonella typhimurium in which a suppressor mutation, at a locus (supQ) substantially distant from the leucine operon, results in the formation of a cytoplasmic product which can substitute for the product of a mutated leuD gene and thereby eliminate leucine auxotrophy. In the wild type, the leuD gene product normally forms a complex with the leuC gene product to form the isopropylmalate isomerase of the leucine biosynthetic pathway. The suppression of leuD mutations by supQ must therefore take place at the level of protein–protein interaction. The rather unusual pattern of suppressor specificity which results is also described and explained. Preliminary reports of this work were presented previously (KEMPER and MARGOLIN 1968a, 1968b). We will use bold face type to indicate the phenotype as illustrated in MATERIALS AND METHODS.

MATERIALS AND METHODS

The media and procedures for transduction used in this work have been described previously (MARGOLIN 1963). All the genetic data are derived from transductions with P22 bacteriophage at a multiplicity of about 15 plaque-forming units per bacterium. For quantitative results the wild-type strain (H1) of P22 bacteriophage was used. For recovery of phage-sensitive (i.e. non-lysogenic) recombinants, used in further crosses, we used a semivirulent mutant VI (ZINDER 1958), or an integration-negative, int4 (SMITH and LEVINE 1967), mutant of P22. As a standard procedure such recombinants were cloned at least twice by single colony isolation. Replica plating (LEDERBERG and LEDERBERG 1952) was used to determine unselected phenotypes of transductant colonies. When testing or selecting for the ara character, the medium contained 0.2% L-arabinose and glucose was omitted. Where required, L-arginine, uracil and/or L-leucine was added to the medium at a concentration of 20 μg/ml.

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We will use the words "cross" and "transduction" interchangeably and a cross $A \times B$ shall always be understood as a transduction with the recipient $A$ and the donor strain $B$. Bold face type with the superscripts + or - (e.g. ara$^+$ leu$^-$) will be used as symbols to describe the phenotype of recombinants as distinct from the italics used for the genotype (ara$^+$ leu$^-$). As to the suppressor terminology, we will define the wild-type allele as supQ$^+$, whereas supQ or supQ1 will indicate the allele with suppressor activity (capable of conferring leucine prototrophy).

The mutant strains used in this work were derived from strain LT-2 (Zinder and Lederberg 1952). Seven of the leu $D$ mutant strains (four digit designations) were generously supplied by Dr. J. M. Calvo who isolated and initially mapped them. The tester strains (SB391, SB392 and SB393) for identifying nonsense mutations (Berkowitz, Hushon, Whitfield, Roth and Ames 1968) were kindly supplied by Drs. D. Berkowitz and B. N. Ames. The following abbreviations will be used: NG for N-methyl-N-nitro-N-nitrosoguanidine, DES for diethyl sulfate, NA for nitrous acid and AP for 2-aminopurine.

RESULTS

Origin and nature of leuD700ara: The mutation leuD700 arose after nitrous acid treatment of a LT-2 strain bearing the ara-9 mutation and was characterized in a complementation test by abortive transduction as a leu $D$ mutation. As shown in Figure 1, the leu $D$ gene is the most operator-distal gene of a cluster of four leu genes which comprise the leucine operon (Margolin 1963; Burns, Calvo, Margolin and Umbarger 1966). Mapping of leuD700 ara-9 showed that no leu$^+$ recombinants were obtained in crosses with the five most operator-distal markers of the leu $D$ gene, at least two of which give substantial leu$^+$ recom-
binants in crosses with each other (see Figure 3). The mutation leuD700, therefore, seems to delete the operator-distal end of the leuD gene. As a part of our standard mapping procedures, leuD700 ara-9 was crossed with the wild-type strain as a donor to obtain a leu D700 (ara+) recombinant strain; more than 10,000 ara- recombinants tested were 100% leu+. This strongly suggests that the deletion leuD700 extends into or through the arabinose gene cluster, and thus does not allow a recombinational separation of the leu and ara markers. Consequently we will refer to this extensive deletion as leuD700ara. It seemed worth testing the possibility that the leuD700ara mutation deleted the entire arabinose gene cluster and extended towards the pyrA locus (see Figure 1). Markers of ara and pyrA are not cotransducible; no cotransductants with the ara-9 marker had been found among approximately 70,000 recombinants for a single marker (pyrA). The chromosomal region carrying both the ara and the pyrA marker is probably too large to be contained in a P22-transducing particle. In the transduction pyrA401 × leuD700ara, however, approximately 0.1% of the more than 100,000 pyrA+ recombinants tested were leu- ara-, indicating that the deletion leuD700ara was cotransducible with pyrA. Apparently it deletes the entire arabinose gene cluster and extends further towards the pyrA locus. As expected, no such cotransductants were found in the reciprocal cross leuD700ara × pyrA401, since the donor strain is wild type as far as the chromosomal distance between markers is concerned.

