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Yeast $tRNA^{Trp}$ genes with anticodons corresponding to UAA and UGA nonsense codons

Daemyung Kim+, Gregory J.Raymond, Shawna D.Clark, Janice A.Vranka and Jerry D.Johnson*
Department of Molecular Biology, Box 3944, University of Wyoming, Laramie, WY 82071, USA

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ABSTRACT

Naturally occurring suppressor mutants derived from $tRNA^{Trp}$ genes have never been identified in *S. cerevisiae*. Oligonucleotide-directed mutagenesis was used to generate potential ochre and opal suppressors from a cloned $tRNA^{Trp}$ gene. In vitro transcription analyses show the ochre suppressor form of the gene, TRPO, accumulates precursors and tRNA in amounts comparable to the parent. The opal suppressor, TRPOP, accumulates 4–5 fold less tRNA. Both forms of the gene are processed and spliced in vitro to produce tRNAs with the expected base sequences. The altered genes were subcloned into yeast vectors and introduced into yeast strains carrying a variety of amber, ochre, and opal mutations. When introduced on a CEN vector, neither ochre nor opal suppressor forms show suppressor activity. Deletion of the CEN region from the clones increases the copy number to 10–20/cell. The opal suppressor form shows moderate suppressor activity when the gene is introduced on this vector, however, the ochre suppressor form exhibits no detectable biological activity regardless of gene copy number. Northern blot analyses of the steady state levels of $tRNA^{Trp}$ in cells containing the high copy-number clones reveal 20–100% increases in the abundance of $tRNA^{Trp}$.

INTRODUCTION

In *Saccharomyces cerevisiae*, numerous nonsense suppressors have been identified by genetic selection for mutations arising *in vivo* (1,2). Anticodon mutations, capable of suppressing amber (UAG) or ochre (UAA) codons, have been identified in $tRNA^{Tyr}$, $tRNA^{Ser}$, and $tRNA^{Leu}$ genes. These same genetic loci are also likely candidates for all known opal (UGA) suppressors (1,2). A normal $tRNA^{Gln}$ which has a CUG anticodon can weakly recognize an amber codon (3). This tRNA, operating via a first position wobble, is the only other nonsense suppressor tRNA identified in *Saccharomyces cerevisiae*.

Suppressor tRNAs are valuable tools for studies of translational processes as well as protein engineering (4,5). At least 8 other types of yeast tRNA genes can be mutagenized to nonsense suppressors by single-base substitutions (6). However, these nonsense suppressor tRNAs have never been identified *in vivo* in spite of extensive screening efforts (7). There are many variables that might restrict isolation of mutant tRNAs by classical suppression of nonsense mutations. As with yeast $tRNA^{Ser}$, an anticodon mutation in a tRNA gene which is present in a single copy may be lethal (8). Highly efficient suppressors can impair cell growth and could be lethal (9). Mutations in the anticodon or adjacent regions may impair the transcription, maturation, or aminoacylation of tRNA (10,11) or cause changes in translation context effects rendering them unable to function as a suppressor (12–15). Very weak suppressors may not be detectable unless the gene is present in multiple copies (16).

Yeast $tRNA^{Phe}$ and $tRNA^{Cys}$ molecules whose anticodons were altered *in vitro* are capable of acting as suppressors (12,13,17). To determine whether the single form of yeast $tRNA^{Trp}$ could act as a suppressor, the CCA anticodon of a cloned $tRNA^{Trp}$ gene was converted, using site-directed mutagenesis, to anticodons corresponding to all three nonsense codons. The mutant genes are transcribed and processed as expected *in vitro*. Suppressor activities of the altered tRNA genes were measured by introducing them, in single or multiple copies, into yeast strains carrying nonsense mutations. The activity of the amber suppressor form of $tRNA^{Trp}$ carried on a single-copy vector has been reported (18). The experiments presented herein demonstrate that $tRNA^{Trp}$ can also act as a weak suppressor of UGA mutations but does not respond to UAA codons.

MATERIALS AND METHODS

Strains and plasmids

Saccharomyces cerevisiae strains used in this work were identified previously (18) except strain JG369-3B (α, *ura3-52*, *met8-1*(a), *trp1-1*(a), *ade2-1*(o), *lys2-1*(o), *can1-100*(o), *leu2-2*(op), and *his4-260*(op)), a generous gift from Audrey Atkin and Dr. John Bell.

E. coli strains JM101 and JM109 were from Vector Cloning Systems. The strain C600SF8 was from Dr. J. Abelson.

The plasmid pJB20m (19) was kindly provided by Dr. J. Abelson. Bluescribe $M13^+$ and $M13^-$ were from Vector Cloning Systems. The yeast centromere plasmid YCp19 was

* To whom correspondence should be addressed

+ Present address: Cold Spring Harbor Laboratory, PO Box 100, Cold Spring Harbor, NY 11724, USA

kindly supplied by Dr. R. Davis. A multicopy version of this plasmid, designated ΔCEN, was created by deleting a 1.6 kb XhoI fragment which includes the CEN4 sequence (20).

