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Construction of an Opal Suppressor by Oligonucleotide-Directed Mutagenesis of a *Saccharomyces cerevisiae* $tRNA^{Trp}$ Gene

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In vitro mutagenesis was used to create putative opal suppressor alleles of a $tRNA^{Trp}$ gene of *Saccharomyces cerevisiae*. The construct with the requisite anticodon change did not result in an active suppressor, whereas when a second change was introduced into the portion of the gene encoding the intron, an active and specific opal suppressor was produced. We propose that the secondary structure of transcripts from the first mutant may prevent efficient pre-tRNA processing, whereas normal processing occurs with the double mutant.

A compilation of the primary sequences of *Saccharomyces cerevisiae* tRNA precursors which contain intervening sequences has been published (11). The introns in these precursors are of variable length and do not have any consensus splicing sequences conserved within the introns or the mature coding sequences, but they all interrupt the anticodon loop 1 base 3′ to the anticodon and contain sequences which are complementary to the anticodon. These conserved features suggest a common structural basis for recognition by the tRNA-splicing enzymes. Splicing follows end maturation of the precursor tRNA transcripts. Excision of introns and ligation of exons are catalyzed by a single endonuclease and tRNA ligase, respectively (13, 14). Suppressor alleles can be isolated from intron-containing genes encoding $tRNA^{Ser}$, $tRNA^{Tyr}$, and $tRNA^{Leu}$ by conventional genetic techniques (for a review, see reference 18), and these alleles no longer have sequences in the intron that are complementary to the anticodon. However, since they encode functional suppressors, this complementarity must not always be essential for intron removal. The majority of conserved bases in intron-containing pre-tRNAs are found in the mature coding sequences (11, 22). This finding has led to a model in which the endonuclease interacts with the sequences in the mature domain of the tRNA precursor. The conserved location of the intron ensures the correct alignment of the splice junctions with respect to the endonuclease (4). Support for this working model for intron removal has come from studies in which drastic alterations to the intron have been made without altering correct excision and splicing (16, 20, 21). Alterations to the intron which do affect the removal of the introns either eliminate the loop at the 3′ splice site or destabilize the secondary structure of the entire precursor (16, 20, 21, 23). In contrast to the scarcity of mutations in the intron which affect intron removal, many mutations which affect removal of introns have been isolated in the mature coding sequences of *S. cerevisiae* tRNA genes (9, 10). A recent elegant study (17) demonstrated the importance of conserved bases in the mature domain of pre-$tRNA^{Phe}$ for removal of introns. In particular, the length of the anticodon stem and loop determines splicing specificity, and a purine is required at the 5′ side of the 3′ splice junction. The structure and sequence of the intron had little effect on splice site selection. Studies of *Schizosaccharomyces pombe* tRNA transcript processing in *S. cerevisiae* also implicate exon mutations as causes of deficient splicing activity (5, 12).

The first example of a yeast suppressor tRNA constructed from a $tRNA^{Trp}$ gene of *S. cerevisiae* was recently described (7). In vitro mutagenesis was used to alter the anticodon of an *S. cerevisiae* $tRNA^{Trp}$ gene from CCA to CTA, and the product of this putative suppressor gene was an efficient and specific suppressor of amber nonsense mutations. The results of others (1) demonstrate that removal of the 13-base-pair intron from precursor transcripts of the *Dictyostelium discoideum* $tRNA^{Trp}$ genes in *S. cerevisiae* can occur only if minimal base pairing of the anticodon with intron sequences is possible. This suggests that the previous attempt to obtain ochre and opal suppressors may have failed (7) because the potential for anticodon-intron base pairing in the transcripts was disrupted. We were able to create a specific opal suppressor from a $tRNA^{Trp}$ gene of *S. cerevisiae* by altering the sequence in the intron of a $tRNA^{Trp}$ gene, containing a previously engineered UCA anticodon, to restore conventional base pairing potential between the intron and the anticodon of precursor transcripts of that gene. We also present evidence that intron-containing precursors tend to accumulate in yeast strains transformed with suppressor constructs having only the requisite anticodon change, and we speculate that this accumulation is due to an altered secondary structure of the precursor which inhibits splicing at the 3′ splice junction.

