Suppressor-specificity of antisuppressors in yeast

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SUMMARY

Eighteen mutations of Saccharomyces cerevisiae, at eight loci, isolated as antisuppressors of SUPQ2, an ochre-suppressing allele of SUP11, were crossed with three other suppressors.

They were found to abolish the ability of SUP2 (inserting tyrosine), to suppress the ochre mutations ade2.1 and can1.100, but not its ability, to suppress his5.2 or lys1.1. When coupled with any antisuppressor, SUPQ5, inserting serine, was also unable to suppress ade2.1, but the suppression of other ochre mutations varied from one aux-SUPQ5 strain to another. No antisuppressor affected the ability of SUP11-am, an amber-suppressing allele of SUP11, to suppress trp1.1, an amber mutation.

1. INTRODUCTION

We have previously described the isolation and genetic analysis of a group of antisuppressor mutations in the yeast, Saccharomyces cerevisiae (McCready & Cox, 1973). Twenty-three such mutations were shown to map at eight loci and to affect suppression by the super-suppressor SUPQ2, of the ochre alleles ade2.1, can1.100, lys1.1 and his5.2. SUPQ2, an allele of SUP11 (Cox, 1971) is a Class I ochre-specific suppressor (Hawthorne & Mortimer, 1968). In all the mutants, suppressor efficiency appeared to be diminished rather than completely abolished. The mutations also counteracted the effect of an extra-chromosomally inherited factor, [psi+], which has been shown to enhance the efficiency of ochre suppression (Cox, 1965, 1971).

The Class I super-suppressors, which all insert tyrosine and are ochre-specific (Gilmore, Stewart & Sherman, 1971), almost certainly code for altered tRNA species (Bruenn & Jacobson, 1972; Capecchi, Hughes & Wahl, 1975). If this is indeed the case, the phenotypic effects of the anti-suppressors could be due to any of several types of mutation:

1. A mutant ribosomal or other component affecting codon recognition, tRNA binding, translation or polypeptide chain release.

2. An enzyme affecting suppressor tRNA structure, for example one affecting the modification of bases or the maturation of the precursor tRNA.

3. A mutant amino-acyl-tRNA synthetase.

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(4) An alteration in the control of tRNA synthesis or of rRNA synthesis such that the ratio of tRNA:rRNA is reduced.

A study of the specificity of the anti-suppressors was undertaken in order to begin to distinguish between these possibilities. Three suppressors were chosen: SUP2, like SUP11, a tyrosine-inserting ochre-specific suppressor (Sherman et al. 1973). SUP11-am, a specifically amber-suppressing allele of SUP11 and SUPQ5, an ochre-specific suppressor inserting serine (Liebman, Stewart & Sherman, 1975). The specificities expected of the kinds of mutations outlined above are described below:

(1) An altered ribosome component affecting tRNA binding or codon recognition would probably be non-specific, except that, where recognition requires ‘wobble’ it would only affect the ochre-specific suppressors (with IUA, possibly, as the anti-codon). An antisuppressor affecting polypeptide chain-termination would be codon-specific rather than amino-acid-specific, if at all.

(2) Antisuppressors affecting tRNA structure could be either specific or not. For example, if the anticodon recognizing UAA is either IUA or SUA (Yoshida, Takekhi & Ukita, 1971) and the modification ($\Delta \rightarrow I$ or $\rightarrow S$) is necessary for its recognition, then an antisuppressor affecting modification would be specific for the ochre suppressors ($SUP2$, $SUP11$ and $SUPQ5$) but would not affect $SUP11$-am. On the other hand, if the iso-accepting tRNA’s are structurally very similar, then they are likely to share certain modifying enzymes, and antisuppressors affecting these would be specific for them ($SUP2$, $SUP11$ and $SUP11$-am) but would not affect $SUPQ5$. By the same token, it can be predicted that antisuppressors specific for the two $SUP11$ alleles, or quite non-specific, may arise by mutation in genes determining the structure of rare, on the one hand or, on the other, more generally utilized, modifying or maturation enzymes. Antisuppressors specific for $SUP2$ or $SUPQ5$ would not, of course, appear in our collection.

