Evidence that the supE44 Mutation of Escherichia coli Is an Amber Suppressor Allele of glnX and that It Also Suppresses Ochre and Opal Nonsense Mutations

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Translational readthrough of nonsense codons is seen not only in organisms possessing one or more tRNA suppressors but also in strains lacking suppressors. Amber suppressor tRNAs have been reported to suppress only amber nonsense mutations, unlike ochre suppressors, which can suppress both amber and ochre mutations, essentially due to wobble base pairing. In an Escherichia coli strain carrying the lacZU118 episome (an ochre mutation in the lacZ gene) and harboring the supE44 allele, suppression of the ochre mutation was observed after 7 days of incubation. The presence of the supE44 lesion in the relevant strains was confirmed by sequencing, and it was found to be in the duplicate copy of the glnX tRNA gene, glnX. To investigate this further, an in vivo luciferase assay developed by D. W. Schultz and M. Yarus (J. Bacteriol. 172:595–602, 1990) was employed to evaluate the efficiency of suppression of amber (UAU), ochre (UAA), and opal (UGA) mutations by supE44. We have shown here that supE44 suppresses ochre as well as opal nonsense mutations, with comparable efficiencies. The readthrough of nonsense mutations in a wild-type E. coli strain was much lower than that in a supE44 strain when measured by the luciferase assay. Increased suppression of nonsense mutations, especially ochre and opal, by supE44 was found to be growth phase dependent, as this phenomenon was only observed in stationary phase and not in logarithmic phase. These results have implications for the decoding accuracy of the translational machinery, particularly in stationary growth phase.

Translation termination is mediated by one of the three stop codons (UAA, UAG, or UGA). When such stop codons arise in coding sequences due to mutations, referred to as nonsense mutations, they lead to abrupt arrest of the translation process. However, the termination efficiency of such nonsense codons is not 100%, as certain tRNAs have the ability to read these nonsense codons. Genetic code ambiguity is seen in several organisms. Stop codons have been shown to have alternate roles apart from translation termination. In organisms from all three domains of life, UGA encodes selenocysteine through a specialized mechanism. In Methanosarcinaeae, UAG encodes pyrrolysine (3). UAA and UAG are read as glutamine codons in some green algae and ciliates such as Tetrahymena and Diplomonads (24), and UAG alone encodes glutamine in Moloney murine leukemia virus (32). UGA encodes cysteine in Euplotes; tryptophan in some ciliates, Mycoplasma species, Spiroplasma citri, Bacillus, and tobacco rattle virus; and an unidentified amino acid in Pseudomicrothorax dubius and Nitotauerus ovalis (30). In certain cases the context of the stop codon in translational readthrough has been shown to play a role; for example, it has been reported that in vitro in tobacco mosaic virus, UAG and UAA are misread by tRNA\textsubscript{Trf} in a highly context-dependent manner (34, 9).

Termination suppressors are of three types, i.e., amber, ochre, and opal suppressors, which are named based on their ability to suppress the three stop codons. Amber suppressors can suppress only amber codons, whereas ochre suppressors can suppress ochre codons (by normal base pairing) as well as amber codons (by wobbling) and opal suppressors can read opal and UGG tryptophan codon in certain cases. As described by Sambrook et al. (27), a few amber suppressors can also suppress ochre mutations by wobbling. The suppression efficiency varies among these suppressors, with amber suppressors generally showing increased efficiency over ochre and opal suppressors. supE44, an amber suppressor tRNA, is an allele of tRNA\textsubscript{Trf} and is found in many commonly used strains of Escherichia coli K-12. Earlier studies have shown that supE44 is a weak amber suppressor and that its efficiency varies up to 35-fold depending on the reading context of the stop codon (8).

Translational accuracy depends on several factors, which include charging of tRNAs with specific amino acids, mRNA decoding, and the presence of antibiotics such as streptomycin and mutations in ribosomal proteins which modulate the fidelity of the translational machinery. Among these, mRNA decoding errors have been reported to occur at a frequency ranging from about 10\textsuperscript{-3} to 10\textsuperscript{-4} per codon. Translational misreading errors also largely depend on the competition between cognate and near-cognate tRNA species. Poor availability of cognate tRNAs increases misreading (18).