Cloning and mutagenesis. A single base change in the CCA anticodon of $tRNA^{Trp}$ should be sufficient to create tRNAs capable of base pairing with the opal (UGA) nonsense codon. To determine whether a $tRNA^{Trp}$ with such an altered anticodon would function as an opal nonsense suppressor, oligonucleotide-directed in vitro mutagenesis was used to change the CCA anticodon of a yeast $tRNA^{Trp}$ to UCA. For this, a 0.41-kilobase-pair *Hin*cII-*Hae*III fragment of yeast DNA from 2BTrp (kindly provided by G. Knapp), which includes the yeast $tRNA^{Trp}$ gene (6), was subcloned into the *Eco*RI site in M13mp19 by using *Eco*RI linkers. The corresponding single-stranded DNA was used as the template for mutagenesis by the double-primer method (19). The presumed secondary structures of the $tRNA^{Trp}$ precursors transcribed from the wild-type and engineered genes are shown in Fig. 1. In addition to a $tRNA^{Trp}$ gene with the requisite anticodon change, a gene that contained an additional change in the intron was also made, resulting in restoration of conventional base-pairing potential between the intron and the anticodon in the precursor product of the

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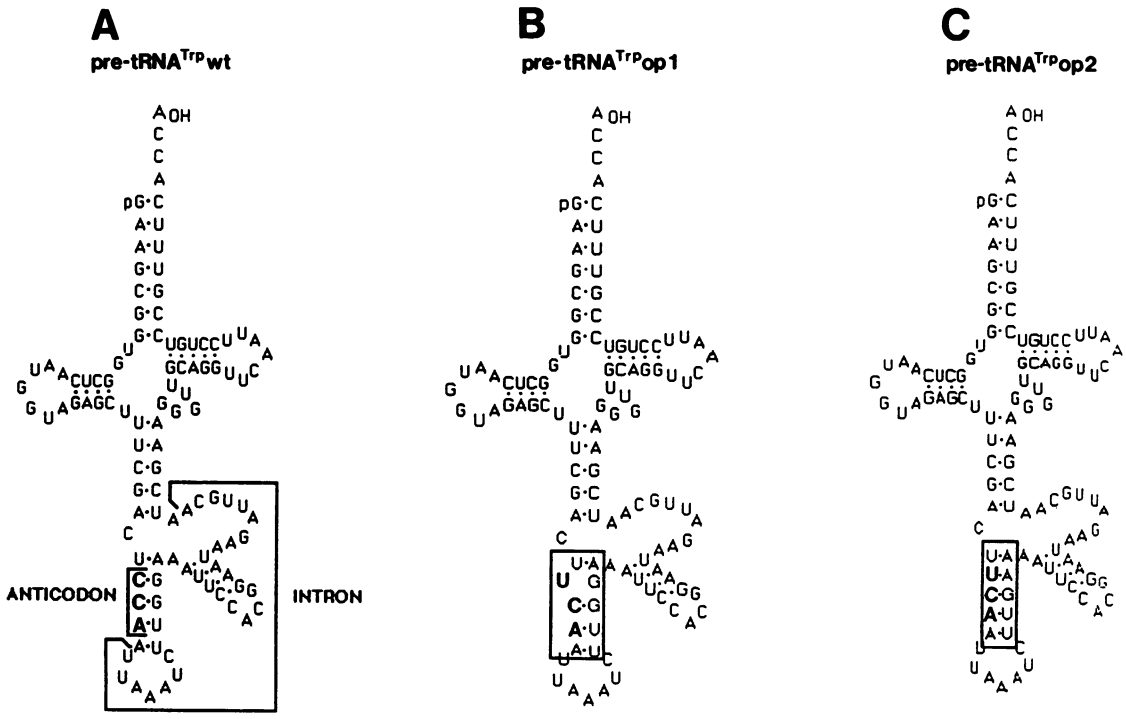

