Identity of *Saccharomyces cerevisiae* tRNA<sup>Trp</sup> Is Not Changed by an Anticodon Mutation That Creates an Amber Suppressor*

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A C35→T mutation in an *Escherichia coli* tRNA<sup>Trp</sup> gene creates an amber suppressor which efficiently inserts glutamine in response to UAG codons *in vivo* (Soll, L., and Berg, P. (1969) *Nature* 223, 1340–1342). We have introduced the same change in a yeast tRNA<sup>Trp</sup> gene and demonstrated that the tRNA acts as an efficient amber suppressor *in vivo*. Amino acid sequence analyses were performed on chitinase produced by cells carrying the corresponding gene with a UAG codon at position 8 of the mature protein plus the mutant tRNA<sup>Trp</sup> gene. In contrast to comparable experiments with *E. coli*, tryptophan is inserted at a frequency ≥80% by the yeast suppressor tRNA<sup>Trp</sup>. Furthermore, *in vitro* charging experiments with the mutant tRNA<sup>Trp</sup> reveal no detectable increase in glutamine acceptor activity results from the C35→T transition. The identity elements in *E. coli* tRNA<sup>Trp</sup> are well characterized (Jahn, M., Rogers, J., and Soll, D. (1991) *Nature* 352, 258–260). Sequence comparisons of the tRNA<sup>Trp</sup> and tRNA<sup>Gln</sup><sub>inr</sub> molecules from *E. coli* reveal that the amber suppressor tRNA<sup>Trp</sup> has four of five identity elements required for glutaminyl-tRNA synthetase recognition. A similar comparison in the yeast system shows only two of the five potential identity elements are present. We conclude that, in spite of substantial structural similarities between yeast and *E. coli* aminoacyl-tRNA synthetases, fundamental differences can exist with regard to tRNA recognition.

In recent years, an interest in elements of tRNA structure which control recognition by cognate and non-cognate aminoacyl-tRNA synthetases (RS) has given rise to a considerable amount of research in this field (reviewed by Schimmel (1989) and Normanly (1989)). The anticodon has been shown to be a strong determinant of identity in numerous tRNAs (Bare and Uhlenbeck, 1986; Schulman and Pelka, 1988, 1989; McClain et al., 1988, 1990, 1991; Jahn et al., 1991; Putz et al., 1991; Himeno et al., 1991; Pallanck and Schulman, 1991; Hasegawa et al., 1992; Nazarenko et al., 1992; Pallanck et al., 1992; Rogers et al., 1992). Any tRNAs in which all members of an isoaccepting group have one or more common bases in the anticodon are candidates to utilize these positions as identity elements. This mode of recognition has been demonstrated for *Escherichia coli* tRNA<sup>Trp</sup> both *in vivo* and *in vitro* (Yaniv et al., 1974; Himeno et al., 1991). Results from Soll and Berg (1969) as well as Celis et al. (1976) have shown that an amber suppressor form of *E. coli* tRNA<sup>Trp</sup> inserts predominantly Gln during translation of a corresponding nonsense codon position *in vivo*. Work by Knowlton et al. (1980) has indicated that *E. coli* tRNA<sup>Trp</sup>, has a dual specificity, charging equally well with either Trp or Gln *in vitro* and *in vivo*. Surprisingly, it appears that the translational apparatus somehow discriminates against the Trp-tRNA<sub>**Trp**</sub>.

