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The functional analysis of nonsense suppressors derived from in vitro engineered *Saccharomyces cerevisiae tRNA^{Trp}* genes

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Abstract

Nonsense suppressors derived from *Saccharomyces cerevisiae* tRNA^{Trp} genes have not been identified by classical genetic screens, although one can construct efficient amber (am) suppressors from them by making the appropriate anticodon mutation in vitro. Herein, a series of in vitro constructed putative suppressor genes was produced to test if pre-tRNA^{Trp} processing difficulties could help to explain the lack of classical tRNA^{Trp}-based suppressors. It is clear that inefficient processing of introns from precursor tRNA^{Trp}, or inaccurate overall processing, may explain why some of these constructs fail to promote nonsense suppression in vivo. However, deficient processing must be only one of the reasons why classical tRNA^{Trp}-based suppressors have not been characterized, as suppression may still be extremely weak or absent in instances where the in vitro construct can lead to an accumulation of mature tRNA^{Trp}. Furthermore, suppression is also very weak in strains transformed with an intronless derivative of a putative *tRNA^{Trp}* ochre (oc) suppressor gene, wherein intron removal cannot pose a problem.

Keywords: processing, intron; Ψ^+, recombinant DNA, mutagenesis, ochre, in vivo

Abbreviations: am, amber; bp, base pair(s); IVS, intron or intervening sequence of *tRNA* gene; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); oc, ochre; oligo, oligodeoxyribonucleotide; op, opal; PAGE, Polyacrylamide-gel electrophoresis; Ψ (psi), extrachromosomal allosuppressor (see section e); *S.*, *Saccharomyces*; tRNA, transfer RNA; $tRNA^{Trp}$, gene encoding tRNA^{Trp}; wt, wild type

Introduction

In theory, many tRNA genes should be able to be mutated to encode a product capable of recognizing termination codons. However, all of the spontaneous nonsense suppressors in *S. cerevisiae* that have been identified by genetic means and characterized to date are alleles of $tRNA^{Tyr}$, $tRNA^{Ser}$, $tRNA^{Leu}$, or $tRNA^{Gln}$ genes (reviewed in Sherman, 1982; Edelman and Culbertson, 1991). In contrast, for *Caenorhabditis elegans* all presently characterized nonsense suppressors are derived from $tRNA^{Trp}$ genes (Hodgkin et al., 1987), so the in vivo conditions influencing the tolerance for various suppressors may vary between species. There are many extant *S. cerevisiae* suppressors which have not yet been fully characterized, and among these it is possible that some may be derived from tRNA genes other than the four known classes (Liebman et al., 1976). There are many possible reasons for the failure to isolate suppressors derived from all of the candidate genes. The copy number of a specific tRNA gene may be too low to tolerate mutational loss of one copy (Brandriss et al., 1975). In other cases, it is possible to invoke deficiencies in the processing of pre-tRNA transcripts from putative suppressor genes (reviewed in Culbertson and Winey, 1989). In addition, anticodon mutations may affect the identity of tRNAs (reviewed in Normanly and Abelson, 1989) and this could lower suppressor efficiency below the detection threshold for most genetic screens, due to a possible impact on aminoacylation.

A single base pair substitution involving the first or second nucleotide in the anticodon of a $tRNA^{Trp}$ gene should be sufficient to produce a tRNA capable of reading an op or am codon, respectively. Kim and Johnson (1988) were the first to demonstrate that it is possible to make a specific and efficient am suppressor by in vitro mutagenesis of a yeast $tRNA^{Trp}$ gene. They have recently demonstrated that the identity of the yeast suppressor tRNA is not changed by the anticodon mutation that permits recognition of the am codon (Yesland et al., 1993), unlike similar mutations in *E. coli* $tRNA^{Trp}$ genes (Soll and Berg, 1969). Furthermore, it has been demonstrated that a reduction in the functional copy number of $tRNA^{Trp}$ genes in haploid *S. cerevisiae* from six to five does not impair viability (Atkin et al., 1992). These reports tend to discount some of the possible reasons why spontaneous tRNA^{Trp} suppressors have not been found and, by elimination, tend to strengthen the notion that processing of precursor tRNAs may play an important role in suppressor efficiency, and thus the ability to detect these suppressors.

