Functional complementation between mutations in a yeast suppressor tRNA gene reveals potential for evolution of tRNA sequences

(tRNA evolution/Schizosaccharomyces pombe/RNase P/nonsense suppression)

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Communicated by Johannes Geiss, July 14, 1986

ABSTRACT Successive rounds of mutagenesis of a Schizosaccharomyces pombe strain bearing the UGA-reading sup3 tRNA\textsuperscript{ome} suppressor have been carried out for two cycles of inactivation and reactivation of the suppressor. The suppressor phenotype at each stage was found to involve different combinations of three mutations, A30, A53, and A67, in the sup3-UGA gene. Single mutations A30 and A53 inactivate the suppressor as does the presence of all three mutations. A67 by itself is phenotypically neutral, but in combination with either A30 or A53 suppressor function is restored. The frequency with which these and other complementation events occur in S. pombe demonstrates a significant potential for nucleotide sequence evolution in tRNA. Differential expression of the S. pombe genes in Saccharomyces cerevisiae suggests that the two yeasts have diverged at the transcripational and RNA processing level. Processing of the mutant tRNA precursors in S. cerevisiae reveals a hierarchy of structural domains within the tRNA that vary in their importance for RNase P cleavage.

The secondary and tertiary structure of different tRNAs is strongly conserved for the interaction of these molecules with a variety of components involved in their biosynthesis (transcription (1), processing of RNA precursors (2), transport from nucleus to cytoplasm (3), nucleotide modification (4)) and in their function during protein synthesis (5). Some conservation of overall tRNA structure is reflected in the primary nucleotide sequences of tRNAs by the occurrence of invariant or semi-invariant nucleotides at specific positions. However, other parts of the molecules must be unique in order to allow specificity in their interactions with proteins, such as aminoacyl-tRNA synthetases. Using nonsense suppression as an assay for tRNA function, we have been examining the biosynthetic consequences of mutations that abolish suppressor activity (6–8). In this work we show that despite the many constraints on tRNA structure, there remains considerable structural flexibility, in that mutations that cause severe defects in specific steps of tRNA biosynthesis can be compensated for by additional sequence changes elsewhere in the tRNA molecule.

MATERIALS AND METHODS

Yeast Strains. All of the Schizosaccharomyces pombe strains carrying sequence alterations in a tRNA gene also contain the UGA nonsense mutation ade6-704 that leads to adenine auxotrophy. The exact genotypes of the basic suppressor strains, which are fully prototrophic, are ade6-704 sup3-UGA and ade6-704 sup9-UGA. The so-called second-site mutations sup3-UGA,A30 (old nomenclature r8) and sup9-UGA,A53 (r9) are auxotrophic and have been described in detail (6, 7). The temperature-sensitive suppressor sup3-UGA,A30,A67 was originally named sup3-UGA,r8,rrl8 (9). The allele sup3-UGA,A30,A53,A67 carrying four mutations was termed sup3-UGA,r8,rr18,rr1 (10). The methods of S. pombe suppressor genetics as well as the growth media have been described in detail (9, 10).

Saccharomyces cerevisiae strains used in this work were 3A84 ade1-UGA hisd4-260 leu2-2 ura3-52 (11) and YH-D5 trp1-1 his4-260 leu2-2 (12).

Cloning of sup3 Genes. Molecular cloning and nucleotide sequence analysis of sup3 genes were carried out as described by Amstutz et al. (13). The allele sup3-UGA,A67 was constructed by primer-directed mutagenesis following the procedures of Stewart et al. (14). This allele, carried on a plasmid, was integrated into the S. pombe genome near the sup3\textsuperscript{+} locus by homologous recombination according to standard techniques (15). Subsequently, the wild-type sup3\textsuperscript{+} gene in S. pombe was replaced with sup3-UGA,A67 by the yeast transformation method with linear DNA (16) and by selection for suppression of the ade6-704 mutation. Correct replacement of the original allele was confirmed by Southern analysis, which indicated that the Hinfl site spanning the sup3\textsuperscript{+} anticodon was no longer present.