DNA

Plasmid DNAs were prepared by the method of Raymond et al. (21) or by the rapid method of Birnboim and Doly (22). Single-stranded DNA was recovered from cells containing Bluescribe M13 plasmids as described by the manufacturer (Vector Cloning Systems). Oligodeoxynucleotides were synthesized in an Applied Biosystems Model 380Å using β-cyanoethyl phosphoramidite precusors. Homogeneity of these preparations was examined by polyacrylamide gel electrophoresis. The fragments used to generate the UAA and UGA suppressors were GCTGAAATT-AATTTAG and GCTGAAGTTAATTTAG respectively. Positions of mismatch with the tDNA^{Trp} are identified by bold face. The sequences used to probe Northern blots for tRNA^{Leu3} and tRNA^{Trp} are TGGTTGCTAAGAGATTCGAA-CTCTT-GCATCTTACGATACCT and AGCTTCCCAACGTCCA respectively. Site-directed mutagenesis and characterization of mutants has been described (18).

Transformation

Transformation of *E. coli* JM109 was performed using the DMSO plus DTT procedure of Hanahan (23). Yeast transformations were performed using the procedure of Ito et al. (24). The final cell pellets were washed twice with 500 μl of 10 mM Tris-Cl(pH 8.0)−1.0 mM EDTA, resuspended in 500 μl of YPD, and incubated at 30° for 30 min with shaking. Aliquots of $5-10\times10^6$ cells were plated on SD agar plates supplemented to select for either complementation or suppression. Suppressor activities were measured by comparing the numbers of colonies appearing on media selecting for suppression to those selecting for complementation (25). Plates were first counted after 2−4 days of incubation then re-examined periodically for 8−10 additional days at 30°. The observation periods reported are the earliest times at which the final colony counts were observed. To determine whether the suppressor activities observed resulted from plasmid borne genes, cells from colonies arising on the original selection plates were transferred, via sterile toothpicks, to plates screening for co-transformation of complementation and suppression.

In vitro analyses

The extracts and conditions used for *in vitro* transcription, splicing, and fingerprinting have been described (18).

Northern blotting

Cultures of YNN217 cells transformed with YCp19 or ΔCEN clones of the tRNA^{Trp} genes were grown in SD media under Ura selection for the plasmids. Cells were harvested in late log phase and total low molecular weight RNAs extracted with phenol as described by Monier et al. (26). Aliquots of these RNA preparations, containing equivalent amounts of UV absorbing material, were subjected to electrophoresis on 10% polyacrylamide gels containing 8 M urea. The RNA was electroblotted to zeta-probe membranes using conditions recommended by the supplier (BioRad Laboratories). Filters were prehybridized in 6× SSPE containing 10× Denhardt's solution for 10−16 hr at 45° then probed with a 41 base oligodeoxynucleotide complementary to the 3′-half of tRNA^{Leu3} or a 16 base fragment complementary to a region of tRNA^{Trp} on the 3′ side of the anticodon. Probes were labeled using polynucleotide kinase and [γ-^{32}P]ATP. After the first experiment, both probes were used simultaneously as no crossreactivity was found. Autoradiograms with 2 and 4 hour exposures were produced from these filters and each was scanned 2−4 times using an LKB Ultrascan densitometer. Areas representing the tRNA^{Leu3} and ^{Trp} signals were integrated and the ratios of the two calculated.

RESULTS

Generation and identification of nonsense suppressor mutations

To mutagenize the anticodon of the yeast tRNA^{Trp} gene to potential nonsense suppressors, synthetic oligodeoxynucleotides 16 bases long were prepared which span the anticodon region and hybridize to the noncoding strand of the tRNA^{Trp} gene with mismatches in the tRNA anticodon. The ochre and opal suppressor mutations destroy a HinfI restriction site normally present in the tRNA^{Trp} gene. The loss of this restriction site was used to screen for mutants. Dideoxynucleotide sequencing of mutants was done to confirm their identity and integrity (data not shown). The sequence and structure of the precursors and tRNAs expected from these constructions, as well as the parental and amber suppressor genes, is shown in Fig. 1.