In *S. cerevisiae*, ten tRNA gene families have been identified which contain intrans (Ogden et al., 1984; Stucka and Feldman, 1988). The enzymology of intron removal from precursor transcripts is established and a recent description is given in Miao and Abelson (1993). For S. *cerevisiae* tRNA genes, the majority of the mutations which affect transcript maturation are located within the mature coding sequences (reviewed by Culbertson and

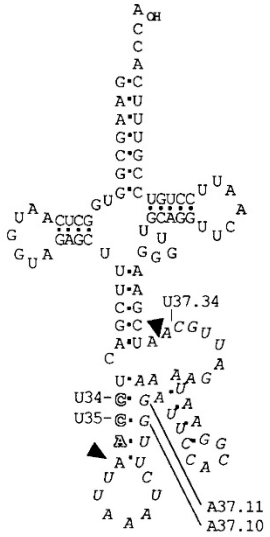
Winey, 1989). Overall, it appears that there is a structural requirement for a single-stranded region at the 3′ splice junction and a base pair between the antipenultimate base in the intron and a base in the anticodon loop for recognition and cleavage by tRNA endonuclease (Szekely et al., 1988; Baldi et al., 1991). Evidence that mutational changes to the anticodon of a yeast *tRNA^{Trp}* gene may influence the accuracy or the rate of intron removal was provided by Atkin et al. (1990) for in vitro constructed op suppressors of a *tRNA^{Trp}* gene. The accumulation of tRNA precursors in strains transformed with *tRNA^{Trp}* genes which encode only the altered anticodon versus no such accumulation in strains transformed with an engineered gene carrying a second compensatory change in the intron correlated well with the respective suppressor activity of these constructs. In the present paper we have examined the ability of a series of in vitro engineered tRNA^{Trp} suppressor constructs to be properly spliced, and concomitantly to see if they can act as effective nonsense suppressors.

Results and Discussion

(a) Construction of putative op, oc, and am suppressors of a S. cerevisiae tRNA^{Trp} *gene*

In vitro constructed am suppressor alleles of a yeast *tRNATrp* gene have been reported (Kim and Johnson, 1988; Atkin et al., 1990) which contained only the requisite anticodon change, and they exhibited efficient and codon-specific suppression. In contrast, in vitro constructed op suppressors (Kim et al., 1990), while codon specific, were very inefficient. However, an op suppressor which has a second change at position 37.11 (tRNA^{Trp}) in the intron of the gene (fig. 1) produced a more efficient suppressor (Atkin et al., 1990). These results support suggestion (Ogden et al., 1984) that the conformation of pre-tRNAs may have an impact on intron splicing in vivo. Specifically, the formation of an aberrant structure in which the altered anticodon and the 3′ splice site are base-paired might result in a splicing defect. In this work, a series of templates was produced (see fig. 1) which together should recognize all of the termination codons. Furthermore, the putative suppressors for each termination codon were made in two or more ways. Some constructs contained only the required changes to anticodon. Others contained additional changes designed to compensate for the respective anticodon change either by recreating potential intron-anticodon pairing or changing the 3′ splice site so as to reduce its complementarity to the anticodon region. An additional derivative, *oc1ΔIVS*, included an oc anticodon and a precise deletion of the intron. This construct provides an independent test of the importance of intron removal in the overall level of suppression since splicing is not required to produce the mature sequence product from this template.

A



B

Mutant allele	Alterations created by site-directed mutagenesis
op1	U34
op2	U34, A37.11
op2'	U34, U37.34
am1	U35
am2	U35, A37.10
oc1	U34, U35
oc2	U34, U35, A37.10, A37.11
oc2'	U34, U35, U37.34
oc1ΔIVS	U34, U35, Δintron

Figure 1. *S. cerevisiae* $tRNA^{Trp}$ suppressors. (A) Primary sequence and secondary structure of *S. cerevisiae* $tRNA^{Trp}$ and intron. The sites in the *$tRNA^{Trp}$* gene which were altered by site-directed mutagenesis are shown on this tRNA diagram. Numbering of the mature domain is by the convention established for $tRNA^{Phe}$ (Schimmel et al., 1979). The bold arrowheads indicate the positions of the splice sites. The anticodon is indicated by larger open letters and the intron sequence (between the arrowheads) is in italics. (B) The combinations of changes which were made in the *$tRNA^{Trp}$* gene to create the putative op, am, and oc suppressor alleles are shown along with the designation for each mutant allele. For the site-specific oligo-directed mutagenesis, a 0.41-kb *Hin*cII-*Hae*III fragment of yeast DNA, containing a cloned *$tRNA^{Trp}$* gene, from plasmid 2BTrp (obtained from Dr. G. Knapp and described in Kang et al., 1980) was subcloned into the *Eco*RI site in M13mp19 using *Eco*RI linkers. The corresponding single-stranded DNA was used as the template for mutagenesis using the double primer method (Stewart et al., 1985). The preparation and storage of plasmid and M13 DNAs, restriction analyses, subclonings, and sequencing were performed according to standard methodology (Sambrook et al., 1989). The oligos used for probes or in vitro mutagenesis were synthesized by the regional DNA synthesis laboratory, University of Calgary or by K.L.R.