(3) Several yeast amino-acyl-tRNA synthetases have been isolated and purified: seryl-, valyl- and lysyl-tRNA synthetases all appear to be homogeneous (Makman & Cantoni, 1965; Lagerkvist & Waldenström, 1865) and a temperature-sensitive mutation in the structural gene for isoleucyl-tRNA synthetase results in 99% loss of the enzyme activity (Hartwell & McLaughlin, 1968). It seems, therefore, that each iso-accepting group of tRNA species is recognized by a single amino-acyl-tRNA synthetase. If an antisuppressor were mutant in the locus determining the structure of tyrosyl-tRNA synthetase, it would be expected to affect only $SUP2$, $SUP11$ and $SUP11$-am, but not $SUPQ5$.

(4) Little is known about the control of production of tRNA. If the amount of tRNA were to affect suppression (perhaps by reducing the competition with termination factors), mutations reducing the production of tRNA might be specific or non-specific antisuppressors, depending on whether it is the control of total tRNA, of a set of iso-accepting tRNAs or of a single locus which is affected.
2. MATERIALS AND METHODS

(i) Yeast strains

The ochre alleles ade2-1, can1-100, his5-2 and lys1-1 and the amber allele trp1-1 were used. All of the antisuppressor strains had the genotype α or α asu, ade2-1, can1-100, lys1-1, his5-2, trp1-1 [psi-]. The other strains were:

| Strain no. | Genotype | Phenotype |
|------------|----------|-----------|
| X2182      | a SUP2, ade2-1, can1-100, lys1-1, his5-2, trp5-48, leu1-12, met1-1, [psi-] | Requiring leucine and methionine for growth |
| 367/8b TRP | α SUPQ2-am, ade2-1, trp1-1 [psi-] | Requires adenine for growth |
| SM135/1c   | a SUPQ5, ade2-1, can1-100, lys1-1, trp1-1 [psi+] | Requires tryptophan for growth |
| 219/7b     | α ade2-1, can1-100, lys1-1, his5-2 [psi-] | Requires adenine, lysine and histidine for growth and is resistant to canavine |

We are indebted to the Yeast Stock Centre, University of California, Berkeley for strain X2182.

(ii) Media and genetic analysis

These have been previously described (McCready & Cox, 1973).

3. RESULTS

(i) Effect of the antisuppressors on SUP2

Table 1 gives the results of tetrad analysis from crosses to X2182, giving the following diploid genotype:

\[
\text{asu} + \text{ade2.1 can1.100 lys1.1 his5.2 trp1.1} + + [\psi^-] \\
+ \text{SUP2 ade2.1 can1.100 lys1.1 his5.2 trp1.1 leu1.12 met1.1}
\]

Where several antisuppressor alleles at the same locus have been tested, the results have been pooled. Omission plates, on which the phenotypes were tested, were examined after two and after four days' incubation at 28 °C and growth recorded as ' + ' (growth) or ' – ' (no growth detectable). Where growth appeared after four days' incubation, there having been none after two, this has been recorded. Since the diploids were all heterozygous for both the antisuppressor locus being tested and for SUP2, it is expected that, if the antisuppressor has no effect on the suppressor, all suppressible phenotypes would segregate 2 suppressed : 2 non-suppressed spores in each tetrad. If the antisuppressor has an effect, a proportion of tetrads will segregate no (0:4) or only one (1:3) suppressed spore. The Table shows that all the antisuppressors were able to prevent suppression by SUP2 of
Table 1. Results of tetrad analysis of crosses involving SUP2 and antisuppressors (all \( \psi^- \))