Nature of the supQ mutation: The leuD700ara strain gave rise, at a very low frequency, to leu+ ara- colonies. Since leuD700ara appeared to be a deletion it was difficult to visualize a reversion mechanism, especially since only the leucine auxotrophy part of the leu- ara- phenotype was changed. Suspecting suppression, tests were made to determine if such leu+ ara- strains contained the original deletion leuD700ara plus an unlinked suppressor mutation. The experiment utilized the fact that the leuD700ara deletion can be cotransduced with pyrA. The transduction pyrA401 × leu+ ara- (tentatively considered to have the genotype: leuD700ara suppressor) was performed, pyrA+ recombinants selected and the arabinose and leucine characters determined by replica plating upon appropriate media. Just as had been found when using the original leuD700ara strain as donor, about 0.1% of more than 100,000 pyrA+ recombinants tested were leu- ara-. Three such leu- ara- recombinants were subjected to the same transduction mapping procedures used to delineate the extent of the original leuD700ara deletion. These revealed that the recombinants were in every respect identical to the original leuD700ara strain, thus establishing that the leu+ ara- strain does in fact contain the leuD700ara deletion plus a mutation which suppresses the leucine auxotrophy. From now on we will refer to the locus whose mutant allele confers leucine prototrophy upon leuD700ara as supQ. The specific mutant allele being used here will be referred to as supQ1. In the above mentioned pyrA401 × leuD700ara supQ1 transduction there was no indication of a cotransduction of leuD700ara and supQ1 since no leu+ ara- recombinants were found. We therefore conclude that the supQ locus is not closely linked to the leucine operon and certainly does not map between the leu-ara region and the
pyrA locus. While these results do not exclude a linkage of supQ and the leu loci on the opposite side of the leucine operon, the following experiment served to eliminate this possibility. The strain leuABCD447, with a deletion of the entire leucine operon (MARGOLIN 1963), was crossed with the donor strain leuD700ara supQ1. A cotransduction of leuD700ara and supQ1, i.e. joint integration of both donor markers into the recipient chromosome, would lead to leu+ ara- recombinants. No leu+ recombinants of any type (ara+ nor ara-) were found in extensive tests, thus confirming that supQ1 is unlinked to the leu-ara region of the chromosome. A high frequency of leu+ ara- recombinants obtained from the transduction leuD700ara × leuD700ara supQ1 proved that the supQ1 marker is, in fact, readily transferred by P22-mediated transduction into an appropriate recipient.

Evidence for specificity of suppression by supQ1: Since supQ1 appeared capable of suppressing a deletion (leuD700ara) it was worth considering that it might have a fairly nonspecific suppressing ability. In the previous section, however, it was shown to be unable to suppress a deletion of the entire leucine operon (leuABCD447) since no leu+ recombinants were obtained when the supQ1 containing strain was used as a donor. To explore the degree of specificity of supQ1 suppression the two strains, leuD700ara and leuD700ara supQ1, were used as recipients in transductions with a series of donor strains carrying different mutations in the leucine operon. The selection was for ara+ (ability to utilize arabinose as a carbon source) and the leucine character was checked by replica plating. The frequencies of leu- colonies are given in Table 1 for a few representative cases. For the genetic map position of the donor leu mutational sites, see Figure 1. These were determined by transduction mapping procedures described previously (MARGOLIN 1963). The frequency of integration of a donor leu marker (cotransduction of leucine auxotrophy with ara+) should represent a measure of the distance between the recipient's deletion (leuD700ara) and the donor marker in those cases in which the suppressor plays no role. The table shows clearly that

<table>
<thead>
<tr>
<th>Donor</th>
<th>leuD700ara</th>
<th>Recipient leuD700ara supQ1</th>
</tr>
</thead>
<tbody>
<tr>
<td>leuA124</td>
<td>73.5</td>
<td>73.9</td>
</tr>
<tr>
<td>leuA428</td>
<td>77.2</td>
<td>79.0</td>
</tr>
<tr>
<td>leuB129</td>
<td>86.1</td>
<td>83.6</td>
</tr>
<tr>
<td>leuB410</td>
<td>86.8</td>
<td>87.3</td>
</tr>
<tr>
<td>leuC724</td>
<td>94.6</td>
<td>93.1</td>
</tr>
<tr>
<td>leuC157</td>
<td>92.6</td>
<td>92.0</td>
</tr>
<tr>
<td>leuD745</td>
<td>96.0</td>
<td>97.5</td>
</tr>
<tr>
<td>leuD128</td>
<td>98.8</td>
<td>0</td>
</tr>
<tr>
<td>leuD491</td>
<td>99.9</td>
<td>99.7</td>
</tr>
<tr>
<td>leuD466</td>
<td>100.0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Established by replica plating a minimum of 1,000 ara+ colonies for each cross.
the frequencies for the recipient strain \( \text{leuD700ara supQ1} \) match those for the strain \( \text{leuD700ara} \) except for the crosses with donor strains \( \text{leuD128} \) and \( \text{leuD466} \). In these two instances the frequency of \( \text{leu}^- \) among the \( \text{ara}^+ \) recombinants is zero, when the \( \text{supQ1} \) allele is present in the recipient.