Acylation of the tRNA<sup>Trp</sup> with both Gln and Trp is perhaps not surprising in view of similarities in the structures of *E. coli* tRNA<sup>Trp</sup> and tRNA<sup>Gln</sup> at positions important for GlnRS recognition (Rogers et al., 1992). Both mutational analyses (Jahn et al., 1991) and the crystal structure determined for a tRNA<sup>Gln</sup>–GlnRS complex (Roud et al., 1989) unequivocally illustrate the importance of both the acceptor stem and anticodon for recognition. The relatively relaxed charging specificity of *E. coli* GlnRS also likely contributes to heterogeneous charging (Engläsch-Peters et al., 1991). After losing a positive identity controlling element at the central position in the anticodon, *E. coli* tRNA<sup>Trp</sup> has a sufficiently lower affinity for TrpRS that the GlnRS becomes an effective competitor for this substrate (Knowlton et al., 1980). In support of this hypothesis, a G1→A mutant in an amber suppressor form of *E. coli* tRNA<sup>Trp</sup> also inserts Gln at UAG codons *in vivo* (Ghysen and Celis, 1974), suggesting that this base, along with U35 and G2:C71, are sufficient for positive recognition by GlnRS.

In prior work, the suppressor activity of yeast tRNA<sup>Trp</sup> has been documented *in vivo* (Kim and Johnson, 1988; Kim et al., 1990). To determine what amino acid this tRNA inserts in response to UAG, a chitinase gene altered to contain an amber codon near its amino terminus (Edwards et al., 1991) was expressed in *Saccharomyces cerevisiae* cells also containing the tRNA<sup>Trp</sup> gene. Amino acid sequence analysis of chitinase isolated from this cell line identified ≥80% Trp at the position specified by UAG. Furthermore, *in vitro* charging assays using tRNAs produced by T7 RNA polymerase from synthetic genes corresponding to and tRNA<sub>**Trp**</sub> show no increase in acceptor activity for Gln with the amber suppressor tRNA.

EXPERIMENTAL PROCEDURES

The construction of YCpTrp and YCpTrpA, carrying the yeast tRNA<sup>Trp</sup> gene and a C35→T variant, respectively, has been reported (Kim et al., 1988). Yeast strain W303Vcts and plasmid constructs pCT28 and pCTam were generously provided by Drs. Paul Schimmel and Catherine Reynolds. The yeast strain W303Vcts is a derivative of W303-1B in which the chitinase gene has been disrupted by a directed integration (Kuranda and Robbins, 1991). The plasmid pCT28 carries the structural gene for chitinase, CTS1-1 (Kuranda and Robbins, 1991), in the vector Yep532 (Hill et al., 1986). The plasmid pCTam contains a version of the CTS1-1 gene in which the codon for Asn<sup>**</sup> of the mature protein has been converted to an amber codon (Trezeget et al., 1991). The W303Vcts cells were transformed...
by the procedure of Ito et al. (1983). Transformants were identified by selecting for nutritional markers and the presence of the expected plasmid(s) confirmed by Southern blot analysis. Chitinase was prepared essentially by the procedure of Kuranda and Robbins (1991). The protein isolated from each cell line was loaded onto a 7% polyacrylamide, 0.1% SDS gel, electrophoresed, and stained with Coomassie Blue for visualization. For amino acid sequence analysis, proteins in SDS-polyacrylamide gels were electroblotted to Immobilon polyvinylidene difluoride membrane (Bio-Rad), then stained with Coomassie Blue. The chitinase bands were cut from the membrane and subjected to sequence analysis using an Applied Biosystems 473A Protein Sequencer.

Charging experiments were done using a commercial yeast aaRS preparation (Sigma). Bulk yeast tRNA was isolated as described by Monier et al. (1960) and decylated following the procedure of Sarin and Zamecnik (1964). Transcripts representing tRNA\textsuperscript{A\textsubscript{p}}, and tRNA\textsubscript{C\textsubscript{UA}} were made using T7 RNA polymerase with cloned oligodeoxyribonucleotide templates as described by Sampson and Uhlenbeck (1988). Nucleotide sequence analyses were done on the templates to confirm their structure. Prior to use in charging reactions, the T7 transcripts were purified by both electrophoresis on 10% polyacrylamide, 8 M urea gels and reverse-phase chromatography, then renatured by slowly cooling from 65 °C in the presence of 20 mM MgCl\textsubscript{2}. Charging reactions were done at 37 °C in 30 mM Hepes (pH 7.5), 25 mM NH\textsubscript{4}Cl or KCl, 15 mM MgCl\textsubscript{2}, 2.5 mM ATP, 4 mM dihydroethanol, and either 12 \mu M [\textsuperscript{3}H]Trp (16 Ci/mM) or 15 \mu M [\textsuperscript{3}H]Gln (26 Ci/mM). The T7 transcripts were present at 1.0 \mu M or the bulk tRNA at 5.8 mg/ml. Aliquots were removed at the times indicated and spotted on GF/C disks (Whatman LabSales Inc., Hillsboro, OR), which were then immersed in cold 10% trichlooroacetic acid. The disks were washed 5 times in cold 5% trichlooroacetic acid, once in 65% ethanol, air-dried, and then radioactivity was measured in 5 ml of a liquid scintillation mixture (Beckman Instruments, Fullerton, CA).