Expression and Analysis of S. pombe tRNA Genes in S. cerevisiae. The 1.0-kilobase HindIII/BamHI fragment containing each sup3 allele was recloned into the S. cerevisiae centromeric plasmid vector YCp50. The allele sup9-UGA,-A53 was recloned into this vector as a 1.5-kilobase ClaI/EcoRI fragment. Recombinant plasmids were used to transform S. cerevisiae strain 3A84 to URA\textsuperscript{+}. Suppressor activity in transformed cells was assayed by visually monitoring colony color during growth at 25°C on minimal media plates lacking or containing limiting amounts of adenine (2.5 μg/ml). Suppression of the histidine and leucine markers was tested subsequently. Suppressor activity at a higher sup3 gene dosage was examined in S. cerevisiae strain YH-D5. The 2.35-kb HindIII fragment containing each sup3 allele was recloned into the multicopy plasmid vector YEp101, and the ability of LEU\textsuperscript{+} transformants to suppress the hisd4 marker was tested. Cultures of these transformants were also grown at 25°C, with selection for the plasmid, immediately preced-

Abbreviation: IVS, intervening sequence.

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ing the preparation of RNA for blot-hybridization analysis (7).

In Vitro Transcription in an S. cerevisiae Extract and Precursor Processing. These reactions have been described in detail (6, 7). Reclones of the various alleles in YCp30 (above) served as the source of template DNA.

RESULTS

Isolation, Characterization, and Sequencing of Multiply Mutated S. pombe Suppressor tRNA Genes. To define intragenic mutational events that affect the function of other mutations in the suppressor tRNA gene, we used the strategy of repeated suppressor inactivation and reactivation outlined in Fig. 1. The structure of the multiply mutated genes thus obtained is shown in Fig. 2. In their wild-type form, the sup3 and sup9 genes code for almost identical serine tRNAs that read the codon UCA (7, 12). In the suppressor genes sup3-UGA and sup9-UGA that encode the UGA-reading suppressor tRNAs, the anticodon TCA replaces the wild-type TGA. This suppressor anticodon is present in all mutant tRNA genes described here.

Upon mutagenesis of the parental suppressor strains, cell lines were obtained that had lost the ability to suppress various S. pombe UGA mutations (10). A large number of these suppressor-inactive mutant genes have been cloned and sequenced (6–8). Two examples relevant to this work are sup3-UGA,A30 and sup9-UGA,A33 (Fig. 2). S. pombe strains carrying these mutant genes are clearly auxotrophic; no residual suppressor activity is observed (10). Analysis of the biosynthesis of these mutants in the heterologous yeast, S. cerevisiae, revealed that neither was able to produce mature tRNA\textsuperscript{ser}\textsuperscript{*}. For sup3-UGA,A30, the primary biosynthetic defect results from a greatly reduced efficiency of 5' end maturation by RNase P (6). In contrast, the major defect of the sup9-UGA,A53 mutant gene is impaired gene transcription (7). In addition, the small amount of transcript that is made in this case also appears to be a poor substrate for RNase P. In the course of this project, we found it necessary (see below) to obtain a further mutation in the sup3-UGA gene, namely a G-to-A transition at position 67. This allele, sup3-UGA,A67, was obtained by primer-directed mutagenesis. Upon integration of the sup3-UGA,A67 gene into the S. pombe genome, a suppressor strain was obtained that was indistinguishable from sup3-UGA in suppressor efficiency. This was deduced from tests with three different UGA mutations in the genes ade6 and ura1, all of which were fully suppressed. This result was confirmed by exact replacement of the sup3* gene on S. pombe chromosome 1 by the sup3-UGA,A67 allele.