(b) In vitro cleavage of pre-tRNA^{Trp} transcripts by tRNA endonuclease

To examine the effects of the engineered changes on cleavage at 5′ and 3′ splice sites by tRNA endonuclease, the *tRNA^{Trp}* genes described in figure 1 were transcribed in vitro by a yeast nuclear extract, under conditions which allow 5′ and 3′ end processing but prevent splicing (Engelke et al., 1985). These precursors were then purified by gel electrophoresis and used as substrates in assays with a partially purified tRNA endonuclease fraction (fig. 2). SUP53, an am suppressor derivative of yeast *tRNA^{Leu3}* gene, served as a positive control, since the pattern of tRNA endonuclease cleavage products for this substrate has been well characterized (Gegenheimer et al., 1983).

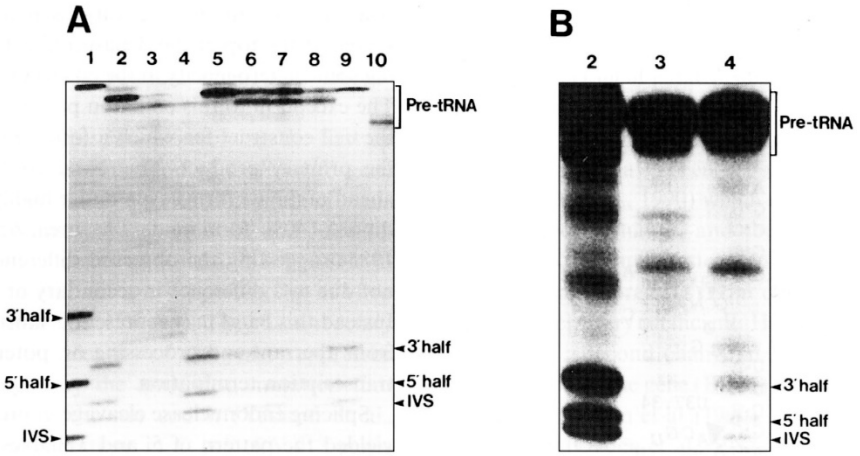


Figure 2. The pattern of cleavage products produced by tRNA splicing endonuclease digestion of gel-purified precursors transcribed from the wt and engineered *tRNA^{Trp}* genes and the *SUP53* control gene. The *S. cerevisiae* tRNA endonuclease used was equivalent to fraction VI of Peebles et al. (1983). The preparation of labeled, end-matured, intron-containing precursor transcripts and the subsequent endonuclease reactions were carried out as previously described (Szekely et al., 1988), and the products were resolved on 12% polyacrylamide/8 M urea gels. Labeled samples were prepared and loaded onto the gel as follows: (A) SUP53 (lane 1), tRNA^{Trp}wt (lane 2), op1 (lane 3), op2 (lane 4), op2′ (lane 5), am1 (lane 6), am2 (lane 7), oc1 (lane 8), oc2′ (lane 9), and oc2 (lane 10). (B) An autoradiograph from another gel showing the pattern of products resulting from tRNA splicing endonuclease cleavage of tRNA^{Trp}wt (lane 2), op1 (lane 3), and op2 (lane 4) precursors.

With one exception, precursors obtained by transcription of the various derivatives comigrated with those observed for the wt template (fig. 2A). However, the relative yield among the various precursor forms, observed at the top of the denaturing gel, differed, suggesting some heterogeneity in the efficiency of end processing. The exception to this common pattern of precursors was the *oc2* construct for which a faster migrating band was the primary product. This band continued to migrate ahead of the wt forms even under highly denaturing conditions (40% formamide/7 M urea, 65°C; Myers et al., 1985) suggesting the observed difference in migration is not due to a difference in secondary or tertiary

structure. Instead this band may represent a shortened form arising from aberrant end processing or, potentially, premature transcription termination.

Splicing endonuclease cleavage of pre-SUP53 (fig. 2A) yielded the pattern of 5′ and 3′ halves plus the IVS expected for this pre-tRNA. Pre-tRNA^{Trp}wt (lane 2) also yielded major bands of the sizes expected for the halves and the IVS from this precursor (Kang et al., 1980). There was considerable degradation of the pre-tRNA^{Trp}op1 and op2 transcripts in the experiment shown (fig. 2A, lanes 3 and 4); however, in other experiments, bands of the expected sizes were observed for op2 transcripts (fig. 2B, lane 4) even though degradation bands were also prevalent in each of the lanes. Both of these precursors were poor substrates for the tRNA endonuclease in comparison to tRNA^{Trp}wt (fig. 2B). However, pre-tRNA^{Trp}op2, with compensating intron/anticodon changes appeared to be cleaved slightly more efficiently than pre-tRNA^{Trp}op1 containing the anticodon change alone. Pre-tRNA^{Trp}op2′, with a change at the 3′ splice site to reduce complementarity to the op anticodon, was an excellent substrate for the tRNA endonuclease (lane 5). Among the am derivatives (*am1* with the anticodon change alone and *am2* with compensating intron/anticodon changes), cleavage by tRNA endonuclease remained efficient (lanes 6 and 7). Among the oc derivatives, pre-tRNA^{Trp}oc1 (lane 8) with the anticodon changes alone was not a substrate for tRNA endonuclease. The oc2 precursor (lane 10), recovered in an apparently shortened form due to altered processing or transcription termination, was also not a substrate for tRNA endonuclease. However, the oc2′ precursor (lane 9) with both the oc anticodon and a noncomplementary 3′ splice site replacement, was an efficient tRNA endonuclease substrate.