| Antisuppressor alleles | Marker | Days of growth on test plates | No. of tetrads with suppressed: non-suppressed spores | Spores suppressed: non-suppressed | Spores suppressed (%) |
|------------------------|--------|------------------------------|-----------------------------------------------|---------------------------------|----------------------|
| asu1, \( (asu1.1, 1.2 \ 1.3, 1.4) \) | ade2.1 | 4 | 3 | 23 | 9 | 41:99 | 29 |
|            | can1.100 | 4 | 23 | 11 | 1 | 45:95 | 32 |
|            | lys1.1 | 2 | 0 | 0 | 35 | 70:70 | 50 |
|            | his5.2 | 2 | 1 | 5 | 29 | 63:77 | 45 |
|            | his5.2 | 4 | 0 | 0 | 35 | 70:70 | 50 |
| asu2, \( (asu2.2, 2.3) \) | ade2.1 | 4 | 2 | 10 | 2 | 14:42 | 25 |
|            | can1.100 | 4 | 2 | 10 | 2 | 14:42 | 25 |
|            | lys1.1 | 2 | 0 | 0 | 14 | 28:28 | 50 |
|            | his5.2 | 2 | 1 | 5 | 8 | 21:35 | 38 |
|            | his5.2 | 4 | 0 | 0 | 14 | 28:28 | 50 |
| asu3, \( (asu3.3, 3.4, 3.5) \) | ade2.1 | 4 | 4 | 17 | 6 | 29:79 | 27 |
|            | can1.100 | 4 | 4 | 17 | 6 | 29:79 | 27 |
|            | lys1.1 | 2 | 0 | 0 | 27 | 54:54 | 50 |
|            | his5.2 | 2 | 2 | 9 | 16 | 41:67 | 38 |
|            | his5.2 | 4 | 0 | 0 | 27 | 54:54 | 50 |
| asu4, \( (asu4.1, 4.2) \) | ade2.1 | 4 | 6 | 8 | 5 | 18:58 | 24 |
|            | can1.100 | 4 | 6 | 8 | 5 | 18:58 | 24 |
|            | lys1.1 | 2 | 0 | 0 | 19 | 38:38 | 50 |
|            | his5.2 | 2 | 2 | 3 | 14 | 31:45 | 41 |
|            | his5.2 | 4 | 0 | 0 | 27 | 34:40 | 45 |
| asu5, \( (asu5.1, 5.2) \) | ade2.1 | 4 | 1 | 15 | 7 | 29:63 | 32 |
|            | can1.100 | 4 | 1 | 15 | 7 | 29:63 | 32 |
|            | lys1.1 | 2 | 0 | 0 | 23 | 46:46 | 50 |
|            | his5.2 | 2 | 0 | 0 | 23 | 46:46 | 50 |
| asu6, \( (asu6.1) \) | ade2.1 | 4 | 2 | 4 | 0 | 4:20 | 17 |
|            | can1.100 | 4 | 2 | 4 | 0 | 4:20 | 17 |
|            | lys1.1 | 2 | 0 | 0 | 6 | 12:12 | 50 |
|            | his5.2 | 2 | 0 | 0 | 6 | 12:12 | 50 |
| asu7, \( (asu7.1, 7.2) \) | ade2.1 | 4 | 5 | 8 | 4 | 16:52 | 24 |
|            | can1.100 | 4 | 5 | 8 | 4 | 16:52 | 24 |
|            | lys1.1 | 2 | 0 | 0 | 17 | 34:34 | 50 |
|            | his5.2 | 2 | 0 | 0 | 17 | 34:34 | 50 |
| asu8, \( (asu8.1) \) | ade2.1 | 4 | 0 | 0 | 0 | 9:27 | 25 |
|            | can1.100 | 4 | 0 | 0 | 0 | 9:27 | 25 |
|            | lys1.1 | 2 | 0 | 2 | 7 | 16:20 | 45 |
|            | lys1.1 | 4 | 0 | 0 | 9 | 18:18 | 50 |
|            | his5.2 | 2 | 0 | 5 | 4 | 13:23 | 36 |
|            | his5.2 | 4 | 0 | 0 | 9 | 18:18 | 50 |
| Control: \( (X2182 \times 219/7b) \) | ade2.1 | 4 | 0 | 0 | 9 | 18:18 | 50 |
|            | can1.100 | 4 | 0 | 0 | 9 | 18:18 | 50 |
|            | lys1.1 | 2 | 0 | 0 | 9 | 18:18 | 50 |
|            | his5.2 | 2 | 0 | 0 | 9 | 18:18 | 50 |
ade 2.1 and can 1.100, but not of lys1.1. Suppression of his5.2 was not prevented either, but in the presence of certain alleles of asu1, asu2, asu3, asu4 and asu8, it was delayed. Segregants from some crosses included cultures in which his5.2 (and in one cross, lys1.1) was not suppressed after two days' incubation, but was after four days (Table 2).