In an attempt to isolate temperature-sensitive nonsense suppressors, Nurse and Thuriaux (9) mutagenized five strains containing different suppressor-inactive alleles of sup3-UGA and tested the progeny for UGA suppression at 25°C and lack of suppression at 35°C (9). Three of the five mutant strains yielded temperature-sensitive suppressors as a result of additional mutations in the sup3 gene. The parental alleles giving rise to these temperature-sensitive suppressors are sup3-UGA,U11, sup3-UGA,A30, and sup3-UGA,A70 (Fig. 2). When we sequenced the temperature-sensitive suppressor derived from the sup3-UGA,A30 allele, the structure sup3-UGA,A30,A67 was found (Fig. 1). This additional mutation in the tRNA (Fig. 2) results in the replacement of the U-G base pair in the acceptor stem with a more stable U-A base pair. This alteration in the structure of the tRNA is the basis for the

![Fig. 2. Secondary structure representation of a tRNA precursor derived from the sup3* or sup9* genes. The two genes differ by a U-to-C substitution at the tip of the extra arm. The intron sequence is bordered by broken lines at the splice sites, and the anticodon is boxed. Bold arrows indicate mutational changes that were found in the mutants described in this paper. Thin arrows indicate two second-site mutations that also can give rise to temperature-sensitive suppressors (18), but these alleles have not been characterized at the molecular level.](http://example.com/f2.png)
ability of this mutation to compensate for the effect of the A30 mutation in the anticodon stem (see below).

Subsequently, various strains were derived from the heat-sensitive suppressor sup3-UGA,A30,A67, which showed loss of suppression at all temperatures. The sup3 gene structure of one of these isolates turned out to be sup3-UGA,A30,A53,A67 (Figs. 1 and 2). Thus, this strain carries a sup3 gene with four different mutations. The newly obtained A53 mutation is known to cause a transcriptional block that, by itself, is sufficient to abolish suppressor activity (see above).

Finally, we isolated spontaneous derivatives of the quadruple-mutant strain of genotype sup3-UGA,A30,A53,A67, which had regained suppressor activity at 25°C. Genetic analysis showed that the reactivating changes had occurred at the sup3 locus and not elsewhere in the genome. Several of these new alleles were subjected to cloning and sequence analysis. One isolate with temperature-sensitive suppression was found to have exactly the same structure as the heat-sensitive suppressor sup3-UGA,A30,A67 described already. Two additional isolates displayed suppression at all temperatures. Their phenotypes are indistinguishable from those of the classical sup3-UGA suppressor (Table 1). Upon cloning and sequence analysis, we found that the molecular structure of these alleles is sup3-UGA,A53,A67 (Fig. 1). Thus, reversion of the A30 mutation in the anticodon stem to the wild-type (G30) enables the phenotypically neutral mutation A67 to rescue suppressor activity from the otherwise inactive sup3-UGA,A53 mutation. How this unexpected compensation occurs at the molecular level in the sup3-UGA,A53,A67 allele as well as in the temperature-sensitive suppressor sup3-UGA,A30,A67 was the subject of the following experiments.