Several studies with E. coli and Saccharomyces cerevisiae have shown the readthrough of nonsense codons in suppressor-free cells. In a suppressor-free E. coli strain, it has been shown in vitro that glutamine is incorporated at the nonsense codons UAG and UAA (26). It has been reported that overexpression of wild-type tRNA\textsubscript{Trf} in yeast suppresses amber as...
MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Genotypes of the E. coli K-12 strains (genetic nomenclature is according to Demerec et al. [6] and Berlyn [4]) and plasmids are listed in Table 1. The medium composition used in the entire study is as described by Miller [21, 22]. All cultures were incubated at 37°C with aeration, and the plates were also incubated at the same temperature. When the plates were incubated for 10 h at 37°C. The Pro− transconjugants were selected by streaking on minimal medium with the appropriate antibiotic and without plaques as described above. supE44 was transduced into desired strains with supE44 allele into desired strains by selecting the transconjugants (Pro− lacZU118 oprA− proB−).

Assay of luciferase activity. The assay of luciferase activity was performed as previously described (28) with minor modifications as follows. Strains for luciferase assay were constructed by transforming the strains MG1655 and MMH1004 with pLUX1, pLUX2, pLUX3, and pLUX4 plasmid clones. Chemical transformation was done as previously described (27). Transformants were purified twice on selective plates before proceeding further. Saturated overnight cultures were diluted 1:10 into the same medium and used directly for the assay. Logarithmic-phase cultures were used without dilution. n-Decyl aldehyde (Sigma-Aldrich, Bangalore, India) was diluted 1:100 in 100% ethanol before use. Two hundred microliters of the cultures was dispensed into a 96-well transparent microwell plate (Tarsons, Kolkata, India), and the optical density at 600 nm (OD600) was measured in a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale, CA); 150 μl of the cultures was then transferred into a 96-well white microwell plate (Nunc, Rochester, NY) to which 15 μl of n-decyl aldehyde was added. Light emission at room temperature was determined using standard settings in the instrument. The luciferase activity was expressed as relative luminescence units normalized with respect to the respective OD values. Suppression efficiency was calculated as the ratio of the activity of the strain containing the luciferase gene with the nonsense mutation to that of the strain containing the wild-type luciferase gene, both with the supE44 allele (28).

RESULTS AND DISCUSSION

Evidence for weak suppression of lacZ ochre mutation by supE44. Studies pertaining to postplating mutagenesis have been under way in our laboratory for quite some time (12, 13, 14, 15). During the course of his investigation, Jayaraman (13) had isolated, mapped, and characterized the mutation named pmm, referring to postplating mutagenesis, and he reported that pmm could enhance the intrinsic misreading of mutant codons and thus promote postplating mutagenesis. It was also reported that colonies of a pmm mutant bearing F−lacZU118(Oc) growing in an IPTG−X-Gal medium appear initially as white colonies but gradually turn blue over a period

TABLE 1. E. coli K-12 strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Source (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td>F− λ− rph-1</td>
<td>M. K. B. Berlyn, CGSC</td>
</tr>
<tr>
<td>LE392</td>
<td>supE44 supF85 hsdR514 galK2 galT2 metB1 trpR55 lacY1</td>
<td>M. K. B. Berlyn, CGSC</td>
</tr>
<tr>
<td>CAG1843</td>
<td>F− aspB3057::Tn10 supE44 − λ− rph-1</td>
<td>M. K. B. Berlyn, CGSC</td>
</tr>
<tr>
<td>JW5437-1</td>
<td>Δ{(araD-araB)567 Δ(lacZ4787::cmlB-3) − Δ(psrS746::kan rph-1) Δ(hsdR514)</td>
<td>M. K. B. Berlyn, CGSC</td>
</tr>
<tr>
<td>AB1157LP</td>
<td>thr-1 leuB6 Δ(gut-lac) hisG4 argE3 rpsL31 zai-3099::Tn10 kan supE44 Δ(lac-pro)</td>
<td>Lab collection</td>
</tr>
<tr>
<td>CSH34</td>
<td>F−128 lacZU118(Oc) proA− proB−</td>
<td>This study</td>
</tr>
<tr>
<td>MG1655LP</td>
<td>Same as MG1655M but has Δ(gut-lac)</td>
<td>This study</td>
</tr>
<tr>
<td>MMH1001</td>
<td>Same as MG1655LP but has supE44 and aspB3057::Tn10</td>
<td>This study</td>
</tr>
<tr>
<td>MMH1002</td>
<td>Same as MG1655LP but has F−128 lacZU118(Oc) proA− proB−</td>
<td>This study</td>
</tr>
<tr>
<td>MMH1003</td>
<td>Same as MMH1001 but has F−128 lacZU118(Oc) proA− proB−</td>
<td>This study</td>
</tr>
<tr>
<td>MMH1004</td>
<td>Same as MG1655 but has supE44 and aspB3057::Tn10</td>
<td>This study</td>
</tr>
<tr>
<td>MMH1005</td>
<td>Same as MMH1004 but has supE44</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
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<tr>
<td>pLUX1</td>
<td>Plasmid with wild-type lacAB</td>
<td>M. Yarus (28)</td>
</tr>
<tr>
<td>pLUX2</td>
<td>Plasmid with lacB13(Am)</td>
<td>M. Yarus (28)</td>
</tr>
<tr>
<td>pLUX3</td>
<td>Plasmid with lacB13(Oc)</td>
<td>M. Yarus (28)</td>
</tr>
<tr>
<td>pLUX4</td>
<td>Plasmid with lacB13(Op)</td>
<td>M. Yarus (28)</td>
</tr>
</tbody>
</table>