Table 2. The delay in the expression of SUP2 as a suppressor of his5.2 as a result of the presence of antisuppressor alleles

| Anti-suppressor allele | Total spore cultures | No. not suppressed (SUP+) | No. suppressed after 4 days' incubation (SUP2) | No. of these not suppressed after 2 days' incubation |
|------------------------|----------------------|--------------------------|-----------------------------------------------|-----------------------------------------------|
| 1.1                    | 36                   | 18                       | 18                                            | 9                                             |
| 1.2                    | 44                   | 22                       | 22                                            | 0                                             |
| 1.3                    | 20                   | 10                       | 10                                            | 0                                             |
| 1.4                    | 40                   | 20                       | 20                                            | 0                                             |
| 2.2                    | 24                   | 12                       | 12                                            | 0                                             |
| 2.3                    | 32                   | 16                       | 16                                            | 7                                             |
| 3.3                    | 44                   | 22                       | 22                                            | 4                                             |
| 3.4                    | 20                   | 10                       | 10                                            | 3                                             |
| 3.5                    | 44                   | 22                       | 22                                            | 6                                             |
| 4.1                    | 28                   | 14                       | 14                                            | 5                                             |
| 4.2                    | 44                   | 22                       | 22                                            | 0                                             |
| 5.1                    | 48                   | 24                       | 24                                            | 0                                             |
| 5.2                    | 44                   | 22                       | 22                                            | 0                                             |
| 6.1                    | 24                   | 12                       | 12                                            | 0                                             |
| 7.1                    | 28                   | 14                       | 14                                            | 0                                             |
| 7.2                    | 40                   | 20                       | 20                                            | 0                                             |
| 8.1                    | 36                   | 18                       | 18                                            | 5                                             |

Note. lys1.1 was suppressed after 2 days' incubation in all those cultures in which his5.2 was suppressed, except that two SUP2 cultures in which asu8.1 was present showed no growth after 2 days', but grew after 4 days' incubation.

With the exception of four cultures, all from the cross involving asu1.2, there was complete coincidence of suppression and non-suppression of ade2.1 and can1.100; that is, all adenine-independent cultures were also sensitive to canavanine.

The non-suppressible markers leu1.2 and met1.1 segregated 2:2 in all tetrads and all cultures required tryptophan for growth.
(ii) Effect of the antisuppressors on SUPQ5

The results of tetrad analysis of crosses to SM135/3c are shown in Table 3. All the diploids had the genotype:

\[
\begin{align*}
\text{asu}^+ & \quad \text{ade2.1 can1.100 lys1.1 his5.2 trp1.1} \quad \text{[psi\textsuperscript{+}]} \\
+ & \quad \text{SUPQ5 ade2.1 can1.100 lys1.1 + trp1.1}
\end{align*}
\]

Since, in these diploids, his5.2 is heterozygous, it is not always possible to be certain whether growth on histidineless plates is due to the activity of the suppressor or occurs because of the presence of the wild-type his5 allele. The segregation of histidine requirement has therefore been omitted from the Table. The