Expression and Analysis of Mutant S. pombe tRNA Genes in S. cerevisiae. The biosynthetic lesions of sup3-UGA and sup9-UGA suppressor-inactive alleles have been analyzed in the past in vivo and in vitro in S. cerevisiae. In this way, a detailed description of the transcriptional characteristics of tRNA genes as well as the processing of the resulting tRNA precursors has been obtained (6, 7, 17). Before applying these procedures to the analysis of the present collection of mutants, we first sought to confirm their suppression phenotype in S. cerevisiae. The various S. pombe tRNA genes were recloned into both centromeric and multicopy plasmid vectors and then transformed into S. cerevisiae. The suppression data are summarized in Table 1. In contrast to the results obtained in S. pombe, neither sup3-UGA,A30,A67 nor sup3-UGA,A53,A67 could suppress any of the UGA mutations in the 3A84 strain when present in only one copy per cell. At increased gene dosage, however, the sup3-UGA,A30,A67 mutation did exhibit temperature-sensitive suppression similar to that observed for this allele in S. pombe. The inability of these alleles to be expressed effectively in single copy in S. cerevisiae is intriguing and may have numerous molecular explanations. The fact that sup3-UGA,A30,A67 is a temperature-sensitive suppressor in S. cerevisiae at elevated gene dosage suggests that the mutations A30 and A67 may affect common processes in the two yeasts but that the magnitude of these effects is different. A similar explanation may also apply for sup3-UGA,A53,A67, but in this case the negative effects of A53 in S. cerevisiae are too severe for suppressor activity to be recovered. Alternatively, or in addition, the mutations A30, A53, and A67 may affect a particular step in tRNA gene expression in a way unique to one or the other yeast. To investigate these possibilities, we analyzed the transcription and processing of the mutant tRNA genes in vitro in S. cerevisiae. The results obtained from measurements of template activity are summarized in Table 1. All of the mutants except those containing the mutation A53 were transcribed with the same efficiency as the wild-type gene. Introduction of the A53 mutation reduced the rate of transcription to 3–6% of the wild-type level. No transcriptional effect was observed for the A67 mutation alone or for this mutation in combination with A53. This result is further supported by the blot-hybridization analysis shown in Fig. 3 and by the coupled transcription/processing experiment shown in Fig. 4. More importantly, however, these latter experiments reveal a pronounced effect of the A67 mutation on precursor processing. A comparison of the hybridization patterns obtained for mutations A30 and A30–A67 (Fig. 3) shows that the latter produces the end-matured precursor to tRNA Ser[ tRNA Ser[ +IVS (intervening sequence)] and presumably mature tRNA Ser[ (which is obscured by cross-hybridization of S. cerevisiae tRNA Ser[ species), whereas the A30 mutation does not. Similarly, in Fig. 4, A30–A67 precursors appear to be 5′ end-matured with essentially the same efficiency as sup3-UGA precursors, while the pattern of products for A30 is characteristic of a defective RNase P substrate (compare the relative amounts of 5′ flanked precursor and tRNA Ser[ +IVS product and note the absence of a 5′ and 3′ end-matured dimer species in the case of A30 products; see also ref. 1). These results indicate that the A67 mutation can compensate for the effect of the A30 mutation on RNase P cleavage. Further examination of the processing of the A30–A67 precursor reveals that splicing

Table 1. Properties of the mutant S. pombe tRNA genes

<table>
<thead>
<tr>
<th>Suppressor allele</th>
<th>S. pombe*</th>
<th>S. cerevisiae†</th>
<th>Transcriptional efficiency‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>sup3+ (UCA)</td>
<td>–</td>
<td>–</td>
<td>wt (100%)</td>
</tr>
<tr>
<td>sup3-UGA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>sup3-UGA,A30</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>sup3-UGA,A53</td>
<td>–</td>
<td>–</td>
<td>3–6%</td>
</tr>
<tr>
<td>sup3-UGA,A67</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>sup3-UGA,A30,A67</td>
<td>+,ts§</td>
<td>–</td>
<td>+,ts§</td>
</tr>
<tr>
<td>sup3-UGA,A30,A67</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>sup3-UGA,A53,A67</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*The ability to suppress the accumulation of red pigment in an ade6-704 background was monitored for all alleles.
†Suppression of UGA mutations in the genes ade1, his4, and leu2 was tested after recloning each allele into the plasmid vector YCP50. For alleles cloned in the multicopy vector YRP101, suppression of the his4 marker was examined.
‡The transcriptional efficiency of each gene was measured in an S. cerevisiae extract as described (1). Wt, wild-type.
§ts, Temperature-sensitive: suppression at 25°C, lack of suppression at 35°C.
Genetics: Willis et al.