These results can be explained if the \( \text{leuD128} \) and \( \text{leuD466} \) mutant sites were integrated into the \( \text{leuD700ara supQ1} \) strain (replacing the deletion \( \text{leuD700 ara} \)) with the same frequency as they were integrated into the \( \text{leuD700ara supQ1} \) strain but the suppressor \( \text{supQ1} \) suppressed the leucine auxotrophy imposed by these two mutations. This hypothesis predicts that most of the \( \text{leu}^+ \text{ara}^+ \) recombinants from, for example, the \( \text{leuD700ara supQ1 } \times \text{leuD128} \) cross should in fact have the genotype \( \text{leuD128 supQ1} \), and thus a suppressed leucine auxotrophy. To test the hypothesis, ten of the \( \text{leu}^+ \text{ara}^+ \) recombinants from the above cross were selected at random, cloned by single colony isolation, and used as donors in transductions with a \( \text{leuD128 ara-9} \) strain as recipient. If, as predicted, most (about 98.8\% according to Table 1) of the \( \text{leu}^+ \text{ara}^+ \) recombinants actually harbored the \( \text{leuD128} \) mutation these strains could donate leucine prototrophy only via their \( \text{supQ1} \) alleles. Since we had previously demonstrated that the \( \text{supQ} \) locus was unlinked by transduction to the \( \text{leu-ara} \) region of the chromosome, it was expected that in most of the ten crosses, 100\% of the \( \text{leu}^+ \) recombinants would retain the \( \text{ara-9} \) allele and be unable to grow on a medium containing arabinose as sole carbon source. More than 10,000 \( \text{leu}^+ \) recombinants from each of the ten crosses were tested by replica plating to an arabinose agar medium. In all ten cases 100\% of the \( \text{leu}^+ \) recombinants were found to be arabinose negative, thus conforming to the prediction. As a further test of the hypothesis, \( \text{ara}^+ \) recombinants were selected in the same ten crosses. Once again the prediction is no linkage of \( \text{ara}^+ \) to the genetic component (\( \text{supQ1} \)) which confers leucine prototrophy. Tests by replica plating of more than 10,000 \( \text{ara}^+ \) recombinants from each cross revealed that in every case, as predicted, they were 100\% leucine auxotrophs.

The above experiments with \( \text{leuD128} \) demonstrated that most \( \text{leu}^+ \) recombinants contained the \( \text{leuD128} \) mutation, which was suppressed by the presence of \( \text{supQ1} \). The complementary experiment, for \( \text{leu} \) mutations not suppressed by \( \text{supQ1} \), would be to demonstrate that the \( \text{ara}^+ \text{leu}^- \) recombinants do, in fact, harbor the \( \text{supQ1} \) allele. For example, an \( \text{ara}^+ \text{leu}^- \) recombinant from the cross, \( \text{leuD700ara supQ1 } \times \text{leuA124} \), should have the genotype \( \text{leuA124 supQ1} \). One such recombinant was used as recipient in the second transduction of the set of three illustrated in Figure 2, the first and third transduction illustrated in the figure served as controls. In all three transductions \( \text{leu}^+ \) recombinants were selected and the frequency of integration of the donor's \( \text{ara-9} \) (arabinose negative) marker determined. In transduction #1 the donor carried a wild-type leucine operon. Only two crossover events were required to produce the 32.3\% \( \text{leu}^+ \text{ara}^- \) recombinants found, as shown in Figure 2, transduction #1. We have demonstrated that \( \text{supQ1} \) suppresses \( \text{leuD128} \). Therefore the presence of \( \text{supQ1} \) in the recipient of transduction #2 would be expected to and, as shown, in fact did result in approximately the same frequency of \( \text{leu}^+ \text{ara}^- \) recombinants (38.0\%). In other words, as far as a \( \text{supQ1} \)-bearing recipient is concerned, a
transductions carrying the leuD128 mutation is the equivalent of a fragment with a wild-type leucine operon. In transduction #3 the recipient lacked the supQ1 allele. As illustrated in the figure, at least four recombinational events were required to produce leu\textsuperscript{+} ara\textsuperscript{-} transductants, resulting in the significantly lowered frequency of 9.0% with which they were found. We feel that the similarity in the frequencies of leu\textsuperscript{+} ara\textsuperscript{-} recombinants from transduction #1 and #2 conforms well to our interpretation.

**Pattern of suppression specificity:** A total of 70 leucine auxotrophic mutations of independent origin were tested for their capacity to be suppressed by supQ1. The tests were the same as described in the previous section. The appearance of zero leu\textsuperscript{-} colonies among the ara\textsuperscript{+} recombinants in crosses of a leucine auxotroph as donor and leuD700ara supQ1 as recipient was taken as evidence for the suppression of the leucine mutation by supQ1. The results are summarized in

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**Figure 2.—** A diagrammatic representation of three transductions in which leu\textsuperscript{+} colonies were selected and the arabinose character determined by replica plating. Frequencies of leu\textsuperscript{+} ara\textsuperscript{-} clones are given in the right-hand column. The top line represents the transducing particle, the lower line the recipient's chromosome and the broken line indicates the simplest crossover pattern that leads to a phenotypic leu\textsuperscript{+} ara\textsuperscript{-} colony. For further details see text.

