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Characterization of the *tRNA^{Trp}* genes of *Saccharomyces cerevisiae*

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Abstract

The purpose of this work was to examine the tRNA^{Trp}-encoding genes *(tRNA^{Trp})* of *Saccharomyces cerevisiae* to gain insight as to why tRNA^{Trp} amber suppressors, isolated by conventional genetic techniques, have not been reported. The results herein indicate that the haploid yeast genome contains six *tRNA^{Trp}* genes which map to five or six chromosomes. Not only do the six genes have identical coding sequences but their introns are also identical. Gene replacement experiments indicate that five copies of *tRNA^{Trp}* are sufficient for cell viability. Thus, mutation of one *tRNA^{Trp}* gene to a suppressor in vivo, lowering the functional number of *tRNA^{Trp}* genes, would not be expected to be lethal.

Keywords: suppressor, recombinant DNA, intron, amber suppressor, mutagenesis, pulsed-field gel electrophoresis, gene replacement, tetrad analysis

Abbreviations: bp, base pair(s); kb, kilobase(s) or 1000 bp; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PCR, polymerase chain reaction; RFLP, restriction-fragment length polymorphism; tRNA, transfer RNA; *tRNA^{Trp}*, gene encoding tRNA^{Trp}; ::, novel junction (fusion or insertion)

Introduction

To date, all of the spontaneous nonsense suppressors in S. *cerevisiae* are alleles of genes encoding tRNA^{Tyr}, tRNA^{Ser}, tRNA^{Leu}, or tRNA^{Gln} (reviewed in Sherman, 1982; Edelman and Culbertson, 1991). Although there are six other tRNA genes which should require only a single base change in the anticodon to encode a functional suppressor, in vivo mutations

involving these tRNA genes have not been isolated. For example, a single base substitution in a *tRNA^{Trp}* gene to change an encoded C to a U in either the first base or the second base of the anticodon should be sufficient to produce a tRNA capable of reading either the opal or amber nonsense codons, respectively. The cloning of a *S. cerevisiae* gene specific for tRNA^{Trp} was originally reported by Kang et al. (1980). An opal suppressor allele of this gene can be constructed by in vitro mutagenesis to change a single base in the anticodon. Suppression by the product of this gene is seen only when the gene is present on a multicopy plasmid (Kim et al., 1990). A gene with changes in both the anticodon and the intron is a more efficient opal suppressor, but this gene must also be present on a multicopy plasmid to observe suppression (Atkin et al., 1990). In contrast, it has been demonstrated that an amber suppressor can be constructed by a single change in the CCA anticodon of this gene to CTA (Kim and Johnson, 1988; Atkin et al., 1990). This gene was found to be an efficient suppressor when expressed from a single copy plasmid. These results suggest that it may not be possible to isolate tRNA^{Trp} opal suppressors by conventional genetic techniques. However, the ability to construct an efficient amber suppressor by a single engineered change raises the question of why it has not been possible to isolate tRNA^{Trp} amber suppressors by conventional genetic techniques.

One species of tRNA^{Trp} precursor accumulates in *rna1* mutants of *S. cerevisiae* (Ogden et al., 1984). This, and the inability to isolate spontaneous amber suppressor alleles of this gene, suggests there could be gene dosage constraints that prohibit mutating a *tRNA^{Trp}* gene to a suppressor. All copies of this gene may be required for tRNA^{Trp} function. Alternatively, there may be several *tRNA^{Trp}* genes with one being transcribed more efficiently than the others, making it indispensable for tRNA^{Trp} function. In this case, mutating one of the other copies to a suppressor-encoding gene would not result in measurable suppression due to inefficient expression of these copies of the gene. To examine these possibilities, the number of functional *tRNA^{Trp}* genes was determined and the consequences of disrupting a functional copy of a *tRNA^{Trp}* gene were examined.

Results and Discussion

(a) Copy number determination for the tRNA^{Trp} *genes of* Saccharomyces cerevisiae

The number of *tRNA^{Trp}* genes in the haploid genome of *S. cerevisiae* was determined by genomic Southern analysis. Genomic DNA was isolated from four haploid yeast strains (RJ293-13C, JG369-3B, N123, and JG 113-5R), and aliquots were digested individually with *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I. The yeast strains used in this study are described in table I. Figure 1 shows the autoradiographs of Southern blots of genomic DNA from two of these strains (RJ293-13C and JG369-3B). The Southern transfers were first probed with an end-labeled oligo complementary to the 21 bases at the 3′ end of the gene (fig. lA,C). Six bands of equal intensity were observed in each genomic digest for each of the haploid yeast strains shown, as well as the other two strains (not shown). RFLPs were observed between each of the four strains analyzed. For example, a single RFLP can be observed between the two strains shown. The other two strains (data not shown) also exhibited polymorphisms.