| Antisuressor | Marker | No. of tetrads with suppressed: | Spores | Spores |
|--------------|--------|-------------------------------|--------|--------|
|              |        | Days of tetrads | non-suppressed | suppressed | (%)     |
|              |        | on test plates | 0:4 | 1:3 | 2:2 | non-suppressed | suppressed |
| asu1 (asu1.1, 1.2, 1.3, 1) - asu2.1 | 4 | 9 | 22 | 5 | 32:112 | 22 |
| lys1.1 | 4 | 8 | 21 | 7 | 35:109 | 24 |
| lys1.1 | 4 | 1 | 12 | 23 | 58:86 | 40 |
| asu2 (asu2.1, 2.2, 2.3) - asu2.1 | 4 | 8 | 21 | 10 | 41:115 | 26 |
| can1.100 | 4 | 7 | 21 | 11 | 43:113 | 28 |
| lys1.1 | 2 | 5 | 12 | 22 | 56:100 | 36 |
| lys1.1 | 4 | 1 | 11 | 27 | 65:91 | 42 |
| asu3 (asu3.3, 3.4, 3.5) - asu2.1 | 4 | 7 | 13 | 7 | 27:81 | 25 |
| can1.100 | 4 | 13 | 13 | 1 | 15:93 | 14 |
| lys1.1 | 2 | 3 | 10 | 14 | 38:70 | 35 |
| lys1.1 | 4 | 0 | 7 | 20 | 47:61 | 44 |
| asu4 (asu4.1, 4.2, 4.3) - asu4.1 | 4 | 5 | 9 | 7 | 23:61 | 27 |
| can1.100 | 4 | 5 | 11 | 5 | 21:63 | 25 |
| lys1.1 | 2 | 5 | 7 | 9 | 25:59 | 29 |
| lys1.1 | 4 | 1 | 6 | 14 | 34:50 | 40 |
| asu5 (asu5.1, 5.2) - asu2.1 | 4 | 5 | 4 | 6 | 16:44 | 27 |
| can1.100 | 4 | 1 | 4 | 10 | 24:36 | 40 |
| lys1.1 | 2 | 0 | 0 | 15 | 30:30 | 50 |
| asu7 (asu7.1, 7.2) - asu7.1 | 4 | 1 | 3 | 3 | 9:19 | 32 |
| can1.100 | 4 | 2 | 4 | 1 | 6:22 | 21 |
| lys1.1 | 2 | 0 | 2 | 5 | 12:16 | 43 |
| lys1.1 | 4 | 0 | 0 | 7 | 14:14 | 50 |
| asu8 (asu8.1) - asu2.1 | 4 | 5 | 4 | 1 | 6:34 | 15 |
| can1.100 | 4 | 2 | 5 | 3 | 11:29 | 28 |
| lys1.1 | 2 | 6 | 2 | 2 | 6:34 | 15 |
| lys1.1 | 4 | 0 | 0 | 10 | 20:20 | 50 |
| Control: | ade2.1 | 4 | 0 | 0 | 8 | 16:16 | 50 |
| SM135/3c x can1.100 | 4 | 0 | 0 | 8 | 16:16 | 50 |
| 219/7b llys1.1 | 2 | 0 | 0 | 8 | 16:16 | 50 |
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suppression of adenine and lysine requirements and of canavanine resistance has been recorded as before. Again, it is clear that all the antisuppressors severely interfere with the ability of the suppressor to suppress ade 2.1 and can1.100. In contrast with their effects on SUP2, they do not do so indiscriminately: it is

Table 4. Results of tetrad analyses of crosses of alleles of the asu4 locus with SUPQ5

| Allele | Marker | Days of growth | 4:0 | 3:1 | 2:2 |
|--------|--------|----------------|-----|-----|-----|
| 4.1    | ade2.1 | 4              | 2   | 4   | 3   |
|        | can1.100 | 4               | 1   | 8   | 0   |
|        | lys1.1 | 2               | 2   | 4   | 3   |
|        | lys1.1 | 4               | 1   | 3   | 5   |
| 4.2    | ade2.1 | 4              | 2   | 3   | 0   |
|        | can1.100 | 4              | 0   | 0   | 5   |
|        | lys1.1 | 2               | 0   | 3   | 2   |
| 4.3    | ade2.1 | 4              | 1   | 2   | 4   |
|        | can1.100 | 4              | 4   | 3   | 0   |
|        | lys1.1 | 2               | 3   | 0   | 4   |
|        | lys1.1 | 4               | 0   | 0   | 7   |

Table 5. Antisuppressor alleles which fail to effect the suppression of (A), lys1.1 or (B) can1.100 by SUPQ5

(In crosses involving these alleles, either lysine requirement or canavanine resistance or both segregated 2:2 in all tetrads.)

| A  | B  |
|----|----|
| 1-2| 1-4|
| 1-4| 1-4|
| 2-2| 2-2|
| 3-3| 3-3|
| 4-3| 4-2|
| 5-1| 5-1|
| 5-2| 5-2|
| 7-1| 7-1|
| 7-2| 7-2|
| 8-1| 8-1|

noticeable that when segregating with asu3 alleles, for example, SUPQ5 suppresses ade 2.1 in more cultures than it does can1.100, while the reverse is true of its combinations with asu5 alleles and asu8.1. In no set of tetrads involving anti-suppressor alleles from any one locus is there complete coincidence of suppression of these markers. However, pooling the data within each locus has obscured the fact that alleles show considerable variation in their effects. This is illustrated in Table 4 in which the tetrads from crosses involving three alleles of asu4 are

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described. Apart from asu4.2, two other asu mutations fail altogether to interfere with the suppression by SUPQ5 of can1.100 (Table 5). On the other hand, three of the four alleles of asul show complete coincidence of suppression of ade2.1 and can1.100 among the segregants. Alleles of asu5, asu7 and asu8 fail to prevent suppression of lysl.1, as do some alleles of the other loci (Table 5). However, the remaining antisuppressors tested clearly interfere with the suppressor's activity in many cultures.