<table>
<thead>
<tr>
<th>Transduction</th>
<th>Leucine</th>
<th>Ara</th>
<th>SupQ</th>
<th>leu\textsuperscript{+} ara\textsuperscript{-}</th>
</tr>
</thead>
<tbody>
<tr>
<td>leuAl24 x ara9</td>
<td><img src="leuAl24_x_ara9.png" alt="Diagram" /></td>
<td><img src="ara9.png" alt="Diagram" /></td>
<td><img src="supQ.png" alt="Diagram" /></td>
<td>32.3%</td>
</tr>
<tr>
<td>leuAl24 supQ1 x leuD128 ara9</td>
<td><img src="leuAl24_supQ1_x_leuD128_ara9.png" alt="Diagram" /></td>
<td><img src="ara9.png" alt="Diagram" /></td>
<td><img src="supQ1.png" alt="Diagram" /></td>
<td>38.0%</td>
</tr>
<tr>
<td>leuAl24 x leuD128 ara9</td>
<td><img src="leuAl24_x_leuD128_ara9.png" alt="Diagram" /></td>
<td><img src="ara9.png" alt="Diagram" /></td>
<td><img src="supQ.png" alt="Diagram" /></td>
<td>9.0%</td>
</tr>
</tbody>
</table>
**TABLE 2**

The pattern of specificity of supQ1 suppression as determined by transduction tests of mutations in the various leu genes

<table>
<thead>
<tr>
<th></th>
<th>leuO</th>
<th>leuA</th>
<th>leuB</th>
<th>leuC</th>
<th>leuD</th>
<th>Multicistronic* deletions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mutations tested</td>
<td>1</td>
<td>14</td>
<td>8</td>
<td>14</td>
<td>25</td>
<td>9</td>
</tr>
<tr>
<td>Number of mutations suppressible by supQ1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>0</td>
</tr>
</tbody>
</table>

leuO mutations: leuO0500.
leuA mutations: leuA32, 120, 124, 125, 158, 428, 430, 469, 475, 511, 517, 526, 552, 554.
leuB mutations: leuB129, 140, 455, 578, 667, 698, 706, 776.
leuC mutations: leuC20, 123, 126, 127, 130, 131, 156, 157, 159, 456, 459, 528, 531, 724.
leuD mutations: see Figure 3.

* Each deletion involves two or more leu genes: leuOABCD447, leuOABC695, leuABCD702, leuABCD5150, leuB0CD39, leuB0CD485, leuCD4068, leuCD5128, leuBC5148.

Table 2. Of 46 mutations involving genes other than leuD none is suppressed. In contrast, supQ1 suppresses about two-thirds of the mutations which only affect the leuD gene. It is quite clear that only mutations in the leuD gene and only certain of these are suppressible by supQ1. The sites of suppressible and nonsuppressible leuD mutations are interspersed among each other as seen in Figure 3.

**leuD**

\[
\begin{align*}
\{3192, 3290, 3329, 3638\} & \quad 3065 \\
503 & \quad 21 \\
538 & \quad 534 \\
745 & \quad 615 \\
125 & \quad 657 \\
449 & \quad 657 \\
503 & \quad 657 \\
438 & \quad 657 \\
594 & \quad 657 \\
491 & \quad 657 \\
474 & \quad 657 \\
479 & \quad 657 \\
466 & \quad 657 \\
\end{align*}
\]

**Figure 3.**—A map of the leuD gene. Black numbers indicate mutations that are not suppressible by supQ. White numbers on a black background indicate mutations that are suppressible by supQ. The four mutations in the top row (3192, 3290, 3329 and 3638) are located in the region indicated by the parentheses. Their exact position and order, however, has not been determined. In all cases in which more than one mutation is listed above a single site, the sites of the members of such a group are either identical or so close that their sequence is difficult to determine with certainty.
Of the 25 original leuD mutations tested, four have been identified as multisite mutations (presumably deletions). The short deletion leuD657, which covers two suppressible and one nonsuppressible mutation, is not suppressed by supQ1. Three deletions which delete the operator-distal end of the leuD gene (leuD700ara, leuD5006 and leuD5115) are suppressible. The two additional deletions, leuD798ara and leuD799ara, shown in Figure 3 are also suppressible. The procedures used for obtaining these two deletions (to be described later) only permitted selection of suppressible leuD mutations. Therefore, they have been omitted from Tables 2 and 3 which characterize 25 leuD mutations whose acquisition was not biased by such selection procedures. It is worth noting that leuD798ara appears to delete most, if not all, of the leuD gene. Transduction tests indicate that leuD798ara covers all the point mutations thus far mapped in the leuD gene, but does not extend into the leuC gene.

Model for the mode of suppression by supQ: The suppression of deletions of the leuD gene indicates that the supQ1 gene codes for a product which can act as a competent replacement for the leuD gene product. The following hypothesis is proposed to account for the fact that certain mutations in the leuD gene are not suppressed, although the supQ1 gene product apparently substitutes for the total leuD gene product.