FIG. 1. Cloverleaf structures of wild-type and engineered pre-tRNA^{Trp}. The structures conform to the generalized secondary structure of yeast tRNA precursors that have intervening sequences (11). (A) Secondary structure of the precursor tRNA^{Trp} encoded by the wild-type gene (6). The position of the intron with respect to the anticodon and the 5-base-pair region of homology between the anticodon loop and the intron are indicated. (B) Possible secondary structure of the precursor tRNA encoded by *op1*, the tRNA^{Trp} gene which has a single anticodon change. The region of homology between the anticodon loop and the intron of the precursor tRNA transcribed from this gene is boxed. The alteration of the tRNA anticodon from CCA to UCA resulted in a partial disruption of the complementary sequence. (C) Most likely secondary structure of the precursor tRNA transcribed from *op2*. *op2* has the anticodon change and a change in the intronic sequence from UUGGA to UUGAA, which would be expected to restore conventional base-pairing potential to the 5-base-pair complementary sequence of the transcripts.

second gene. The expected secondary structure of this precursor is illustrated in Fig. 1C.

Analysis of putative suppressors in vivo. The 0.41-kilobase-pair fragments of yeast DNA containing the wild-type or mutant forms of the yeast tRNA^{Trp} gene were subcloned into YRp17 (a high-copy-number plasmid) and YCp50 (a single-copy plasmid). The YRp17 constructs were transformed into JG113-5R, JG369-3B, and RJ293-13C, and the YCp50 constructs were transformed into JG369-3B and RJ293-13C (8). The genotypes of these yeast strains are listed in Table 1, footnotes *c* and *d*. Transformants were replica plated onto selective media to test for suppression of nonsense markers. The suppressor phenotype of JG113-5R transformed with the wild-type and engineered tRNA^{Trp} genes is presented in Table 1. As expected, no suppression was observed for transformants transformed with YRp17 or the construct containing only the wild-type tRNA^{Trp} gene. Suppression of the opal markers, *leu2-2* and *his4-260*, was seen when JG113-5R was transformed with pYRtRNA^{Trp}op2, the plasmid with the tRNA^{Trp} gene that has both the altered anticodon and a correspondingly altered intron. These transformants were unable to suppress amber or ochre alleles also resident in the tester strains (Table 1, footnotes *c* and *d*; results not shown). Therefore, tRNA^{Trp}op2 is a specific suppressor of opal alleles. Transformants with the plasmid containing the tRNA^{Trp} gene which has only the anticodon change (pYRtRNA^{Trp}op1) were unable to suppress opal alleles in JG113-5R, even after extended incubations of up to 2 weeks. Thus, both the anticodon and the intron changes appear to be required to obtain a specific opal suppressor from this yeast tRNA^{Trp} gene. The ability to suppress UGA alleles, conferred on the transformants by the presence of pYRtRNA^{Trp}op2, was confirmed by demonstrating that spontaneous loss of tryptophan prototrophy by plasmid loss resulted in a concurrent loss of the ability to suppress the *leu2-2* and *his4-260* mutations in all cases (results not shown). Therefore, the ability of these transformants to grow in the absence of leucine or histidine results only when a functional opal suppressor tRNA encoded by the altered tRNA^{Trp} gene on the pYRtRNA^{Trp}op2 plasmid is present.