(c) Northern analysis of precursor and mature tRNA^{Trp} isolated from yeast strains transformed with the putative suppressor constructs

RNA was isolated from yeasts transformed with individual putative suppressor constructs and was subjected to a Northern hybridization analysis to see if any constructs caused an accumulation of precursor and/or mature tRNA^{Trp} in vivo. The oligo used as a probe to identify the precursors (fig. 3A) is complementary to 10 nt of intronic sequence and 10 nt of exonic sequence surrounding the 3′ splice junction, and thus will hybridize to the precursors transcribed from the six endogenous *tRNA^{Trp}* genes as well. Quantitation (data not shown) of the levels of pre-tRNA^{Trp} in yeasts transformed with the various constructs consistently showed that a higher level of precursor accumulated in strains transformed with pYRtRNA^{Trp}op1 than those transformed with the pYRtRNA^{Trp}wt control construct. None of the other constructs reproducibly caused any significant accumulation of precursors above the control levels. In addition, yeasts transformed with pYRtRNA^{Trp}oc2 reproducibly showed precursor accumulations lower than those transformed with pYRtRNA^{Trp}wt. This is the same construct which produced the aberrantly short pre-tRNA^{Trp} for the in vitro splicing assay. This fragment may be labile in vivo and the precursors detected (fig. 3A, lane 9) are likely contributed entirely by the endogenous *tRNA^{Trp}* genes.