In vitro transcription and processing

Cloned DNAs representing the four forms of tDNA^{Trp} and a yeast tRNA^{Leu3} gene (27) were linearized then used as templates in coupled transcription-splicing reactions. Transcription reactions were performed using [α-^{32}P]UTP to label the transcripts from

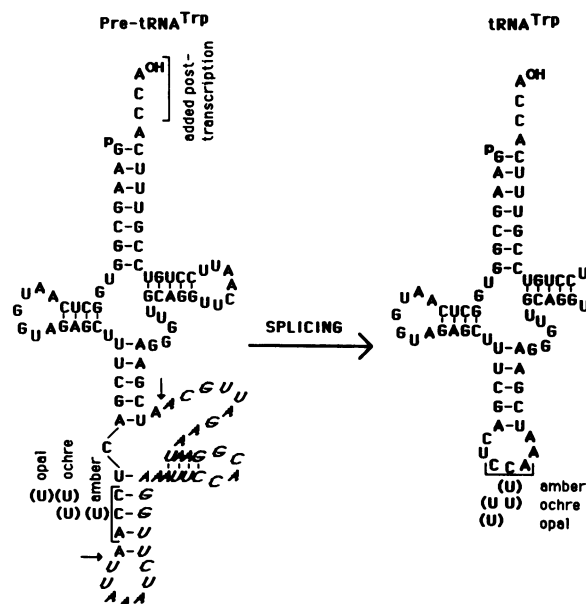
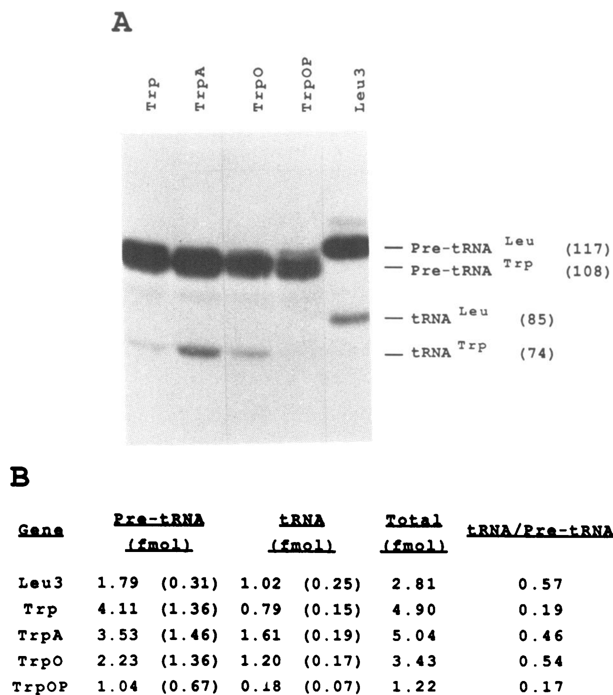


Figure 1. Transcription products from yeast tRNA^{Trp} genes. The primary transcript from tDNA^{Trp} includes the complete sequence of the mature tRNA plus extensions at both the 5′ and 3′-ends and a 34 base intervening sequence (IVS). End-trimming and addition of the 3′-terminal -CCA$_{OH}$ are accomplished prior to removal of the IVS. The end-matured precusor is depicted in the usual secondary structure expected for these precursors. The IVS boundaries are marked by arrows and IVS bases are italicized. The anticodon region is bracketed, and substitutions representing the three different mutants are shown in parentheses adjacent to bases in the parent form which are replaced. Mature tRNA^{Trp}, resulting from the splicing reaction is also presented.

the tRNA genes. Radioactive RNAs were resolved by electrophoresis and Fig. 2A is an autoradiogram of such a gel. The precursors from all $tRNA^{Trp}$ forms, whose expected structures are depicted in Fig. 1, have the same electrophoretic mobility as the parent indicating that 5'- and 3'-end processing of the mutant genes is not affected by the mutations. The expected structures of the spliced tRNA derived from these precursors are also included in Fig. 1. Again, the size of these molecules does not vary as a result of the mutations, indicating that the size of intron removed is not altered by the mutations.