*The supE44 allele in this strain was confirmed by us by sequence analysis, though it is not reported in CGSC database.
of 7 to 10 days (14). This observation triggered us to study this phenomenon more deeply and to investigate the reason for the suppression or misreading of the ochre codon. The report by Jayaraman (14) was not very clear whether this is due solely to ppm or to something else also. In order to determine the involvement of supE44, if any, in this process, the following was done. ∆LP [Δ(gpt-lac)] was introduced into E. coli strain MG1655 along with the linked Kan’ marker (see Materials and Methods for details). In order to construct the MG1655∆LP supE44 strain, a linked Tet’ (asnB3057::Tn10) was transduced into strain CSH34 harboring supE44. The transductants obtained were screened for the retention of supE44 allele by the plaque assay method. The plaque assay method involves support of phage growth (λNK1316) bearing an amber mutation in a replication gene (P). Therefore, the phage was propagated in the host strain LE392. Since supE44 will suppress the amber mutations, transductants that retained the supE44 allele during transduction will support the growth of the phage, λNK1316. Out of 30 transductants which were screened, all of them turned out to be supE44. A survey of the literature (29) on the construction of CAG18433 showed that the parental strain, P2217, also harbored the supE44 allele. Hence, we confirmed the presence of the supE44 allele in CAG18433 also by sequencing (see below). Since CAG18433 itself is asnb3057::Tn10 and supE44, we directly used the lysate made on CAG18433 to transduce asnb3057::Tn10 supE44 into MG1655∆LP. The Tet’ transductants obtained on appropriate selective plates containing tetracycline were checked for acquisition of supE44 allele by the plaque assay method. Thus, MG1655 asnb3057::Tn10 supE44 and supE44’ isogenic strains were constructed. In order to check whether supE44 was involved in the weak suppression of lacZU118 ochre allele, the following was done. F’ bearing lacZU118 was introduced into MG1655∆LP supE44’ and supE44 strains, and the transconjugants were selected on Pro’ selection (see Materials and Methods for details). When these two strains were streaked on relevant minimal plates with X-Gal and IPTG, one could clearly see blue-centered colonies in supE44 strains after 7 days of incubation, and this was not seen in the wild-type supE44’ strain (Fig. 1). These results favor the notion that the amber suppressor supE44 might somehow play a role in the suppression of the lacZU118 ochre allele.

**Sequence analysis of supE44 regions from relevant strains.**

Before proceeding further in investigations about the nature of suppression by supE44, it was imperative to verify the presence of an amber suppressor mutation in the expected tRNA gene of the supE44 strain. For this purpose, strains CSH34 and CAG18433 were chosen. In these two strains, the relevant chromosomal region expected to harbor the supE44 allele (15-min region having the seven-tRNA operon) was PCR amplified using Fidelity polymerase and was sequenced. As was expected, in both strains we could see the amber suppressor mutation in the glnX tRNA. The mutation defines a GC → AT transition in the third nucleotide position of the anticodon.