Isopropylmalate isomerase, the second enzyme specific to the biosynthesis of leucine, probably consists of two different polypeptide subunits coded by the leuC and leuD genes (Gross, Burns and Umbarger 1963). Figure 4 illustrates the leucine biosynthetic pathway and the related genes and enzymes. Accordingly, the leuC and leuD gene products would normally combine (Figure 5a) to form an active isomerase. Since the leuD product is substituted for by the supQ1 product we conclude that the latter is also able to combine with the leuC product and that the complex thus formed has isomerase activity (Figure 5b). An obvious requirement would be the availability of free leuC product to combine with the supQ1 product. It seems reasonable to expect that a substitute, such as the supQ1 product, created by a single mutational event, would compete poorly with the normally evolved leuD product in terms of complex formation affinity for the

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**Figure 4.**—Map of the leucine operon showing the enzyme products of the genes and the site of activity of each enzyme in the leucine biosynthetic pathway. IPM: isopropylmalate.
**Figure 5.**—Diagram of the proposed mode of interactions of leuC, leuD and supQ1 protein products to explain the pattern of supQ1 suppressor specificity described in the text. (a) non-mutant leuD gene: formation of active isomerase from subunits coded for by wild-type leuC and leuD genes. (b) suppressible leuD gene mutation: formation of active isomerase from leuC gene and supQ1 gene protein products. (c) non-suppressible leuD gene mutation: formation of an inactive isomerase complex between leuC and mutant leuD gene protein products thereby preventing the formation of an active leuC-supQ1 protein complex.
leuC product. Some leuD mutations might result in synthesis of mutant peptides which retain affinity for the leuC product, but form complexes lacking enzyme activity. Such leuD peptides would exclude the supQ1 product and thus prevent formation of an enzymically active complex of the products of leuC and supQ1 (Figure 5c). Hence the two classes of leuD mutations, suppressible and nonsuppressible, can be explained by whether or not they result in the availability of free leuC product. On the basis of this hypothesis suppressible mutations would include all those which result in the absence of a leuD product or in any alteration which eliminates the ability to combine with leuC product. The types of leuD mutations expected to have such an effect would consist mainly of major deletions, nonsense mutations and frameshift mutations. In addition, certain specific missense mutations might affect the complexing ability. In contrast, the nonsuppressible leuD mutations would be those which result in formation of a mutant peptide capable of combining with leuC product. These would be expected to consist of missense mutations or small deletions which do not create a frameshift and are outside the region governing the complexing ability.

It is interesting to note that all mutations (both deletions and point mutations) of the most operator-distal portion of the leuD gene are suppressible by supQ1 (see Figure 3). Conceivably this portion of the leuD gene codes for a segment of the peptide product which has an important role in the ability to complex with the leuC gene product. If true, most mutations in this region which cause leucine auxotrophy might also reduce the capacity for complex formation and thereby be susceptible to suppression by supQ1.

Experimental tests of the model: The hypothesis requires that most leuD deletions and nonsense mutations be suppressible by supQ1, whereas nonsuppressible mutations would largely consist of missense mutations. Table 3 summarizes the results of examining the 25 available leuD mutations with regard to these characteristics. A mutation was considered a deletion if it produced no leu+ recombinants in crosses with at least two other leuD mutations having sites separable by recombination. A positive response to a test for la nonsense mutation consisted of a capacity for reversion-induction by NG plus a positive response to amber and ochre tester strains (Berkowitz, Hushon, Whitfield, Roth and Ames 1968).

**TABLE 3**

Characterization of leuD mutations

<table>
<thead>
<tr>
<th>leuD mutations</th>
<th>Test for nonsense types among point mutations</th>
<th>Deletions</th>
<th>Not testable*</th>
<th>Positive response</th>
<th>Negative response</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-suppressible by supQ1</td>
<td></td>
<td>1</td>
<td>0</td>
<td>7†</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Suppressible by supQ1</td>
<td></td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

* Mutations giving no reversion response to N-methyl-N'-nitro-N-nitrosoguanidine; nonsuppressible: leuD3638; suppressible: leuD128, 466, 474, 479.
† leuD438, 444, 491, 615, 745, 3192, 3329.
‡ leuD21, 449, 505, 534, 538, 594, 603, 3065, 3290.
Nonsuppressible deletion: leuD657.
Suppressible deletions: leuD5115, leuD5046, leuD700ara.
The third known nonsense suppressor type, UGA, was not tested for. The data in the table clearly satisfy the specific requirements of the model. Three out of four deletion mutations are suppressible. Most of the other suppressible mutations gave positive responses to the test for nonsense mutations. In contrast, none of the nonsuppressible leuD mutations gave a positive response in these tests.