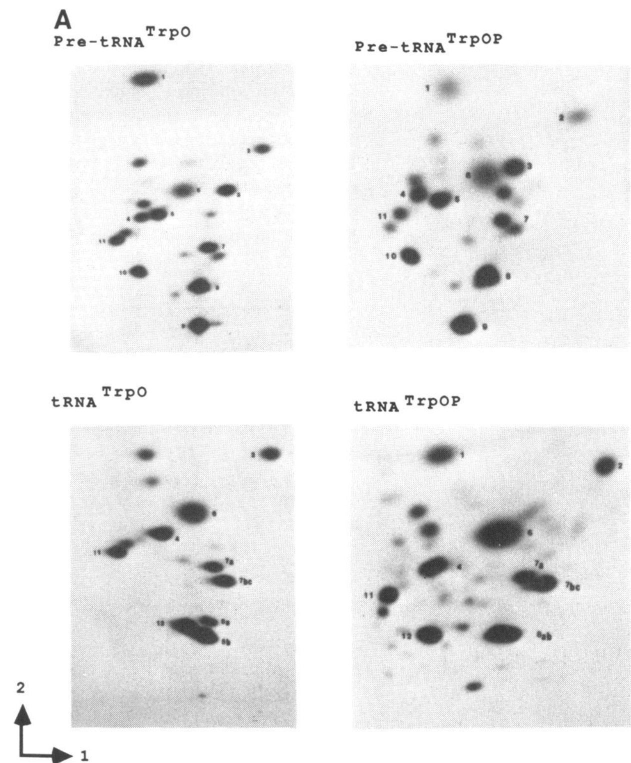
In Fig. 2B, the molar amounts of transcripts produced from $tDNA^{Leu3}$ and each of the four forms of $tDNA^{Trp}$ are presented. The efficiency of transcription of the amber suppressor form, measured by the total number of transcripts accumulated, is almost identical to the parent. However, the opal and ochre suppressor forms produce only 25% and 70% as many transcripts as the parent, respectively. The splicing efficiencies of the precursors were estimated by comparing the ratios of tRNA to precursor. Amber and ochre mutants are spliced with 2–3 fold greater efficiency than the parent while the opal suppressor precursor is converted as efficiently as the parent molecule. However, because of the smaller amount of precursor, the amount of $tRNA^{TrpOP}$ accumulated is 4 fold lower than that for the parental tRNA. Incubation of RNAs from all forms of $tRNA^{Trp}$ in the transcription-splicing extracts indicate no differences in



B

Gene	Pre-tRNA (fmol)	tRNA (fmol)	Total (fmol)	tRNA/Pre-tRNA
Leu3	1.79 (0.31)	1.02 (0.25)	2.81	0.57
Trp	4.11 (1.36)	0.79 (0.15)	4.90	0.19
TrpA	3.53 (1.46)	1.61 (0.19)	5.04	0.46
TrpO	2.23 (1.36)	1.20 (0.17)	3.43	0.54
TrpOP	1.04 (0.67)	0.18 (0.07)	1.22	0.17

Figure 2. *In vitro* transcription and splicing of the $tRNA^{Trp}$ genes. Bluescribe M13 clones of the parental and three nonsense suppressor forms of the $tRNA^{Trp}$ gene were used as templates in coupled transcription-splicing reactions. The reactions were done as described in Methods using linearized plasmid DNA as template and [α-^{32}P]UTP to label reaction products. The transcripts were separated by electrophoresis on a 10% polyacrylamide gel containing 8 M urea. (A) An autoradiogram of such a gel with transcripts from a yeast $tDNA^{Leu3}$ template included as markers. Positions of end-processed pre-tRNA and mature tRNA are indicated. The values in parentheses are the number of nucleotides in the molecule. (B) The molar amounts of precursor and tRNA from the $tRNA^{Leu3}$ gene and each of the four forms of the $tRNA^{Trp}$ gene are presented. The values are averaged from three separate experiments; standard deviations are in parentheses.



B

SPOT NUMBER	FRAGMENT (common)	MOLAR RATIO	RNase A PRODUCTS
1	G	1.85(3)	G
2	UUG	0.75(1)	U
4	CUCAAUG	2.07(2)	C, AAU
6	UCCG	1.11(2)	G
7	CUUUCG	3.17(3)	2)U, C
8	UUCAAUUCCUG	5.00(5)	U, C, G, AAU
11	UUUCACCA	1.48(2)	2)U
	(Pre-tRNA specific)		
3	AUUG	1.55(2)	AU
5a,b	CAAUCG	2.05(1)	AAU
	AAUAAG	(1)	AAU
9(O)	ACUUUAAUUAAAUCUUG	9.31(8)	3)U, C, AC, AAU, AAAU
9(OP)	ACUUCAAUUAAAUCUUG	7.14(7)	2)U, C, AC, AAU, AAAU
10	AAAUUCCACG	1.68(2)	AAAU
	(tRNA specific)		
12(O)	ACUUUAAAUCG	3.77(4)	2)U, AC, AAAU
12(OP)	ACUUCAAAUCG	3.04(3)	U, AC, AAAU

Figure 3. Fingerprint analysis of pre-tRNA and tRNA molecules produced *in vitro*. Radioactive RNA molecules were eluted by diffusion from the gel depicted in Fig. 2 and recovered by ethanol precipitation. About 10^4–10^5 CPM of precursor or mature tRNAs were recovered, digested with RNase T1, and the resulting oligonucleotides separated by two dimensional polyacrylamide gel electrophoresis. The first dimension was 10% polyacrylamide in 25 mM citric acid (pH 3.5). The second dimension was 22% polyacrylamide in TBE (pH 8.3). Top and bottom panels in part A are autoradiograms of oligonucleotide patterns resulting from analysis of precursors and tRNA molecules respectively. Spots corresponding to fragments which differ only by base modification are represented by a single number except 5a and 5b which represent two different fragments that are not resolved in this system. Arrows indicate the direction of electrophoresis. Part B presents the identification of the RNaseT1 oligonucleotides numbered in part A. Identities were deduced from their electrophoretic mobilities, molar yields, and RNaseA digestion products.