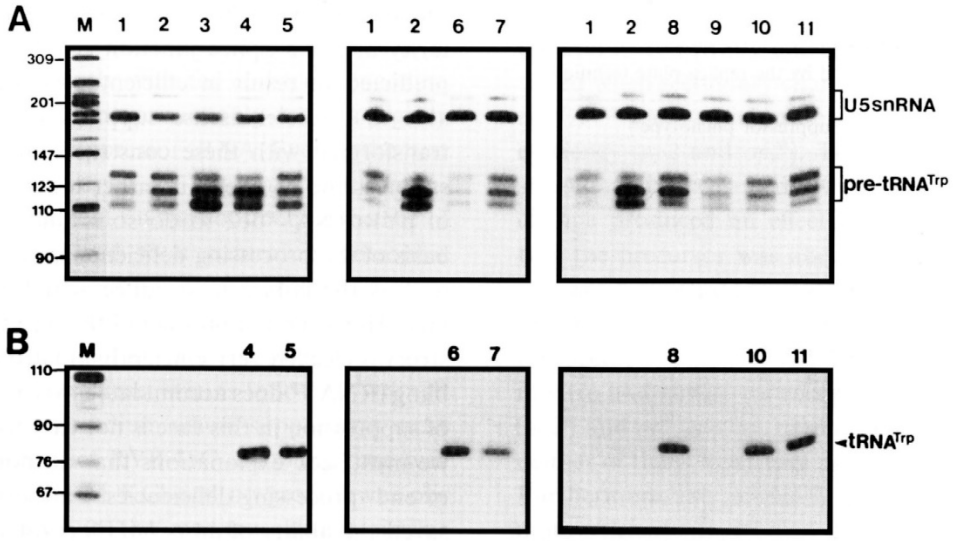


Figure 3. Northern hybridization analysis of the $tRNA^{Trp}$ transcripts isolated from transformants expressing the putative suppressor constructs. The RNA used to load the gels was isolated by the hot phenol method (Leeds et al., 1991). Samples of RNA (10 mg) and 0.1 mg of denatured *Msp*I-digested pBR322 DNA (as marker) were resolved on 10% polyacrylamide/8.3 M urea gels and transferred onto GeneScreen Plus membranes using the electroblot protocol recommended by the manufacturer (duPont Co.). The samples were loaded as follows: RNA isolated from cells transformed with YRp17 (lane 1); or transformed with pYR$tRNA^{Trp}$wt (lane 2); pYR$tRNA^{Trp}$op1 (lane 3); pYR$tRNA^{Trp}$op2 (lane 4); pYR$tRNA^{Trp}$op2′ (lane 5); pYR$tRNA^{Trp}$am1 (lane 6); pYR$tRNA^{Trp}$am2 (lane 7); pYR$tRNA^{Trp}$oc1 (lane 8); pYR$tRNA^{Trp}$oc2 (lane 9); pYR$tRNA^{Trp}$oc2′ (lane 10), and pYR$tRNA^{Trp}$oc1ΔIVS (lane 11). (A) The $tRNA^{Trp}$ precursors shown were probed with an end-labeled oligo complementary to 10 nt of intronic sequence and 10 nt of exonic sequence surrounding the 3′ splice side. U5 snRNA was used as an internal control (detected by an end-labeled oligo probe based on known sequence, O'Connor and Peebles, 1991) and the *Msp*I-digested DNA served as a size marker in lane M. (B) The same Northern blots were stripped and reprobed with 20-nt oligos complementary to the op (left panel), am (center panel), or oc (right panel) mature tRNA transcripts under stringent conditions which minimize mismatches between RNA/DNA hybrids, by including excess cold competitor oligo complementary to the wt $tRNA^{Trp}$. The blots were quantitated on a Betascope Blot Analyzer and cpm for pre-$tRNA^{Trp}$ relative to the cpm of the pre-$tRNA^{Trp}$ in lane 1 (transformed with YRpl7) were determined, all standardized to the amount of U5 snRNA in each lane. The Northern blots were hybridized as previously described (Atkin et al., 1990), with some modifications for the competitive hybridizations. Varying amounts of wild type competitor oligo were used; only 5 × cold competitor was used in hybridizations to detect mature op $tRNA^{Trp}$ while 100 × cold competitor was necessary in the hybridizations to detect am and oc $tRNA^{Trp}$. Blots were prehybridized and hybridized at 50°C and then washed six times for 5 min each at room temperature. Marker DNA was detected using end-labeled *Msp*I-digested pBR322 as probe.

The ability of the constructs to promote the production of mature-sequence tRNA^{Trp} is shown in figure 3B. For these experiments, a radioactive probe complementary to nt 25-45 (spanning the splice junction) was used for each of the respective putative mature tRNA^{Trp} molecules. Competitive hybridizations, using the relevant labeled oligo as probe and an excess of cold competitor oligo complementary to the wild type tRNA^{Trp} sequence, were done using the same Northern blots as in figure 3A. Autoradiographs of these blots show that a RNA similar in size to mature tRNA^{Trp} can easily be detected in strains transformed with plasmids pYRtRNA^{Trp}op2 and pYRtRNA^{Trp}op2′ (fig. 3B, lanes 4 and 5). Mature-sized tRNA^{Trp}op1 was not detected in these experiments (lane 3). This is consistent with the inability of tRNA endonuclease to cleave in vitro the pre-tRNA^{Trp} derived from tRNA^{Trp}op1, as well as the accumulation in vivo of tRNA^{Trp} precursors derived from this construct. RNAs similar in size to tRNA^{Trp} were also detected in strains transformed with pYRtRNA^{Trp}am1 or pYRtRNA^{Trp}am2 (fig. 3B, lanes 6 and 7), in agreement with the in vitro cleavage results (fig. 2A, lanes 6 and 7). Three of the four putative oc suppressor constructs (fig. 3B, lanes 8, 10, and 11) can also direct the accumulation of spliced tRNA^{Trp}. The failure of the pYRtRNA^{Trp}oc2 construct (fig. 3B, lane 9) to do so is consistent with the production of the aberrant shorter precursor from this construct in vitro (fig. 2A, lane 10). In other gels (results not shown) where a radioactive oligo complementary to the wt sequence of tRNA^{Trp} was used as a probe, and no competitor oligo was added, a tRNA product identical in size that detected in figure 3B lanes 4–8, 10, and 11 was detected in all lanes. This is expected, since the wt product from the six wt *tRNA^{Trp}* genes would always be present.

The results from the in vitro cleavage assay (fig. 2) generally are consistent with the in vivo accumulation of RNA resembling mature-sized tRNA^{Trp} (fig. 3), but there are two constructs which exhibit differences. The pYRtRNA^{Trp}op2 transcript was not cleaved very efficiently in vitro (fig. 2B, lane 4) yet in vivo this construct directed the accumulation of an RNA resembling mature-sized tRNA^{Trp} (see fig. 3B, lane 4) as well as pYRtRNA^{Trp}op2′ did. The other discrepancy is that the pYRtRNA^{Trp}oc1 transcript was not cleaved by the tRNA endonuclease in vitro (fig. 2A, lane 8), but yeast transformed with this transcript did accumulate mature tRNA^{Trp} in vivo. The differences between the in vitro and in vivo treatment of transcripts suggest that there are auxiliary factors important for the overall efficiency of processing tRNA precursors in vivo which are missing or seriously depleted in the partially purified tRNA endonuclease preparation used in the in vitro experiments. Further, it is possible that these factors might distinguish between precursors with subtle conformational differences, as might be expected from the various experimental constructs herein. This is consistent with the overall increase in processing in vivo (fig. 3 vs. fig. 2). Several candidate factors exist, which include the products of the *STP1*, *SEN1*, *LOS1*, *TPD1*, and *PTA1* genes (Hurt et al., 1987; Wang and Hopper, 1988; Van Zyl et al., 1989; DeMarini et al., 1992; O'Connor and Peebles, 1992).