To determine whether the suppressor phenotype of the transformants is influenced by plasmid copy number or genetic background, the YRp17 and YCp50 constructs were transformed into additional *S. cerevisiae* strains, JG369-3B and RJ293-13C. JG369-3B was constructed independently of JG113-5R but has the same markers as JG113-5R, with two additions: *lys2-1*, a suppressible ochre allele, and *ura3-52*, a nonsuppressible mutation. RJ293-13C has the suppressible *his4-260* opal allele in a *ura3-52 leu2-3,112* background. The suppressor phenotypes of JG369-3B and RJ293-13C transformed with wild-type and engineered tRNA^{Trp} genes cloned into YRp17 and YCp50 are also presented in Table 1. When transformed with the pYRtRNA^{Trp}op2 plasmid, the JG369-3B strain grew on leucine and histidine omission media within 3 to 5 days at 30°C but no suppression of the ochre or amber alleles was observed (results not shown). The pYRtRNA^{Trp}op2 transformants of RJ293-13C also grew on the histidine omission medium. Thus, these genetic back-

TABLE 1. Suppressor phenotypes of yeast strains transformed with wild-type and engineered $tRNA^{Trp}$ genes

Strain and plasmid[a]	Suppression of opal allele[b]	
	leu2-2	*his4-260*
JG113-5R[c]		
YRp17	–	–
pYRtRNA^{Trp}wt	–	–
pYRtRNA^{Trp}op1	–	–
pYRtRNA^{Trp}op2	+	+
JG369-3B[d]		
YRp17	–	–
pYRtRNA^{Trp}wt	–	–
pYRtRNA^{Trp}op1	–	–
pYRtRNA^{Trp}op2	+	+
YCp50	–	–
pYCtRNA^{Trp}wt	–	–
pYCtRNA^{Trp}op1	–	–
pYCtRNA^{Trp}op2	–	–
RJ293-13C[e]		
YRp17	NA	–
pYRtRNA^{Trp}wt	NA	–
pYRtRNA^{Trp}op1	NA	–
pYRtRNA^{Trp}op2	NA	+
YCp50	NA	–
pYCtRNA^{Trp}wt	NA	–
pYCtRNA^{Trp}op1	NA	–
pYCtRNA^{Trp}op2	NA	–

[a] The YRp17 plasmid carries the *TRP1* and *URA3* genes. Complementation of the *trp1-1* allele was used to select for the presence of the YRp17 plasmid and its derivatives in JG113-5R and JG369-3B. Complementation of the *ura3-52* allele was used to select for RJ293-13C transformants that have the YRp17 and YCp50 constructs and for the transformants of JG369-3B that have the YCp50 plasmid and its derivatives.

[b] NA, Not applicable (since RJ293-13C does not have an opal-suppressible *leu2* allele). The suppressor phenotypes were determined after incubation of 3 to 5 days at 30°C. –, No suppression observed within the 3- to 5-day incubation period; +, suppression observed during the 3- to 5-day incubation period. See text for unusual results which were observed after extended incubation periods.

[c] The genotype of JG113-5R is *MATa ade2-1* (UAA) *can1-100* (UAA) *met8-1* (UAG) *trp1-1* (UAG) *leu2-2* (UGA) *his4-260* (UGA).

[d] The genotype of JG369-3B is *MATa ade2-1* (UAA) *can1-100* (UAA) *lys2-1* (UAA) *met8-1* (UAG) *trp1-1* (UAG) *leu2-2* (UGA) *his4-260* (UGA) *ura3-52* (nonsuppressible).

[e] The genotype of RJ293-13C is *MATa his4-260* (UGA) *ura3-52* (nonsuppressible) *leu2-3,112*.

grounds did not alter the suppressor phenotype of $tRNA^{Trp}$op2. No suppression of opal alleles was observed in JG369-3B and RJ293-13C strains transformed with pYCtRNA^{Trp}op2 (a single-copy plasmid) or the pYRtRNA^{Trp}op1 or pYCtRNA^{Trp}op1 constructs, even after prolonged incubation at 30°C. Since the gene for $tRNA^{Trp}$op2 must be present at a high copy number to achieve opal suppression, $tRNA^{Trp}$op2 must be a relatively weak suppressor. In all three genetic backgrounds tested, both the anticodon change and the intron change are required to obtain an opal suppressor from the $tRNA^{Trp}$ gene. However, others have reported that constructs with only the anticodon change do suppress opal markers (J. Johnson, personal communication). This may reflect differential sensitivity in the assays.