Initial characterization of the supE allele of E. coli was done by isolation of supE tRNA followed by its sequencing. The analysis had showed the presence of duplicate genes for tRNA with anticodons corresponding to glutamine codon CAG and to the amber codon UAG (11). The authors had suggested that supE can be in one of the two genes for tRNA. Theoretically, supE can be an allele of either glnV or glnX. However, the exact location of the supE allele is not very clear from previous reports. According to the CGSC database, supE is an allele of glnV tRNA, and there are no alleles reported here for glnX tRNA. In our sequence analysis, the supE lesion was found to be in the duplicate copy of tRNA for glnX. The glnV tRNA was found to be the wild-type copy, as expected.

**Efficiency of suppression of nonsense mutations by supE44.**

*supE44 also suppresses ochre and opal mutations.* To quantify the efficiencies of suppression of all the three nonsense mutations by supE44, the in vivo luciferase assay was employed. The strategy behind the use of the luciferase assay to quantify the suppression is as follows. Plasmids pLUX1, pLUX2, pLUX3, and pLUX4 harbor luxAB genes coding for bacterial luciferase. While pLUX1 carries wild-type copies of both genes, pLUX2 bears an amber mutation in luxB, pLUX3 bears an ochre mutation in luxB, and pLUX4 bears an opal mutation in luxB. All the mutations are in the same position in luxB, changing the 13th amino acid codon into the nonsense codon. Depending on the extent of suppression of these nonsense codons by the suppressor allele, the luciferase activity will vary from strain to strain. In this investigation, we employed this strategy, originally developed by Schultz and Yarus (28), to quantify the extent of suppression of amber, ochre and opal mutations by supE44. Therefore, the supE44’ and supE44 strains were transformed with all the plasmids, and the transformants in each case were selected on appropriate selective plates. Two transformants in each case were purified by streaking on antibiotic plates, and from that, cultures were grown in LB medium with relevant antibiotics and luciferase assays were performed as described in Materials and Methods. As can be seen from the data presented in Fig. 2A, in supE44 strains, the efficiency of suppression of the amber codon (pLUX2) is high. Unexpectedly, we also could see a good level of suppression of ochre and opal mutations in the supE44 genetic background (Fig. 2A). A careful analysis of this observation indicates that the efficiency of amber suppression by supE44 is higher than previously reported. This is perhaps due to the sensitivity of the assay reported here. Moreover, in the logarithmic growth phase, the suppression efficiency of supE44 was 65.12%, whereas in the stationary growth phase it was increased to 92.68% (compare Fig. 2A and B).

The assays were done in stationary phase, as the lacZ ochre suppression by supE44 was observed only after 7 days of incubation.
The luciferase assay was preferred over the β-galactosidase assay because the efficiency of suppression was expected to be low in this case. A basal-level readthrough of nonsense codons was seen in suppressor-free cells, perhaps due to misreading. supE44 has been reported to be a weak amber suppressor for which the efficiency of suppression was calculated based on the ability to suppress nonsense mutations in either lacZ or lacI genes or lacI-lacZ fusions using the β-galactosidase assay (17). Earlier experiments, reported by Shultz and Yarus (28), on such a comparative analysis of the two techniques have reported that the suppression efficiencies derived from luciferase assays were higher than those from β-galactosidase assays. The in vivo luciferase assay has been reported to be better than the classical β-galactosidase assay for measuring the suppression efficiency of nonsense suppressors, especially weak suppressors, in terms of its sensitivity (28), and our results also support this notion. The efficiency of suppression of suppressor tRNAs has been reported to be dependent on various factors (8).

**Growth phase dependence of the supE44 effect.** The supE44 effect on the suppression of nonsense mutations was found to be growth phase dependent. The suppression of nonsense codons by supE44 was pronounced in the stationary phase compared to logarithmic growth phase (compare Fig. 2A and B). The efficiency of suppression is determined largely by the competition between release factors, resulting in termination, and a near-cognate tRNA, resulting in suppression (31). The intracellular concentrations of tRNAs and release factors are dependent on the growth phase (1, 7). In our case, supE44 is the near-cognate tRNA which we believe reads through the stop codons in a growth phase-dependent manner.