RESULTS

A paucity of naturally occurring nonsense suppressor tRNAs in yeast (Sherman, 1982), compared to \textit{E. coli} (Raftery and Yarus, 1985), encouraged an attempt to create via \textit{in vitro} genetics tRNAs which could function in this capacity. A C35→T mutant of the \textit{S. cerevisiae} tRNA\textsubscript{C\textsubscript{UA}} gene was created, tRNA\textsubscript{C\textsubscript{UA}}\textsuperscript{T\textsubscript{p}}, and its ability to suppress a number of amber mutations \textit{in vivo} was demonstrated (Kim and Johnson, 1988). To determine whether this tRNA actually inserts Trp \textit{in vivo}, yeast tRNA\textsuperscript{A\textsubscript{p}} and the suppressor tRNA\textsuperscript{A\textsubscript{p}} were used as substrates for the yeast GlnRS, an amino acid was recovered at position 8 with protein from the pCTam+ tRNA\textsuperscript{A\textsubscript{p}} system (Fig. 2B). Low levels (≤10% the amount of Trp) of Gln and Asp were recovered in this cycle. Similar amounts of background were also observed in the other sequencing cycles. We can therefore conclude that ≥80% of the amino acid inserted by tRNA\textsubscript{C\textsubscript{UA}}\textsuperscript{T\textsubscript{p}} is indeed Trp.

Knowleson et al. (1980) demonstrated that, both \textit{in vivo} and \textit{in vitro}, approximately equal amounts of Trp and Gln were esterified to \textit{E. coli} tRNA\textsubscript{A\textsubscript{p}}. To determine whether \textit{S. cerevisiae} tRNA\textsubscript{C\textsubscript{UA}}\textsuperscript{T\textsubscript{p}} was a substrate for the yeast GlnRS, an \textit{in vitro} system was established to allow direct measurement of charging. Following the methods developed by Sampson and Uhlenbeck (1988), synthetic oligodeoxyribonucleotides were synthesized and cloned such that T7 RNA polymerase could be used to produce transcripts representing mature-sized tRNA\textsubscript{A\textsubscript{p}} and tRNA\textsubscript{C\textsubscript{UA}}\textsuperscript{T\textsubscript{p}}. These transcripts were used as substrates for a crude aaRS preparation from yeast with either [\textsuperscript{3}H]Trp or [\textsuperscript{3}H]Gln present. Bulk, deacylated tRNA from yeast was used as a positive control for the activity of both RS enzymes (Fig. 3A). The results demonstrate that the C35→T transition which generates the amber suppressor does

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**Fig. 1.** SDS-polyacrylamide gel electrophoresis of chitinase preparations. Chitinase was isolated from YPD media of stationary phase cultures of W303\textit{Vcts} transformed with either pCT28, pCTam+YcpTrp, or pCTam+YcpTrpA, by complexing the protein with powdered chitin. The chitinase-chitin complexes were isolated by filtration, then disrupted by heating at 65 °C or 90 °C in 2% SDS, 5% β-mercaptoethanol. Equal amounts of protein from each sample were electrophoresed on a 7% polyacrylamide, 0.1% SDS gel. Protein bands, stained with Coomassie Brilliant Blue, are identified, and values in parentheses are the corresponding molecular masses in kDa.
not measurably affect the Gln acceptor activity of tRNA\textsuperscript{Trp} (Fig. 3, B and C). The levels of the Gln charging in Fig. 3 (B and C) are equal and represent ±2 times background.