(d) Suppressor phenotype of yeasts transformed with the in vitro engineered tRNA^{Trp} genes

Both strains utilized as transformation hosts, when transformed with either pYRtRNA^{Trp}-op2 or pYRtRNA^{Trp}op2′ exhibited inefficient op-specific suppression, whereas pYRt-

RNA^{Trp}op1 did not produce any detectable suppression in our hands (see table I). The suppression seen with the *op2* and *op2′* constructs was weak, as it was only observed when strains were transformed with pYRtRNA^{Trp}op2 or pYRtRNA^{Trp}op2′ (high copy number plasmids) but not with pYCtRNA^{Trp}op2 or pYCtRNA^{Trp}op2′ (single copy plasmids). Thus, even though pre-tRNA^{Trp}op2′ was cleaved more efficiently by tRNA endonuclease in vitro, suppressor activity was not increased relative to tRNA^{Trp}op2, suggesting that in vivo splicing levels of the two pre-tRNAs are similar. Alternatively, it may imply that accurate splicing is necessary but not sufficient to allow efficient suppression by these tRNAs. The failure of pYRtRNA^{Trp}op1 to promote even low levels of suppression correlates with the inability of its transcript to be spliced in vitro as well as the failure to accumulate RNA resembling mature tRNA^{Trp} in vivo. The anticodon change in pYRtRNA^{Trp}am1 does not predict any obvious difficulty in anticodon/intron interactions that would influence the important parameters of intron splicing, and the additional change in pYRtRNA^{Trp}am2 does not predict an improvement in this process. Consistent with this notion, both of these constructs direct the production of a tRNA^{Trp} which can act as an efficient and codon-specific suppressor (table I). The putative oc suppressor allele of *tRNA^{Trp}* (pYRtRNA^{Trp}oc1) containing the requisite anticodon changes does not promote suppression of oc nonsense alleles in replica plate experiments (table I), consistent with previous reports (Kim and Johnson, 1990). Additional changes in the intron sequence, intended to restore complementarity between the anticodon loop and the intron (pYRtRNA^{Trp}oc2) or to decrease complementarity at the 3′ splice junction (pYRtRNA^{Trp}oc2′), were predicted to result in efficient processing, and thus able to direct nonsense suppression. However, strains transformed with these constructs were also unable to suppress the oc alleles in either tester strain. The fail of pYRtRNA^{Trp}oc2 to do so is understandable on the basis of the processing deficiency in the in vitro assay as well as the failure to produce a mature-sized tRNA in vivo. However, the product of the pYRtRNA^{Trp}oc2′ construct is cleaved very efficiently in vitro and RNA resembling tRNA^{Trp} does accumulate in vivo. Thus, the absence of suppression in this case is not easy to understand, and we must seek explanations that do not involve splicing or end-processing deficiencies. Furthermore, we also assayed the ability of an *oc1ΔIVS* construct to direct suppression. Cells transformed with single or multiple copy plasmids carrying this intronless gene were tested. Suppression was observed but only with a 2μ-based multiple copy plasmid (table I), and this suppression was very weak. Again splicing difficulties cannot be invoked, and this emphasizes the need to seek other explanations for the nonexistent or very low levels of suppression observed for putative oc suppressors based on tRNA^{Trp}.

Table I. Suppressor phenotype of yeast strains when transformed with wt and engineered *tRNA^{Trp}* genes as assayed by the replica plate technique

	Suppressor phenotype[b]		
Putative suppressor alleles of a *tRNA^{Trp}* gene[a]	am	oc	op
wt	–	–	–
op1	–	–	–
op2	–	–	+
op2′	–	–	+
am1	++	–	–
am2	++	–	–
oc1	–	–	–
oc2	–	–	–
oc2′	–	–	–
oc1ΔIVS	–	+/–	–
oc1ΔIVS[c]	–	+	–

a. The suppressor phenotypes of strains JG369-3B and JG113-5R transformed with the putative suppressor alleles of the *tRNA^{Trp}* gene were determined as described in Atkin et al. (1990). The genotypes of these two strains are as follows: JG369-3B; *MATα*, *ade2*-1 (UAA); *can1*-100 (UAA), *lys2*-1 (UAA), *met8*-1 (UAG); *trpl*-1 (UAG), *leu2*-2 (UGA); *his4*-260 (UGA), *ura3*-52. JG113-5-R; *MATα*; *ade2*-1 (UAA); *can1*-100 (UAA); *met8*-1 (UAG); *trpl*-1 (UAG); *leu2*-2 (UGA); *his4*-260 (UGA). These strains were both provided by Dr. J.-Paul Gelugne. The letters in brackets following the mutant genotypes identify the nonsense alleles.