FIG. 3. Blot-hybridization analysis of RNA from S. cerevisiae transformants. Transformants of S. cerevisiae strain YH-D5, each containing a mutant S. pombe tRNA gene on plasmid YRp101, were grown at 25°C (Table 1) prior to the preparation of RNA. Each lane contained equal amounts (50 µg) of RNA. The parental suppressor sup3-UGA is designated 3e.

of the IVS occurs at only a fraction of the rate observed for the sup3-UGA precursor. Possibly differential processing at this step in S. pombe and S. cerevisiae may contribute to the observed difference in the expression of the sup3-UGA, A30, A67 allele in these two yeasts (Table 1). Evidence for divergence in the tRNA structural requirements of the splicing endonucleases from S. pombe and S. cerevisiae has been described (18). Given the dramatic effect of the A67 mutation on the processing of A30 precursors, it was somewhat surprising that a comparison of the processing of sup3-UGA and sup3-UGA, A67 precursors did not reveal a similar difference. The steady-state concentrations of dimeric precursors and monomeric tRNASer precursors in vivo (Fig. 3) appear to be the same for these two mutations as do the amounts of these products that are produced in vitro (Fig. 4). Thus, it seems that the effect of the A67 mutation on the rate of RNase P may be largely restricted to substrates that are impaired in this process. A further example of this effect is demonstrated by the double mutation A33–A67: in comparison to mutation A33, an increase in the rate of RNase P cleavage is indicated by the appearance of the third dimeric tRNA precursor and of the end-matured tRNASer–IVS species (Fig. 4). Notably, these products are absent in transcriptions of both the sup3-UGA, A30, A67 and sup9-UGA, A53 genes.

DISCUSSION

Despite the numerous interactions of tRNAs with other molecules and the resulting constraint on evolutionary change, tRNA sequences and their gene families show considerable differences even between related organisms (19, 20). All members of an isoacceptor-family (codon family) have evolved together (concerted evolution), divergent from members of the same gene family in other organisms. In this communication we reveal an evolutionary potential for tRNA sequences which may, in part, solve the dilemma to understand on one hand the sequence divergence, while maintaining the functional constraints of a tRNA molecule. We have documented two cases where a single base substitution in the sup3 gene, which is incompatible with suppressor function, can be functionally compensated by a second unrelated base change that itself is phenotypically neutral. In general terms these observations may be stated as follows: single-point mutations in any member of a multigene family are not lethal to an affected cell because the remaining wild-type members of the family are still able to fulfill their function. Thus, a limited number of mutations can be accumulated. Neutral mutations like the A67 base change in the sup3 gene are believed to be steadily accumulating in the genome (21). This type of mutation could hit the primarily mutated gene and activate it, as for the A30–A67 and A33–A67 combinations in the sup3 gene. The sequential addition of mutations to a particular tRNA gene, as with this scheme, is not the only way in which multiply mutated tRNA genes may arise, however. The ability of members of a tRNA gene family to recombine with each other leaves open the possibility that mutations in different genes may be brought together by intergenic conversion (13, 22). The new combination of two mutations is now subject to being spread or eliminated in the gene family. Thus, this series of events has the potential to change the sequence of all the members of an isoacceptor family, provided that the resulting tRNA satisfies the cellular requirements placed upon it. The coevolution of isoacceptor gene families in this way is clearly constrained, for example, by the need to conserve those features of tRNA sequence and structure that assure their interaction with the cognate aminocacyl-tRNA synthetase.