An additional surprising result was obtained for both the JG369-3B and RJ293-13C genetic backgrounds. In extended incubations of up to 2 weeks at 30°C, weak growth was seen for pYRtRNA^{Trp}wt transformants on media selecting for opal suppression but no suppression was observed on plates selecting for suppression of ochre or amber nonsense mutations. This result was not observed with the JG113-5R genetic background. Thus, the wild-type $tRNA^{Trp}$ can act as an extremely weak opal suppressor, but only in the genetic backgrounds of JG369-3B and RJ293-13C. These strains may have a modifier gene(s) which allows rare pairing of the C in the first position of the anticodon with the A in the UGA opal nonsense codon. Modifier genes have been selected in *S. cerevisiae* which enhance or decrease suppressor efficiency (for a review, see reference 18) and that result in UAG suppression in a UAA suppressor genetic background (3) or that permit suppression of opal and amber nonsense mutations when wild-type $tRNA^{Gln}_{CAA}$ and $tRNA^{Gln}_{CAG}$ are present on high-copy-number plasmids (15, 26). The latter class of modifier genes may be analogous to the modifier genes we postulate to be present in JG369-3B and RJ293-13C.

Northern blot analysis of $tRNA^{Trp}$ transcripts from transformants. Bulk tRNAs from untransformed JG113-5R and from transformants of that strain were analyzed to determine whether any changes in transcription or processing of $tRNA^{Trp}$ precursors could be detected. Yeast tRNA was resolved on polyacrylamide-urea gels and transferred onto GeneScreen Plus membranes by the electroblot protocol recommended by the manufacturer (du Pont Co.). The Northern blots were hybridized by a standard protocol (24), with modifications (25). The Northern hybridization blots were first probed with a 21-base oligonucleotide complementary to the 3′ end of the mature *S. cerevisiae* $tRNA^{Trp}$. This oligonucleotide will also hybridize to all $tRNA^{Trp}$ precursors. By utilizing this probe, a predominant band that migrated slightly faster than the 77-nucleotide pCp-labeled yeast $tRNA^{Phe}$ was detected (Fig. 2A, lane M [left]). Mature yeast $tRNA^{Phe}$ migrated only slightly more slowly than the 75-nucleotide mature $tRNA^{Trp}$. Therefore, we feel that this predominant band corresponds to the mature yeast $tRNA^{Trp}$. A band indicating hybridization to trace amounts of $tRNA^{Trp}$ in the yeast $tRNA^{Phe}$ preparation was also observed (Fig. 2A, lane M [right]). The autoradiogram was purposely overexposed to detect smaller amounts of precursors. Lighter bands, which migrate considerably more slowly, were detected for all bulk tRNA preparations (Fig. 2B). These bands correspond to precursor transcripts and processing intermediates of the $tRNA^{Trp}$ genes. There appear to be increased levels of these transcripts in the yeast transformed with either the wild-type or the engineered $tRNA^{Trp}$ genes cloned into YRp17. This is expected, since YRp17 is a high-copy-number plasmid. Thus, there will be a great many more $tRNA^{Trp}$ genes in these transformants than the six copies (7; our unpublished results) found in the haploid yeast genome. However, pYRtRNA^{Trp}op1 transformants appear to accumulate even more precursor transcripts than either pYRtRNA^{Trp}wt or pYRtRNA^{Trp}op2 transformants (Fig. 2A).