b. For the suppression data a plus (+) symbol indicates that suppression was observed only when strains were transformed utilizing a high-copy number plasmid vector (Yrp17 or YEp352). The + + symbol indicates that suppression was observed when strains were transformed with either a high-copy number plasmid or a single-copy plasmid (YCp50). A minus (–) symbol indicates that suppression was not observed. A +/– indicates very weak suppression and observed only when strains were transformed with a YEp352-based vector. The results with each yeast strain were identical so the results are combined.

c. In the case of the *oc1ΔIVS*, strain RVB-45C (Ψ$^+$) was also transformed with pYEtRNA^{Trp}oc1ΔIVS. The genotype of this strain is *MATα; ade2*-1 (UAA); *lys1*-1 (UAA); *trpl*-1 (UAG); *his3*-11; *leu2*-3; *ura3*-52; (Ψ$^+$); obtained from R. C. von Borstel.

(e) Additional assays for oc suppression

We attempted to devise more sensitive tests for oc suppression following the observation that mature-sized tRNA hybridized to a tRNA^{Trp} oc-specific probe (fig. 3B, lanes 8, 10, 11) in the presence of a cold competitor. A "drop test" was used, since this had previously been shown to be an effective method for detecting weak suppression (Shaw and Olson, 1984). Essentially, this method involved concentrating the cells and then plating dilutions in 10 ml spots on selective media. Using this method, very weak oc suppression was detected using pYRtRNA^{Trp}oc1, pYRtRNA^{Trp}oc2′, and pYRtRNA^{Trp}oclΔIVS transformants (results not shown) and these are the three oc constructs which produced a mature-sized tRNA. The relative efficiency of suppression in *oc1* versus *oc1ΔIVS* transformants was monitored by colony color development in a RVB-45C genetic background. RVB-45C (a Ψ$^+$ strain) has a suppressible oc *ade2*-1 marker. Colonies will be white if the oc nonsense mutation in *ade2*-l is suppressed whereas they will be pink if suppression does not occur, due to accumulation of a precursor in the adenine biosynthetic pathway. The psi (Ψ$^+$) factor is an extrachromosomal allosuppressor specific for oc nonsense mutations (Cox, 1977), thus

we would expect an enhanced level of suppression in this background. By this assay, the colonies containing the pYEtRNA^{Trp}oclΔIVS construct were white compared to pinkish white colonies of yeasts transformed with the pYEtRNA^{Trp}oc1 construct (results not shown). This indicated that the *ade2*-1 oc allele in a Ψ$^+$ background is suppressed more efficiently with the construct encoding the intronless *tRNA^{Trp}* gene, presumably due to a slightly higher level of mature suppressor tRNA. Even in the best case the suppression is very weak, but it has been demonstrated that it is dependent on the continued presence of the Ψ$^+$ factor. Compared to a control prototrophic strain, RVB-45C (harboring Ψ$^+$) transformed with pYEtRNA^{Trp}oc1LΔIVS was shown to be very sensitive to high salt in liquid culture (fig. 4). Hypertonic salt conditions are known to cause yeast strains to lose Ψ$^+$ (Singh et al., 1979). In liquid medium where suppression must occur to allow growth of RVB-45C, doubling time was greatly extended with 1 M KCl and no growth was observed with 2 M KCl in the medium. Under similar growth regimes, the control strain was not affected significantly. The difference in the growth kinetics of the two strains with no added KCl reflects prototrophic growth versus growth due to weak suppression. All of these results indicate that, while suppression is very weak, it is genuine oc suppression. Suppression is maximal when Ψ$^+$ is present, and cannot be measured in liquid cultures when Ψ$^+$ activity is eliminated. However, none of these results provide a satisfactory explanation of why oc suppression is so weak in situations where it can be detected.