Previous studies had shown an absolute correlation between the two yeasts regarding the phenotypes of sup3 and sup9 second-site mutants (M.N., unpublished data). Therefore, it was surprising to find that the expression of sup3-UGA, A30, A67 suppressor activity was gene dosage-dependent, whereas sup3-UGA, A33, A67 was inactive even at an increased gene dosage in S. cerevisiae. Recent work by Kaufer et al. (23) has highlighted another difference in gene expression between S. cerevisiae and S. pombe, namely mRNA splicing. Together with other comparisons (cited in ref. 23) it seems that S. pombe more closely resembles higher eukaryotes than the two yeasts resemble each other. Thus, clues to the differential expression of the tRNA genes described here may be found by examining the effects of similar mutations in other systems. From such an analysis, a transcriptional role for nucleotide 67 seems unlikely: nucleotide 67 lies outside the 3’ internal transcription control.
region, the 3' boundary of which is generally believed to be at nucleotide 62 (1) but may extend as far as nucleotide 64 (24); and transcription of a suppressor-inactive mutation of SUP4-0 tRNA^{51-7} (767) in *Xenopus laevis* or yeast extracts revealed no effect on template activity (refs. 25 and 26; see also Table 1). Two examples of mutations at the otherwise invariant position 53 in tRNA have been reported in addition to the A53 mutation in the *sup3/sup9* genes described here. Interestingly, the *C. elegans* tRNA^{51-7} mutation T53 is transcribed at 43% of the wild-type rate in HeLa cell extract (24) in contrast to the 3-6% observed in *S. cerevisiae* extracts for the *S. pombe* mutants containing A53 (Table 1). In the other case, a human tRNA^{Met} mutation (A53), template activity has not been quantified, but sufficient tRNA^{Met} could be recovered from *Xenopus* oocyte transcriptions to permit a study of its transport kinetics (3). Thus, it seems likely that the primary factor, although perhaps not the only factor (see below), contributing to the inactivity of *sup3-UGA,A53,A67* in *S. cerevisiae* is the severe transcriptional defect resulting from the A53 mutation. Conversely, the activity of this mutation in *S. pombe* may be due to a far less severe effect of A53 on transcription, which persists but is compensated for by an improvement in the efficiency of other biosynthetic processes (such as 5'-end maturation, Fig. 4) as a result of the A67 mutation. To account for the different transcriptional effects of identical mutations in different genes, it has been suggested that the importance of any base in promoting transcription is context-dependent (24). In addition, it is likely, based on the above explanation, that the strength of any contact between a tRNA gene and a transcription component (in this case, nucleotide 53 and transcription factor IIC; see ref. 27) may vary from one system or organism to another.

The recognition and cleavage of tRNA precursors by RNase P is dependent upon the conservation of overall tRNA structure. Point mutations in every stem region and in the D and T loops have been found to affect this process (e.g., refs. 6 and 24). Even changes in the structure of the anticodon/ intron domain of the precursors can influence the rate of RNase P cleavage (17). The three mutations described in this study are located in different helices of the tRNA and do not make contact in the tertiary structure. Thus, the massive improvement in the rate of RNase P cleavage brought about by the introduction of the A67 mutation into A30 and A53 precursors suggests that within the tRNA, there is a hierarchy of structural domains that vary in their importance for RNase P cleavage. The dominant effect of the acceptor stem structure over the T- and anticodon stem base-pair mismatch indicates that this region is of primary importance. This is consistent with the observation that mutations in this stem, unlike those elsewhere in the tRNA, can abolish RNase P cleavage (6). Of course, compensatory third-site mutations in tRNA need not be restricted to a particular domain of the molecule. Indeed, studies with *Escherichia coli* su'3 tRNA^{51-7} have shown that tRNA function may also be restored by repairing the mismatched base pair (28).

We have presented several explanations for the functional complementation in *S. pombe* of *sup3-UGA,A30* and *sup3-UGA,A53* mutations by the A67 mutation and for the lack of complementation, as the case may be, in *S. cerevisiae*. Thus far, these descriptions have been limited to biosynthetic processes. It is important to recognize, however, that additional contributions towards the suppressor phenotype may be made at the level of aminoacylation, at the ribosome, or by effects on tRNA half-life.

This work was supported by a grant from the National Institutes of Health and by Switzerland-U.S. Collaborative Research Grant NSF-INT 8017923.