**Effect of rpoS mutation on supE44 effect.** The elevated suppression of all three nonsense codons during stationary phase indicated that σ70 might play a role. Therefore, we checked whether the increased readthrough of nonsense codons in stationary growth phase by supE44 was dependent on the stationary-phase sigma factor σ70. The results reported here indicated that only a slight reduction of the suppression efficiency was observed in the ΔrpoS mutant. Based on these results, one cannot conclude that rpoS might be involved in this phenomenon (Fig. 2C). The seven-tRNA operon in the 15-min region of the E. coli chromosome is under the control of the vegetative σ70 promoter. Zaslaver et al. (33) has reported the presence of a second promoter in the 35-nucleotide region between glnU and glnW. The nature of this promoter has not been elucidated. It can be hypothesized that the second promoter might be controlled by the RpoS sigma factor. Increased expression of supE44 can be hypothesized as one of the causes of increased suppression of nonsense codons, specifically by supE44 in stationary phase. This can be confirmed by studying the efficiency of suppression by overexpressing supE44 tRNA, and studies pertaining to these aspects are under way.

**Possible explanation for the suppression of ochre and opal nonsense codons by supE44.** In vitro experiments with suppressor-free E. coli have shown that glutamine is inserted in the place of UAG and UAA (26). Hence, the readthrough of amber and ochre mutations in wild-type E. coli, as shown by the in vivo luciferase assay, might possibly be due to tRNA\(^{\text{Gln}}\). Opal mutations have been previously reported to be leaky due to their suppression by near-cognate tRNAs in suppressor-free cells (24). Since the discovery of the genetic code, the rules of codon-anticodon pairing have been stretched by the subsequent wobble hypothesis developed by Crick (5) and its expansion in the later years (23). Codon-anticodon pairing is critically influenced by the anticodon loop-and-stem structure (2) and the codon-anticodon interactions with the decoding center of the ribosome (20). The increased suppression of all three nonsense codons specifically by supE44 can be due to the misreading effect of nonsense codon-supE44 anticodon base pairing. In the CUA-UAA codon-anticodon duplex, the mismatch occurs in the third codon position. Neither the wobble hypothesis nor its modifications support C-A base pairing.
Lagerkvist (19) had proposed the “two out of three” hypothesis, an alternative method of codon reading, according to which only the first two codon nucleotides are recognized by the anticodon. He had proposed based on previous experimental evidence that ochre codons can be read by amber suppressors by using the “two out of three” method. Recently, Lim and Curran (20) have proposed rules of misreading based on stereochemical and thermodynamic analyses of the ribosome decoding center, according to which the C-A base pairing with A in the third codon position can promote misreading. This accounts for the misreading of UAA by supE44, which is not possible with wild-type tRNA^Gln, as there are two mismatches at the first and third codon positions (Fig. 3). In the CUA-UGA codon-anticodon duplex, there are two mismatches at the second and third codon positions. The PyUA anticodon can form correct duplexes with a U-G mismatch at codon position two if there is a canonical base pair at position one and thereby mediate readthrough. The readthrough of UGA by supE44 can be explained by this model. Although the first rule of codon misreading states that misreading errors should occur only at one of the three codon positions, the additional mismatch of C-A base pairing at codon position three does not seem to affect the increased opal suppression seen in a supE44 strain.

Here we have reported a novel effect shown by an amber suppressor tRNA, supE44, which shows suppression of ochre and opal nonsense mutations. Several questions remain unanswered. The reason for the stationary-phase specificity of this observation is still unclear. Identification of suppressors for this phenotype may provide some insight into the mechanism behind this effect. Another question that can be asked is whether supE44 can suppress missense mutations apart from nonsense mutations. Studies on the leakiness of the leuB6 allele of E. coli strain AB1157 (proved to be missense by sequencing) shows that the leakiness is independent of supE44 (B. Singaravelan and M. H. Munavar, unpublished data). However, more experiments are necessary to relate supE44 to a general translational ambiguity phenotype. If so, the readthrough of nonsense codons by supE44 can be considered to be due to increased mistranslation by supE44 in the stationary growth phase. Increased mistranslation has been shown to result in a transient mutator phenotype, a phenomenon termed translational stress-induced mutagenesis (TSM). TSM was initially observed in cells expressing a mutant glycine tRNA with an anticodon mutation. Thereafter, it has been shown to occur in cells with mutations in genes encoding other tRNAs, tRNA-modifying enzymes such as miaA, and ribosomal proteins such as msPD bearing the Ram (ribosomal ambiguity mutation) and also in cells exposed to streptomycin (10). Besides these findings, previous studies in our laboratory have identified a mutagenic ochre suppressor which maps to around 15 min on the E. coli chromosome (14). However, the lesion responsible for this phenotype has not been identified. We are currently investigating whether supE44 shows a mutator phenotype in aging cells, as the supE44 effect is observed only in the stationary phase.

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REFERENCES