Table I. **(A)** Activities of tRNA^{Trp} genes cloned in a single copy vector. Yeast cells were transformed with the indicated DNAs which contain both the *ura3* marker and one of the four forms of tDNA^{Trp}. Equal numbers of cells from each transformation were plated on SD media selecting for *ura3* gene complementation(c) or suppression of amber(a), ochre(o), or opal(op) markers. Presence or absence of growth was scored after 2 days of incubation at 30°. The number of colonies appearing with each type of selection plate is averaged from two separate experiments. **1.** =SL797-1A; **2.** =YNN217; **3.** =RJ293-13C. **(B)** Appearance of unselected characters in yeast cells transformed with tRNA^{Trp} genes. Cells from colonies appearing on the selective plates in panel **A** were transferred to SD plates supplemented to allow detection of unselected characters. Presence or absence of growth was scored after 2 days incubation at 30°.

A

	Number of colonies on selective SD plates			
DNA	*ura3-52*(c)	*met8-1*(a)	*tyr7-1*(a)	*leu2-1*(o)
1. None	0	97	10	13
Trp	100	60	8	8
TrpA	452	478	405	8
TrpO	656	109	12	23
TrpOP	414	72	13	19
		lys2-801(a)	*ade2-101*(o)	
2. None	0	13	0	
Trp	207	17	0	
TrpA	1245	802	0	
TrpO	1414	24	6	
TrpOP	996	22	0	
		his4-260(op)		
3. None	0	5		
Trp	24	1		
TrpA	201	1		
TrpO	279	3		
TrpOP	272	1		

B

	First	Second selection			
DNA	selection	*met8-1*(a)	*tyr7-1*(a)	*lys2-801*(a)	*ura3-52*(c)
TrpA	-Ura	50/50	50/50	50/50	-
TrpA	-Met	–	–	–	47/50
TrpA	-Tyr	–	–	–	46/50
TrpA	-Lys	–	–	–	49/50
		leu2-1(o)	*ade2-101*(o)	*ura3-52*(c)	
TrpO	-Ura	6/50	0/50	-	
TrpO	-Leu	–	–	0/21	
TrpO	-Ade	–	–	2/6	
		his4-260(op)	*ura3-52*(c)	*leu2-2*(op)	
TrpOP	-Ura	0/50	–	–	
TrpOP	-His	–	1/5	–	
TrpOP	-Ura	0/50*	–	0/50*	

* in strain JG369-3A

stability except the precursor from tDNA^{TrpOP} which is about 20% less stable than the others (data not shown).

Fingerprint analyses

Precursor and spliced tRNA molecules were recovered from the polyacrylamide gels depicted in Fig. 2A, digested with RNase T1, and the resulting oligonucleotides separated by two-dimensional polyacrylamide gel electrophoresis. Fig. 3A shows the analysis of precursor and tRNA forms of the ochre and opal suppressors. Comparable data for the parent gene and amber suppressor forms has been presented (18). All numbered oligonucleotides on the fingerprint gel were eluted and their RNase A digestion products analyzed (Fig. 3B). All expected fragments are present indicating that the nonsense suppressor mutations generate the expected precursor and tRNA molecules as depicted in Fig. 1.

Precursors differ only in the mobility of spot number 9, the fragment containing the anticodon of the tRNA. As expected, the replacement of C by U in the opal suppressor mutant slightly increases the mobility of this spot in the first dimension, relative to that of the parental form. The replacement of an additional C by U in the ochre suppressor mutant further increases the mobility of this fragment (Fig. 3A). The only difference in RNase A digestion products is a reduced amount of U in the opal suppressor (Fig. 3B).

Fingerprints of the tRNAs produced by *in vitro* splicing of the

Table II. Transformation of yeast cells with tRNA^{Trp} genes cloned in a multicopy vector. Yeast cells were transformed as described in Methods with YCp19ΔCEN clones of the tRNA^{Trp} genes. Aliquots of the transformed cells were plated on SD media supplemented to select for complementation by the *ura3* gene carried on the vector. The presence or absence of growth was scored after 3–4 days of incubation at 30°. The data for Ura$^+$ colonies is averaged from two independent experiments. Selections for suppressor activity were done with cells from colonies prototrophic for Ura. Presence or absence of growth was scored after 3–4 days of incubation at 30°. 1. = YNN217; 2. = RJ293-13C; 3. = JG369-3B.