All the segregants required tryptophan for growth.

Table 6. Tetrad data from crosses involving SUP11am and antisuppressors

| Antisuppressors | No. of tetrads with trp+:trp- spores | Spores suppressed: | Spores non-suppressed | Spores suppressed (%) |
|-----------------|----------------------------------|------------------|----------------------|----------------------|
|                 | 0:4                             | 1:3              | 2:2                  |                      |
| asu1.1          | 0                               | 0                | 9                    | 18:18                | 50 |
| asu1.2          | 0                               | 0                | 5                    | 10:10                | 50 |
| asu1.4          | 0                               | 0                | 12                   | 24:24                | 50 |
| asu2.2          | 0                               | 0                | 3                    | 6:6                  | 50 |
| asu2.3          | 0                               | 0                | 8                    | 16:16                | 50 |
| asu3.1          | 0                               | 0                | 4                    | 8:8                  | 50 |
| asu3.2          | 0                               | 0                | 1                    | 2:2                  | 50 |
| asu3.3          | 0                               | 0                | 2                    | 4:4                  | 50 |
| asu4.1          | 0                               | 0                | 6                    | 12:12                | 50 |
| asu4.2          | 0                               | 0                | 5                    | 10:10                | 50 |
| asu5.1          | 0                               | 0                | 12                   | 24:24                | 50 |
| asu6.1          | 0                               | 0                | 7                    | 14:14                | 50 |
| asu7.1          | 0                               | 0                | 8                    | 16:16                | 50 |
| asu8.1          | 0                               | 0                | 4                    | 8:8                  | 50 |

(iii) Effect of the antisuppressors on SUP11am

Table 6 summarizes the results of crosses of the antisuppressors to 367/8b TRP+, which produces diploids of genotype:

\[
\begin{align*}

\text{asu} & + \quad \text{ade2.1 \ lys1.1 \ can1.100 \ his5.2 \ trp1.1} & \text{[psi-]} \\
+ \quad \text{SUP11am \ ade2.1} & + \quad + & + \quad \text{trp1.1}
\end{align*}
\]

No antisuppressor has any effect on the ability of SUP11am to suppress trp1.1.

4. DISCUSSION

(1) It is noticeable about all the antisuppressors described here that none of them completely prevents suppression by any of the suppressors with which they have been combined. SUP2 continues to suppress lys1.1 and his5.2 in the presence of any antisuppressor and in [psi+] strains, SUPQ5 combined with some of the mutations suppresses lys1.1 occasionally and does so invariably when combined with the others. We have previously shown that SUPQ2 (SUP11) sup-
presses all these ochre mutations when coupled with antisuppressors in a [psi\(^+\)] background: it is however, no longer a recessive lethal. These observations reinforce our earlier conclusion that the antisuppressors depress the level of suppression, rather than completely inactivate the suppressor. This means that no tRNA is removed from production or prevented from maturation completely by any of these suppressor mutations.

(ii) None of the suppressors is specific to the isoinserting suppressors SUP11 and SUP2; both these suppressors and SUPQ5 are affected. This eliminates the possibility that any of them represents an altered activating enzyme.

(iii) No effect on suppression by SUP11am of trp1.1 could be detected. This may have been because amber suppression is more efficient than ochre suppression (Sherman et al. 1973) and that a reduction in suppressor efficiency would go undetected in the system chosen. However, if the antisuppressors are indeed all ochre-specific, this would suggest that they affect codon recognition and that the loci involved determine the structure or amounts of certain ribosomal proteins, of ochre-specific termination factors or of enzymes affecting tRNA structures or bases that are involved in the recognition of ochre codons. The fact that eight different loci can mutate to have this effect makes the last two possibilities rather unattractive explanations.

If a more sensitive test shows that amber suppression is indeed affected, this would enlarge the range of possible modes of action of the antisuppressors to include factors involved in total tRNA production; tRNA maturation; the modifications of tRNA bases required generally for tRNA function; ribosomal structures that affect tRNA binding and conformation and possibly polypeptide chain elongation and termination factors. At this point no more firm conclusion can be reached.

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