The Northern blots were also probed with an end-labeled 20-base oligonucleotide which is complementary to 10 bases 3′ to the intron and 10 bases 5′ to the intron in the yeast $tRNA^{Trp}$ gene product. Under the hybridization conditions used, this probe will recognize only transcripts which do not contain the intron. With this probe, only the band which corresponds to the mature $tRNA^{Trp}$ transcript was detected (Fig. 2C). Thus, the precursors which hybridized to the first probe appear to contain intervening sequences. Alteration of the CCA anticodon seems to affect the ability of the yeast

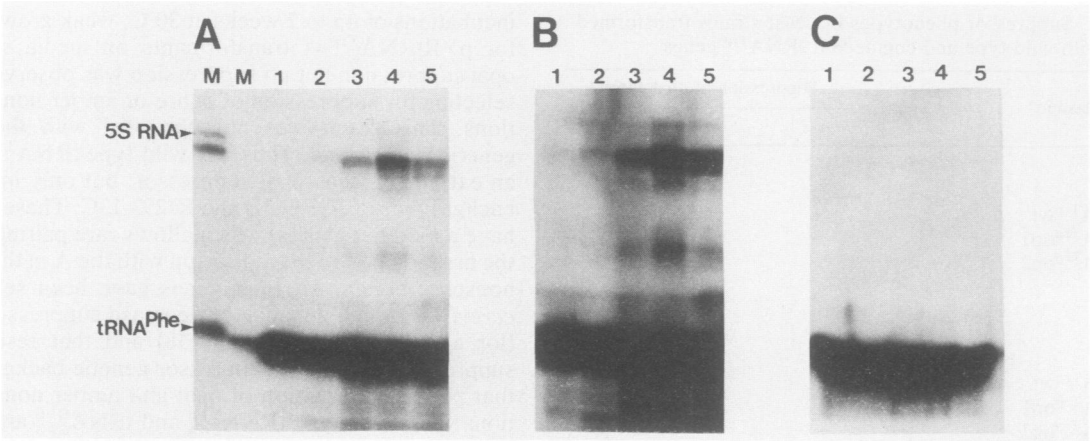

FIG. 2. Northern hybridization analysis of $tRNA^{Trp}$ transcripts isolated from the transformants. Five micrograms of partially purified tRNA was electrophoretically resolved on a 10% polyacrylamide–8.3 M urea gel. Lanes 1, tRNA isolated from untransformed JG113-5R cells; lanes 2, tRNA from JG113-5R transformed with YRp17; lanes 3, tRNA isolated from pYRtRNA^{Trp}wt transformants; lanes 4, tRNA from pYRtRNA^{Trp}op1 transformants; lanes 5, tRNA from pYRtRNA^{Trp}op2 transformants; M (left), *Escherichia coli* 5S RNA and yeast $tRNA^{Phe}$ which were 3′ end labeled with pCp (5′-^{32}P). The fastest-migrating band in this marker lane corresponds to the 77-nucleotide end-labeled $tRNA^{Phe}$. The most slowly migrating band corresponds to the 121-nucleotide end-labeled 5S RNA. In ethidium bromide-stained gels, this band migrates with the yeast 5S RNA. The band which migrates slightly faster than the end-labeled 5S RNA is likely a breakdown product of the 5S RNA. Lane M (right), second marker lane with 1 μg each of unlabeled yeast $tRNA^{Phe}$ and *E. coli* 5S RNA. Shown are 24-h (A) and 48-h (B) exposures of a Northern blot probed with a 5′-end-labeled oligonucleotide complementary to the sequence at the 3′ end of the transcripts and a Northern blot probed with a 5′-end-labeled oligonucleotide 20 bases in length and complementary to 10 bases in the mature domain immediately 5′ to the intron and 10 bases in the mature domain 3′ to the intron, under conditions sufficiently stringent that only transcripts which do not contain the intron will hybridize (24, 25). The amount of RNA loaded into each lane was monitored by the presence of the 5S rRNA band in ethidium bromide gels. Equal amounts of 5S RNA were observed in each lane in ethidium bromide-stained gels.

cell to splice the intervening sequences out of the precursor $tRNA^{Trp}$ transcripts effectively. Since the accumulation of intron-containing precursors in the yeast transformed with pYRtRNA^{Trp}op2 is comparable to that in the yeast with extra copies of the wild-type $tRNA^{Trp}$ gene (Fig. 2A), the ability to process the precursor efficiently is apparently restored when the second change is made in the intron.