The second test concerned a prediction of the model, that nonsuppressible leuD mutations would show a significantly higher frequency of reversion to the leu+ phenotype in the presence of the supQ1 allele. This was based upon the hypothesis that nonsuppressible leuD mutations are leu- in the presence of supQ1 because a leuD mutant protein product is formed which ties up all available leuC product. One might expect that certain secondary mutations in the leuD gene would either prevent the formation of a leuD product or further alter the leuD product so that it could no longer complex with the leuC product. The occurrence of any such secondary leuD mutation would make leuC product available to combine with the supQ1 product and result in a leu+ phenotype. To test this prediction, the leu+ reversion frequency of the nonsuppressible leuD491 mutation was determined in a strain bearing the supQ1 allele and compared to that in a strain harboring the supQ1 allele. As predicted, the reversion frequency in the presence of supQ1 was at least 100-fold higher than in its absence.

An even more stringent test examined the effect of supQ1 upon the reversion frequency of the nonsuppressible deletion mutation leuD657. Platings, under selective conditions, of more than 10^11 cells of the leuD657 supQ+ strain had produced no leu+ colonies. When a strain was constructed with the genotype leuD657 supQ1, a plating of about 2 x 10^8 cells gave rise to more than 200 leu+ colonies. According to the model the leu+ colonies which arose in the leuD657 supQ1 strain should be the result of secondary leuD mutations, such as deletions and nonsense mutations, which eliminate the formation of a mutant leuD product capable of combining with leuC product.

To examine many of the leu+ clones for the presence of such secondary leuD deletion mutations would have involved extensive recombination tests with many leuD point mutations. To avoid this, we considered the possibility that some secondary deletion mutations might extend into or through the arabinose operon. Such deletions would be easily detected by the simultaneous occurrence of ara- and leu+ phenotypes. Seven such clones were readily found. These apparent long deletions were then examined to see if they extended far enough towards pyrA to allow them to be cotransduced with pyrA+, as had been found for the original leuD700ara deletion. Three of them, leuD798ara, leuD799ara and leuD800ara, showed cotransduction frequencies with pyrA+ ranging from 0.2% to 0.4%.

Using the cotransduction with pyrA+, we constructed leucine auxotrophic strains bearing the secondary leuD798ara and leuD799ara deletions plus, presumably, the original leuD657 deletion but lacking the supQ1 allele. These essentially nonreverting leucine auxotrophic strains were then used as recipients in crosses with many of the available leuD mutant strains to determine the extent of the deletion in the leuD gene. As mentioned in a previous section, leuD-798ara appears to be a deletion of the entire leuD gene. Therefore, the original,
small deletion, \textit{leuD657}, can no longer exist in this strain. In contrast, the deletion \textit{leuD799ara} seems to involve only the operator-distal segment of the \textit{leuD} gene and the original \textit{leuD657} deletion is retained (see Figure 3). We conclude that some of the \textit{leu}+ revertants of strain \textit{leuD657 supQ1} do, in fact, result from secondary deletions in the \textit{leuD} gene and their occurrence satisfies the prediction of the model.

Nonsense mutations were another major type of secondary \textit{leuD} mutation expected to be the cause of \textit{leu}+ clones arising in the \textit{leuD657 supQ1} strain. To test for the occurrence of such secondary nonsense \textit{leuD} mutations, a strain with the genotype \textit{leuD657 supQ1 trpA50} was constructed. The \textit{trpA50} mutation had previously been identified as an \textit{amber} nonsense mutation (Bauerele and Margolin 1966). The \textit{leuD657 supQ1 trpA50} strain had a \textit{leu}- \textit{trp}- phenotype. Twenty \textit{leu}+ revertants of independent origin were selected and cloned by single colony isolation. These strains with a \textit{leu}+ \textit{trp}- phenotype presumably arose as a result of secondary \textit{leuD} mutations which eliminated the formation of mutant \textit{leuD} products and allowed the \textit{supQ1} product to combine with the resulting free \textit{leuC} product. If any of these secondary mutations were an \textit{amber} mutation, its phenotypic effect could theoretically be reversed (to \textit{leu}-) by the presence of an \textit{amber} suppressor mutation. The occurrence of such an event would identify the secondary \textit{leuD} mutation as an \textit{amber} mutation. Furthermore the reversal of the \textit{leu} phenotype by a nonsense suppressor would provide substantial support to our explanation of the mechanism of \textit{supQ1} suppression. We have conjectured that the suppressibility of a \textit{leuD} mutation by \textit{supQ1} is determined by the presence or absence of a mutant \textit{leuD} protein product capable of combining with \textit{leuC} product rather than by the structure of the actual mutational site in the \textit{leuD} gene. A nonsense suppressor acting at the translation level allows us to overcome the termination of protein synthesis without altering the mutant character of the \textit{leuD} gene.