DNA	Ura$^+$ colonies	second selections			
		lys2-801(a)		*ade2-101*(o)	
1. TrpΔCEN	1420	0/100		0/100	
TrpAΔCEN	21	39/42		0/42	
TrpOΔCEN	604	1/100		0/100	
TrpOPΔCEN	646	0/100		4/100	
		his4-260(op)			
2. TrpΔCEN	45	19/90			
TrpAΔCEN	2	0/4			
TrpOΔCEN	21	0/42			
TrpOPΔCEN	30	20/60			
		ade2-1(o)	*lys2-1*(o)	*leu2-2*(op)	*his4-260*(op)
3. TrpΔCEN	300	0/50	1/50	34/50	0/50
TrpAΔCEN	0	–	–	–	–
TrpOΔCEN	300	3/50	3/50	2/50	0/50
TrpOPΔCEN	151	1/50	2/50	45/50	48/50

pre-tRNA molecules differ in the mobility of spot number 12, the fragment containing the anticodon. Again, first dimension mobilities of these fragments is increased by the additional U residues and the RNase A products differ in the yield of U (Fig. 3B).

Suppressor activity

To test the biological function of the nonsense suppressor forms of tDNA^{Trp}, their ability to cause phenotypic suppression was measured in yeast strains carrying amber, ochre, or opal mutations that confer nutritional requirements. Cells from three different yeast strains, each carrying different nonsense mutations, were transformed with YCp19 clones of each form of the tRNA^{Trp} gene. Following transformation, aliquots of the cells were plated on SD media supplemented with nutrients such that complementation as well as suppression of amber, ochre, or opal markers could be measured.

Table IA shows all three amber mutations, *met8-1* and *tyr7-1* in strain SL797-1A and *lys2-801* in strain YNN217, are clearly suppressed by the presence of the TRPA form of tDNA^{Trp}. The ochre suppressor form of the gene was not able to suppress either of two ochre mutations, *leu2-1* in strain SL797-1A or *ade2-101* in strain YNN217. This gene also has no effect on any of the amber or opal mutations. Transformation with the UGA suppressor mutant gene does not relieve the His- phenotype resulting from *his4-260*(op) in either of two cell strains (Table IB). The *leu2-2*(op) marker in strain JG369-3A is also unaffected. The TrpOP gene also has no effect on any of the amber or ochre mutations in the other strains.

To confirm that the suppressor activity observed is due to the presence of tRNA^{Trp} genes carried on the plasmid, transformed cells from colonies appearing on the selective plates were transferred to SD plates supplemented to allow detection of unselected characters (Table IB). Cells transformed with the amber suppressor form of tDNA^{Trp} and initially selected for uracil prototrophy uniformly show suppression of all amber markers. Those selected for suppression of *met8-1*, *tyr7-1*, or *lys2-801* have also simultaneously lost the requirement for uracil. Coappearance of both characters makes it unlikely that the observed prototrophies are due to host cell reversions. However, cells transformed with ochre or opal suppressor forms of tDNA^{Trp} and selected for suppression have not lost their requirement for uracil and, conversely, cells initially selected for uracil do not show suppression of ochre or opal markers indicating that the colonies appearing on ochre and opal selection plates are due to host cell reversions rather than the presence of plasmid borne suppressor mutants of the tRNA^{Trp} gene. This screening for suppressor acitivity in cells first selected for complementation also provides a more sensitive assay for weak suppressor activity because cells carrying TRPO and TRPOP genes have additional time to accumulate the mutant tRNAs before selection is applied. However, no additional supressor activity is revealed by this approach. Since it is known that weak suppressors can be detected if the cognate gene is present in multiple copies (16), we wished to determine whether the ochre and opal suppressor forms of tRNA^{Trp} might show biological activity if present in higher levels. The plasmid YCp19 has a centromere sequence (CEN4) which causes the plasmid to be carried at 1–2 copies per cell (28–29). By removing a 1.6 kb XhoI fragment which includes the CEN4 gene, copy number control is lost and the plasmid is carried at 10–20 copies per cell when maintained under selective pressure.