DISCUSSION

An amber mutation in theCTS11 gene of yeast can be suppressed at about 5–10% efficiency by tRNA\textsuperscript{Gln} (Fig. 1), and Trp is inserted at ≈80% of the suppression events (Fig. 2). The nearly quantitative insertion of Trp in this system is in clear contrast to a comparable result in E. coli, where approximately ∼90% Gln is found at positions specified by a UAG codon when tRNA\textsuperscript{Gln} is present (Celis et al., 1976). This is quite surprising as other work (Knowlton et al., 1980) demonstrated that the kinetics of charging of tRNA\textsuperscript{Gln} by the E. coli GlnRS and TrpRS enzymes in vitro are nearly identical. Also, in vivo the accumulation of glutaminyl and tryptophanyl forms of the tRNA is nearly equivalent. This implies a very unusual selection must exist for the Gln tRNA\textsuperscript{Gln} in some aspect of E. coli translation. Again, the yeast system behaves rather differently as in vitro charging experiments indicate no measurable Gln acceptor activity for the tRNA\textsuperscript{Gln} (Fig. 3). The anticodon mutation does impede charging with [\textsuperscript{3}H]Tryp by 10-20-fold. This may provide a partial explanation for the reduced amount of chitinase produced by yeast cells carrying pCTam-YCyTrpA (Fig. 1). The reduction in extent of [\textsuperscript{3}H]Tryp charging observed in vitro with the amber suppressor mutant is likely due to a non-enzymatic deacylation reaction (Bonnett and Ebel, 1972).

A comparison of the sequences of yeast and E. coli tRNAs for Trp and Gln is presented in Fig. 4. The sequence elements that specify the charging identity for the E. coli system have been established (Himeno et al., 1991; Jahn et al., 1991) and are boxed in the figure. The C35→T transition in E. coli tRNA\textsuperscript{Gln} results in five of six identity elements for recognition by GlnRS being present in this molecule. Two of these similarities, U35 and G73, are known to be the predominant recognition sites (Ghysen and Celis, 1974; Jahn et al., 1991). Comparing the two yeast tRNAs in these putative recognition regions suggests a possible explanation for the different charging behavior. Only two of the five positions are equivalent between the yeast tRNAs corresponding to tRNA\textsuperscript{Gln} and tRNA\textsuperscript{Gln} (Fig. 4). This suggests that the recognition elements used by the TrpRS and GlnRS enzymes have evolved differently in yeast and perhaps other eukaryotes (Nagel and Doolittle, 1991). This also appears to be the case for TyrRS, LeuRS, and MetRS (Edwards et al., 1991; Lee and Raj-Bhandary, 1991). Alternatively, the differences may reflect an intrinsic misacylation tendency of the E. coli GlnRS (Englsch-Peters et al., 1991). An important caveat emptor to these arguments is that the recognition sites for the yeast
Fig. 4. Sequence comparisons of yeast and E. coli tRNAs for Trp and Gln. The cloverleaf structures of yeast and E. coli tRNATp are presented (Keith et al., 1971; Hirsh, 1970) along with the tRNA\(^{Gln}\) and tRNA\(^{Gln}\) sequences for both organisms (Roulul et al., 1989; Tschumper and Carbon, 1982; Weiss and Friedberg, 1988). Nucleotides known to be involved in recognition of the E. coli versions of these tRNAs by their cognate aaRS are boxed. The corresponding yeast sequences are also boxed. The U35 which converts tRNA\(^{Tyr}\) to an amber suppressor as well as bases which differ in tRNA\(^{Gln}\) and tRNA\(^{Gln}\) are presented in brackets.

GlnRS and TrpRS enzymes have not been mapped. Experiments to identify these sites are currently in progress.

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