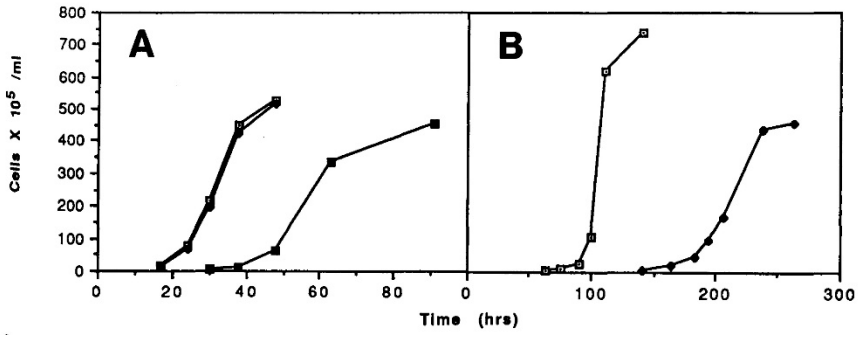


Figure 4. Effect of high salt concentration on the ability of a Ψ$^+$ yeast strain to promote oc suppression, as monitored by growth. (A) Growth kinetics of a Ura$^+$·Lys$^+$ control strain (JG113-5R) growing in Ura$^-$ Lys$^-$ media containing no added KCl (–□–), 1 M KCl (–◆–), or 2 M KCl (–■–). (B) Growth kinetics of RVB-45C (Ψ$^+$) transformed with pYEtRNA^{Trp}oc1ΔIVS in identical Ura$^-$ Lys$^-$ media containing no KCl (–□–) or 1 M KCl (–◆–). This strain was unable to grow in medium containing 2 M KCl. The complete genotype of the two yeast strains is given in table I.

(f) Conclusions

(1) The failure of a putative op suppressor, with only the requisite anticodon change, to promote efficient suppression may be explained by the relative inability of the splicing machinery to remove the intron (from *tRNA^{Trp}op1*), whereas intron removal, accumulation of a mature-sized tRNA, and suppression are all readily detected when secondary changes are incorporated into the *tRNA^{Trp}* genes which were designed to enhance splicing efficiency

(*op2* and *op2′*). The results with the *oc2* construct may be explained if we assume that the secondary change produced an effect on overall processing, such that the precursor was aberrantly short. This precursor was not a substrate for the tRNA endonuclease, mature tRNA did not accumulate and, of course, suppression did not occur. The tRNA^{Trp}oc2′ precursor was cut efficiently in vitro and strains transformed with both this construct (*oc2′*) and *oc1* accumulated a mature-sized tRNA^{Trp}; yet neither of these constructs promoted suppression in vivo. Furthermore, the *oc1ΔIVS* construct also failed to promote efficient suppression. Thus, oc suppression as well as op suppression are both very weak even in situations where they can be demonstrated. In contrast, am suppression is efficient and processing difficulties were neither invoked nor demonstrated.

(2) An unanswered question is why do these suppressors operate with such vastly different levels of efficiency in situations where we were able to demonstrate suppression? The am suppressors are efficient and apparently the gene identity has not been changed (Yesland et al., 1993). It is a formal possibility, until experimentally clarified, that the op and oc changes (which both alter the first position of the anticodon) have a more drastic effect on tRNA identity or function than the change in the second position that was sufficient to make the am suppressor. For example, the base modification 2′-*O*-methylcytidine is at the 5′ position of the wt tRNA^{Trp} anticodon (Sprinzl et al., 1989). The efficient am suppressors derived from *tRNA^{Trp}* do not involve changes that would necessarily affect this base modification, whereas the inefficient oc and op suppressors both must have a uridine residue at this position and thus do affect this modification. The significance of this correlation has not yet been explored, but there are precedents for a nucleoside modification deficiency in the anticodon being correlated with decreased suppressor efficiency.

(3) Given that it is possible to create a codon-specific and efficient am suppressor in vitro which functions efficiently in vivo when introduced on a single copy plasmid, why are not am suppressors based on tRNA^{Trp} recovered in a genetic screen? Although it is possible that tRNA^{Trp}-derived suppressors do exist among the collections of partially characterized and unmapped suppressors, this possibility has yet to be fully explored. Nevertheless, it is difficult to map a redundant gene lacking a specific tag such as suppressor ability provides, so no mapping of *tRNA^{Trp}* genes has been reported for *S. cerevisiae* except for linkage group assignments (Atkin et al., 1992; Yesland et al., 1992). It is also possible that suppression mediated by a *tRNA^{Trp}* gene in its natural location (in vivo) is too inefficient to be detected due to an inherently low transcriptional activity, whereas the same gene on a plasmid is more active and suppression is detected.

Several lines of experimentation could help resolve the issue. The flanking sequences of the six endogenous *tRNA^{Trp}* genes are not conserved (Riazi, 1992). Thus it will be possible to identify all *tRNA^{Trp}* genes individually and systematically disrupt them as has already been done for one copy (Atkin et al., 1992). The cloning of each gene also provides the means to study its in vitro transcriptional level as influenced by the respective flanking sequences. It should also be possible to replace any specific copy of a *tRNA^{Trp}* gene in its natural chromosomal context with the appropriate in vitro constructed am allele. This should allow us to determine whether each or every endogenous *tRNA^{Trp}* gene can function as a suppressor. Finally, the gene disruption experiments as done in Atkin et al. (1992)

introduce a reliable marker gene at the site which provides a way to map individual targeted *tRNA^{Trp}* genes genetically, and this will eventually allow comparisons with mapped but otherwise uncharacterized nonsense suppressors in *S. cerevisiae*.

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