Cells from three yeast strains were then transformed with ΔCEN clones representing each type of tRNA^{Trp}. Transformed cells were first selected for complementation by the *ura3* gene carried on the vector (Table II). The transformation efficiency of the ΔCEN amber suppressor tRNA gene is very low in all strains analyzed implying that high levels of the amber suppressor tDNA^{Trp} has a deleterious effect on cell viability. Cells from colonies selected for the presence of the *ura3* gene of the YCp19ΔCEN vector were transferred to SD plates supplemented with nutrients to measure suppressor activity (Table II). By first selecting for complementation then subsequently screening for suppressor activity, the identification of weak suppressors is facilitated. This is presumably because the tRNAs are stable and accumulate to functional levels if given sufficient time. Although

transformation efficiency with the multicopy, amber suppressor form of tDNA^{Trp} is significantly reduced, the amber mutation *lys2-801* is clearly suppressed in cells which survive. Growth of these surviving cells, under Ura selection, is clearly impaired (data not shown). The *ade2-101* ochre mutation remains unaffected by the presence of the TRPO gene in two different genetic backgrounds. A second UAA mutation, *lys2-1* is also unaffected. Two UGA mutations, *his4-260* and *leu2-2*, in strain JG369-3B are suppressed by the opal suppressor form of tRNA^{Trp} when the gene is present in multiple copies. In a different genetic background, strain RJ293-13C, the *his4-260* marker is only partially suppressed (Table II).

tRNA^{Trp} levels in transformed cells

To verify that the presence of the ΔCEN versions of the genes increased the levels of tRNA in the transformed cells, low molecular weight RNA was isolated from transformed YNN217 cells, electrophoresed on 10% polyacrylamide gels containing 8 M urea, and the RNA blotted to nylon membranes. The blots were probed with ^{32}P-labeled oligonucleotides complementary to a region of tRNA^{Trp} common to all forms of the gene just 3′ from the anticodon. A second probe, complementary to the 3′-half of tRNA^{Leu3}, was included to provide an internal reference for comparing tRNA^{Trp} levels. An autoradiogram, shown in Fig. 4, indicates that the two species of tRNA are well separated and clearly identified by the probes. Results from quantitative densitometry of several such films is presented in Table III. Cells transformed with the ΔCEN versions of the tRNA^{Trp} genes contain 20–100% more tRNA^{Trp}, relative to tRNA^{Leu3}, than do cells transformed with the vector only or with YCp19 clones of the same genes. The opal suppressor form of the gene seems to have the largest effect on *in vivo* tRNA levels (Fig. 2).

DISCUSSION

Tryptophan is specified by a single codon, UGG, which is only one base different from the amber (UAG) and opal (UGA) codons and two bases from the ochre codon (UAA). The lack of any naturally occuring yeast tRNA capable of inserting tryptophan at any of the nonsense codons prompted the engineering of the cognate suppressor mutations in a cloned copy of a tRNA^{Trp} gene to determine whether any functional tRNAs could be formed *in vitro* and *in vivo*. Using site-directed mutagenesis, the C to T transitions were introduced at the central, the 5′ and central, or the 5′-position of anticodon of the tRNA gene, generating amber, ochre, and opal suppressor forms of tRNA^{Trp} gene respectively. We have previously reported that the amber suppressor form of tRNA^{Trp} is expressed *in vitro* and acts as a strong suppressor when the gene is present in yeast cells on a CEN vector (18).

In vitro transcription and splicing reactions indicate that both the ochre and opal suppressor forms of the gene also produce transcripts which are spliced and processed to form mature tRNA albeit at a reduced level for the TRPOP gene (Figs. 2 and 3). However, neither of two ochre mutations, *leu2-1* and *ade2-101* nor two opal mutations, *his4-260* and *leu2-2* were suppressed by the presence of the cognate mutant tDNAs when present on plasmids at 1–2 copies per cell (Table I).

To determine whether the observed lack of biological activity of the ochre and opal suppressor forms resulted from the low copy number of YCp19 derived plasmids, multicopy versions

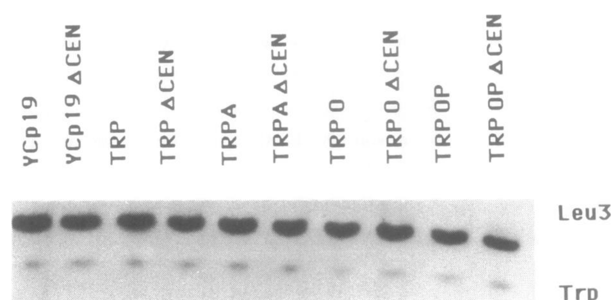


Figure 4. Northern blotting of tRNA from *S. cerevisiae* YNN217 transformed with YCp19 and ΔCEN clones of tRNA^{Trp} genes. Extracts of whole yeast cells containing unfractioned, low-molecular weight RNAs were separated by electrophoresis on 10% polyacrylamide gels containing 8 M urea. RNA in the gel was electroblotted to a nylon membrane and probed with ^{32}P-labeled oligodeoxynucleotides complementary to tRNA^{Trp} and tRNA^{Leu3}. The autoradiogram shown was exposed for 2 hours. The identities of the clones used to transform the YNN217 cells are indicated above the lanes. Positions of tRNA^{Leu3} and tRNA^{Trp} are marked.