The \textit{trpA50} amber mutation in the twenty \textit{leu}+ \textit{trp}- strains enabled us to obtain nonsense suppressor mutations by simply selecting for \textit{trp}+ revertants on a minimal agar medium supplemented with leucine. The revertants obtained were then replica plated to unsupplemented minimal agar medium to see if any of the \textit{trp}+ colonies had simultaneously become \textit{leu}- Four of the twenty \textit{leu}+ \textit{trp}- strains gave rise to such \textit{leu}- \textit{trp}+ colonies. Several of these \textit{leu}- \textit{trp}+ colonies from three of the strains were cloned by single colony isolation and checked with the amber and ochre tester strains for the expected presence of nonsense suppressors. In all three cases \textit{leu}- \textit{trp}+ colonies were found which gave positive responses to the tests for the presence of nonsense suppressors. We do not believe that these tests allowed us to positively discriminate between amber and ochre suppressors in all cases. However, we feel that they clearly indicate that these \textit{leu}- \textit{trp}+ clones contain nonsense suppressors capable of suppressing the \textit{trp}- phenotype of the \textit{trpA50} amber mutation, the \textit{leu}+ phenotype imposed by a secondary \textit{leuD} nonsense mutation and the \textit{lac} phenotype of the \textit{lacX82} amber mutation on the \textit{Flac} episome from the SB391 tester strain (Berkowitz, Hushon, Whitfield, Roth and Ames 1968). We feel fairly confident in identi-
fying the nonsense suppressor mutation in one of the leu- trp+ clones as an amber suppressor. The genotype of this clone, therefore, would be leuD657 leuDamber supQ1 trpA50 supamber (F'lacX82).

The above tests indicate that, as predicted by the hypothesis, nonsense leuD secondary mutations cause a leu+ phenotype in the leuD657 supQ1 strain. They also confirm that nonsense suppressor mutations, acting at the level of translation, can reverse this effect, as expected if the supQ1 suppression occurs at the level of protein-protein interaction.

We should point out that, according to the model, the leu phenotype in strains such as those described above is the result of a complex interaction between the nonsense suppressor efficiency and the efficiency of the supQ1 product in its competition with the mutant leuD product for combining with leuC product. One might expect, therefore, that some nonsense suppressors are efficient enough to give rise to a trp+ phenotype without completely reversing the leu+ character of the clone. In support of this prediction we can report that the strain identified as bearing a secondary leuDamber mutation gave rise to clones with nonsense suppressor mutations which caused a trp+ lac+ phenotype and a partial leucine auxotrophy. This indicates a rather sensitive response of the leu phenotype to the efficiency of nonsense suppression. It modifies the preliminary interpretation of our finding that only four of the original twenty leu+ trp- strains gave rise to leu- trp+ clones. Initially we had assumed that only those four strains contained nonsense secondary leuD mutations. Since we tested only a limited number of trp+ clones from each leu+ trp- strain, the evidence just described suggests that some of the remaining sixteen strains might contain nonsense secondary leuD mutations which were suppressed so inefficiently that a leu- phenotype was not readily detected.

The chromosomal location of supQ: Normally following infection by P22 bacteriophage, surviving cells of strain LT-2 of Salmonella typhimurium are lysogenic. Such lysogens are resistant to lysis by superinfecting P22 phage and are not useful as recipients in transduction experiments. The lysogenic bacteria ordinarily do not cure spontaneously. It was found, however, that after infection by P22 phage, spontaneously cured clones could readily be recovered from all strains carrying the supQ1 allele. This suggested the possibility of a relationship between the P22 attachment site and the site of the supQ1 allele. A further indication of such a relationship came from the observation that suppressor mutations of the supQ1 type arose with a significantly higher frequency following phage infection, than among uninfected cells.

The above observation focused our attention upon the region of the chromosome bearing the P22 attachment site as a possible location for the supQ locus. The P22 attachment site has been shown to be closely linked to the proA gene (Young and Hartman 1965; Smith and Levine 1966) and most likely maps between proA and proC (Miyaaki and Demerec 1960; Itikawa and Demerec 1967; Sanderson 1967) (see also Figure 1). To test for the presence of the supQ locus in this region of the chromosome a strain, leuD700ara proA575, was used as a recipient in transduction with a donor strain leuD700ara supQ1. A minimal
agar medium supplemented with proline was used to select for leu+ transductants representing recombinants which had integrated the supQ1 allele. The leu+ colonies were replica plated to medium lacking proline to determine whether any of them had simultaneously acquired the pro+ phenotype conferred by the pro+ allele of the donor. Approximately 99% of the leu+ colonies were pro+, indicating an extremely close linkage of supQ1 and proA.

We have collected a large number of strains, of independent mutational origin, bearing supQ alleles which show the same pattern of suppression of leuD mutations as supQ1. These have arisen in several strains bearing suppressible leuD mutations; namely leuD466, leuD5046, leuD798ara as well as leuD700ara. The mutagens NG, DES, AP and NA have been tested and all act to significantly increase the frequency of mutations of the supQ locus to a form which allows it to substitute for the leuD gene. We have found that different supQ alleles vary in the efficiency of their suppressor activity as measured by growth rate. The suppressor efficiencies of all the alleles tested show a distinct temperature sensitivity, the least efficient alleles showing the greatest sensitivity. Mutation to supQ suppressor alleles capable of suppression at 24°C with a low efficiency, but unable to suppress at 37°C occurs with relatively high frequency when compared to the frequency of mutation to efficient suppressors capable of suppression at 37°C. A total of 28 supQ suppressor alleles of various efficiencies and modes of origin have been tested and all have been found to be linked by transduction to the proA locus. Details on the efficiency of suppression, the linkage to proA and the nature of the origin of the suppressor alleles of supQ will be presented in a subsequent paper.