There are two alternative secondary structures possible for the anticodon stem, loop, and intron of the wild-type pre-$tRNA^{Trp}$ (11). The change in the anticodon from CCA to UCA in tRNA^{Trp}op1 resulted in a U · G base pair in the anticodon-intron complementary sequence in the first conformation (Fig. 1B), thereby destabilizing the precursor in this confirmation (2). In the alternative conformation, the 5′UUCAAU3′ sequence from the anticodon loop could base pair with the underlined bases in the 3′AACGUUA5′ sequence at the 3′ splice junction. The 3′ splice site of this structure would then be located in a double-stranded region. Mutations in the introns of pre-$tRNA^{Leu}_3$ and pre-$tRNA^{Pro}$ which reduce or eliminate removal of the intron have been found to affect the 3′ splice site. These mutations all reduce the size of the single-stranded loop at the 3′ splice junction. Precursors which have a fully double-stranded structure at the 3′ splice site were not cleaved by the endonuclease, although cleavage could still occur at the 5′ splice junction (16, 20, 23). The precursors of tRNA^{Trp}op1 may accumulate because cleavage at the 3′ splice junction is inhibited. The second change made in the tRNA^{Trp}op2 gene restores the potential for conventional base pairing between the anticodon loop and the 5-base-pair sequence in the intron. Pre-tRNA^{Trp}op2 in this conformation should be a suitable substrate for endonucleolytic cleavage of the intron, since it places the 3′ splice junction in a single-stranded region. As a result, mature tRNA^{Trp}op2 would be expected. Consequently, strains transformed with pYRtRNA^{Trp}op2 should be able to suppress *leu2-2* and *his4-260* opal alleles, and growth on leucine and histidine omission medium would be expected, as was observed. It is possible that a requirement for a single-stranded 3′ splice junction is the most essential characteristic for the correct processing of tRNA introns in *S. cerevisiae*.

If the above hypothesis is true, it would predict that $tRNA^{Trp}$ genes with the anticodon sequence altered from CCA to CTA should encode an amber suppressor tRNA which would be efficiently processed into a mature tRNA. This prediction has been tested. It has been reported (7) that a single change in the CCA anticodon sequence (to CTA) is sufficient to create an efficient and specific suppressor of amber nonsense mutations. We have confirmed this result by observing that when the gene for this suppressor tRNA is present on a single-copy plasmid, colonies appear on media selecting for amber suppression within 1 to 2 days at 30°C. No suppression of the ochre or opal alleles was observed in either JG369-3B or JG113-5R transformants. However, when yeast cells were transformed with the $tRNA^{Trp}$ amber suppressor gene on a high-copy-number plasmid, they grew very slowly (results not shown). Thus, this suppressor seems to be toxic when present on a high-copy-number plasmid, presumably by interfering with the normal chain termination process of the cell, resulting in reduced cell viability. This argument also predicts that maturation of transcripts will be inhibited for putative ochre suppressors in which the CCA anticodon sequence of the $tRNA^{Trp}$ gene has been altered to TTA. As predicted, this putative ochre suppressor is inactive in vivo (7; our unpublished results). A second change in the 5-base intronic sequence that restores complementarity between the anticodon loop and the intron may result in more-efficient processing, because the second site changes would favor the formation of a conformation with a single-stranded 3′ splice site. This, in turn, may result in an active suppressor, if the ochre tested alleles can be suppressed by

tryptophan insertion at the premature stop codons. Experiments to test this prediction are in progress.

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