TABLE III: Tryptophan tRNA content of *S.cerevisiae* YNN217 cells transformed with single and multicopy vectors carrying tRNA^{Trp} genes. Autoradiograms produced from filters as shown in Fig. 4 were scanned with a densitometer and the areas of peaks corresponding to tRNA^{Leu3} and tRNA^{Trp} compared for each isolate. The tRNA^{Trp}:tRNA^{Leu3} ratio in cells carrying only the YCp19 vector was adjusted to 1.00. The relative amount of tRNA^{Trp} found in the other cell types is presented with standard deviations in brackets. Values are averaged from 2–4 scans of 3 different filters.

PLASMID	tRNA^{Trp}
YCp19	–1.00–
YCp19ΔCEN	1.06[0.15]
TRP	1.07[0.17]
TRPΔCEN	1.45[0.19]
TRPA	1.05[0.08]
TRPAΔCEN	1.29[0.13]
TRPO	0.98[0.05]
TRPOΔCEN	1.21[0.10]
TRPOP	1.29[0.06]
TRPOPΔCEN	2.02[0.57]

of the tRNA^{Trp} gene clones were generated and introduced into the same yeast strains. When the amber suppressor form of the tRNA^{Trp} gene was introduced on a multicopy plasmid, transformation efficiency is significantly reduced in the three strains analyzed (Table II). This result suggests that a high level of tRNA^{TrpA} has a deleterious effect on cell viability. This is corroborated by the poor growth characteristics of surviving transformants. The result is also consistent with that of Masson et al. (17) in which a multicopy version of a synthetic yeast amber suppressor tRNA^{Phe} gene affected viability.

The two ochre mutations, *leu2-1* and *ade2-101*, were not affected by the presence of tDNA^{TrpO} even at the higher copy number (Table II). It has been reported that a synthetic yeast ochre suppressor form of tRNA^{Phe} gene, when present on a multicopy vector, weakly suppresses ochre mutations and has a deleterious effect on cell viability (17). Transformations using the multicopy version of the ochre suppressor form of the tRNA^{Trp} gene showed normal numbers of transformed cells (Table II). Also, growth curves of these transformants were identical to those of cells transformed with the vector only (data not shown).

In contrast, the opal suppressor form of the gene gave moderate to efficient suppression with both *his4-260* and

leu2-2(Table II). Surprisingly, an apparent suppression of the *his4-260* mutation was observed in the RJ293-13C strain when the parental tRNA^{Trp} gene was present in multiple copies (Table II). Such an interaction would require an A·C base-pair between the anticodon of the parental tRNA^{Trp} (CCA) and opal codon of the messenger RNA (UGA) at the wobble position. If this result is due to the increased abundance of normal tRNA^{Trp} (Table III), it implies that fidelity of translation may be sensitive to the intracellular balance of tRNAs. Supporting this concept is the evidence that *S. cerevisiae* tRNA^{Gln} genes which normally decode CAG or CAA can act as suppressors of UAG and UAA respectively when present on multicopy plasmids (30,31). It also has been shown that *E. coli* tRNA^{Trp} with the normal CCA anticodon can suppress UGA mutations and an A→G mutation in the dihydrouridine stem increases suppressor efficiency (32).

While there is a positive correlation between tRNA gene copy number and tRNA abundance (6), direct evidence of a causal relationship is lacking. To determine whether the high copy number vector actually led to an increase in the tRNA^{Trp} content of transformed cells, Northern blots of total low molecular weight RNAs from such cells were probed with oligodeoxynucleotides to detect both tRNA^{Leu3} and tRNA^{Trp}. Assuming that the level of tRNA^{Leu3} was not affected by the additional copies of tDNA^{Trp}, Table III shows that the abundance of tRNA^{Trp} was increased 20−100% by the ΔCEN clones. The TRPOP version of the gene caused the largest increase even though *in vitro* studies indicated that this gene was transcribed and processed at a 5 fold lower rate than the other forms (Fig. 2). The dramatic increase in abundance of this tRNA *in vivo* does, however, provide an explanation for the appearance of suppressor activity.

The level of activity of the opal suppressor form of tRNA^{Trp} demonstrated herein indicates this construction will be a useful tool for creating amino acid substitutions in proteins expressed in yeast.

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Note added in proof

A manuscript by Atkin et al., 'Construction of an Opal Suppressor of an *S. cerevisiae* tRNA^{Trp} Gene by Oligonucleotide Directed Mutagenesis', (Mol. Cell. Biol., in press) presents results and conclusions which are substantially in accord with those in this work.

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