DISCUSSION

The experiments clearly indicate that supQ is a substitute gene for leuD. A suggested alternative hypothesis postulates that the leuC gene product alone has isomerase activity which is inhibited by mutant leuD peptide product as well as by supQ+ gene product. The suppression would then be the result of mutations which in some way inactivate the supQ+ gene and eliminate the formation of its product. This alternative hypothesis is made unlikely by evidence that a strain bearing leuD798ara and a deletion, proAB47 (MIYAKE and DEMEREC 1960; SMITH and LEVINE 1965), of the region of the chromosome where the supQ gene normally resides is still a leucine auxotroph. The absence of the supQ gene in this strain is indicated by its inability to give rise to any supQ suppressor alleles.

We have considered the possibility that supQ originated as a translocational duplication of the mutated leuD gene with a simultaneous alteration which overcame the leuD mutational defect. This possibility was excluded since supQ suppressor mutations arose in a strain with a deletion of the entire leuD gene (leuD798ara). Alternatively supQ+ might be thought of as a preexisting dormant duplication of the leuD gene which becomes functional as a result of the supQ suppressor mutation. This mode of origin seems unlikely for several reasons. It requires that the dormant leuD gene had resisted evolutionary pressures for
extensive periods, since we have been able to demonstrate the potential for mutation to a supQ suppressor allele in strain LT7 of S. typhimurium. Furthermore, the supQ gene product competes very poorly with leuD mutant peptide products for combination with leuC product. Therefore, it seems unlikely that the supQ product and the leuD product are identical. This is also in agreement with our failure to demonstrate recombination between supQ1 or supQ+ and a mutant leuD gene. Although extensively tested, no leuD+ recombinants were obtained from transductions using the leuD657 strain (a small deletion within the leuD gene) as recipient and strains leuABCD447 supQ1 or leuABCD447 supQ+ as donors. The donor's leucine mutation, leuABCD447, is a deletion of the entire leucine operon so that leu+ recombinants could only occur if pairing and recombination could take place between the leuD and supQ DNA regions.

We should point out that it is highly improbable that the supQ+ locus is normally involved in any way with leucine biosynthesis. Among hundreds of mutations affecting leucine biosynthesis none except supQ suppressor mutations have been mapped near the proA−attP22 region of the chromosome. We do not know the normal function of the supQ locus. Mutations to the suppressor form of supQ do not cause any other noticeable change in nutritional requirements or growth characteristics. Presumably supQ normally has a function which is dispensable in growth media and under conditions thus far tested, or the suppressor form of the supQ product can also carry out is original function.

The supQ suppressor system is, to our knowledge, the first case of a gene substitution in an amino acid synthesizing system. The suppression of proA and proB mutations by arg mutations (Bacon and Vogel 1963; Itikawa, Baumberg and Vogel 1968) at first glance appears to be very similar to the supQ suppression, especially since deletion mutations are suppressed (Kuo and Stocker 1969). The suppressor mechanisms, however, differ substantially. The suppressor of the proline mutations acts by supplying the missing intermediate of the proline pathway (Bacon and Vogel 1963; Itikawa, Baumberg and Vogel 1968). In contrast, the supQ suppressor acts by providing a substitute for the missing leuD gene product. Furthermore, since the supQ suppression affects an enzyme consisting of nonidentical subunits, it occurs at the level of protein-protein interaction and results in a unique pattern of suppressor specificity.

The mutation of supQ to a form which allows it to act as a substitute for leuD has interesting evolutionary aspects. In the case where the original leuD gene is completely deleted, the presence of supQ1 results in a strain which, in essence, possesses the wild-type phenotype. In effect, supQ is a new leuD locus. The genes of the leucine biosynthetic pathway are no longer all members of a single operon. We do not know if the regulatory mechanisms involving the clustering of genes of a single metabolic pathway into an operon represent a primitive form of control, or an advanced and specialized one. However, the process which evolved a supQ1 gene certainly demonstrates a procedure for dispersing the genes of an operon. It will be interesting to see if we can devise procedures which will select mutations which bring the functioning of supQ1 into some form of parallel regu-
lation with the remaining leucine pathway genes. Hopefully we will eventually be able to identify the normal function of supQ. This would be of interest with respect to the evolution of enzyme specificity.

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SUMMARY

A suppressor gene, supQ, was found to be specific for leuD gene mutations. It suppressed a deletion of the entire leuD gene indicating that it was acting as a substitute gene and is thus a “no nonsense” suppressor. However only 16 out of 25 leuD gene mutations were suppressed. The sites of suppressible and nonsuppressible mutations were interspersed among each other. Since the isopropylmalate isomerase seems to consist of two different subunits coded for by the leuC and leuD genes, the supQ gene product must suppress at the level of protein-protein interaction. The pattern of suppressor specificity is explained by evidence suggesting that some leuD gene mutations result in formation of mutant peptide products which interfere with the interaction between the leuC and supQ gene products. The supQ locus was found to be closely linked to the proA gene by P22-mediated transduction.

LITERATURE CITED