Table I. Yeast strains used in this study

Strain[a]	Genotype	Source
RJ293-13C	*MAT*a, *his4*-260, *leu2*-3,112, *ura3*-52	J. Johnson
JG369-3B	*MAT*α, *ade2*-1, *lys2*-1, *can1*-100, *met8*-1, *trp1*-1, *leu2*-2, *his4*-260, *ura3*-52	J. P. Gelugne
JG113-5R	*MAT*a, *ade2*-1, *can1*-100, *met8*-1, *trpl*-1, *leu2*-2, *his4*-260	J. P. Gelugne
N-123	*MAT*a, *his1 (rho +, ome –, CHL^s, ERY^s, OLI^s)*	R. C. von Borstel
ALA1-12B	*MAT*α, *lys2*-1, *met8*-1, *trpl*-1, *his4*-260, *ura3*-52	This study
ALA2-28C	*MAT*a, *ade2*-1, *met8*-1, *trpl*-1, *ura3*-52, *ilv1*	This study
ALA11	ALA1-12B × ALA2-28C	This study

a. The above strains were constructed using standard genetic techniques (Ausubel et al., 1989).

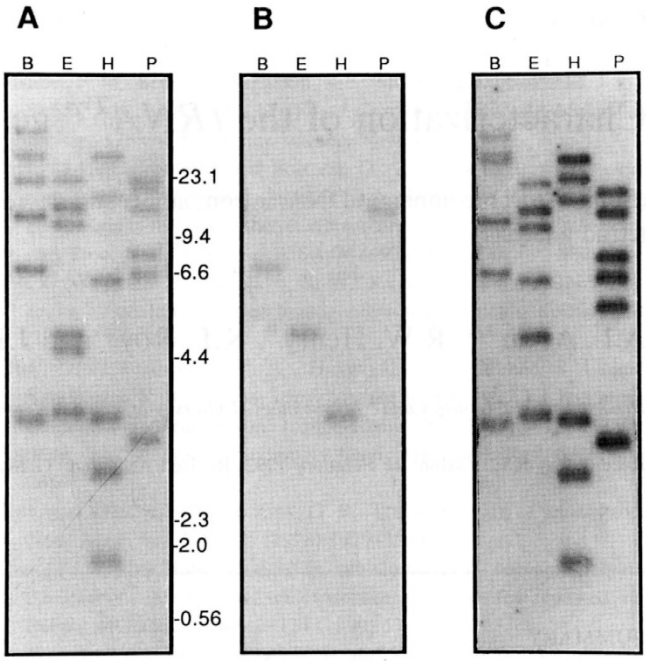


Figure 1. Southern analysis of genomic DNA from *Saccharomyces cerevisiae* strains RJ293-13C and JG369-3B. (Panel A) The Southern transfer of genomic DNA from RJ293-13C digested with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), and *Pst*I (P) was hybridized with an end-labeled oligo probe which is complementary to 21 nt of the antisense strand at the 3′ end of the gene. (Panel B) The same Southern transfer hybridized to a probe which is complementary to 15 nt, extending 5′ from 12 nt upstream from the mature coding sequences of the *tRNA^{Trp}* gene cloned by Kang et al. (1980). (Panel C) An autoradiograph of a Southern transfer of genomic DNA from JG369-3B digested with *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I hybridized with the same probe as in A. The location of the λ *Hin*dIII size markers (in kb) has been indicated. Methods. Yeast genomic DNA was prepared by the method of Ausubel et al. (1989). These DNAs, digested with the appropriate restriction enzymes, were resolved on 1% agarose gels and subsequently transferred to GeneScreen Plus following the manufacturer's (duPont Co.) instructions. The Southern transfers were hybridized to

the 5′ end-labeled oligo as described in Van Tol et al. (1988), with modifications (Wallace and Miyada, 1987). The design of the oligos used as probes was based on the sequence of the previously cloned *S. cerevisiae tRNA^{Trp}* gene (Kang et al., 1980).

To determine whether each of the six *tRNA^{Trp}* genes contains an intron, Southern blots were probed with an end-labeled oligo which is complementary to 10 nt 5′ and 10 nt 3′ to the intron, or an oligo complementary to 10 nt of intronic sequences and 10 nt of exonic sequence surrounding the 3′-splice junction. These hybridizations were done under stringent conditions. A blot with mature tRNA^{Trp} was included in the experiment as a control. We have previously shown that the first end-labeled oligo hybridizes specifically to mature tRNA^{Trp} and not intron-containing precursors (Atkin et al., 1990), while the second end-labeled oligo hybridizes only to intron-containing precursors under the experimental conditions used. In agreement with these previous experiments, the first end-labeled oligo hybridized to only the mature tRNA^{Trp}. The second end-labeled oligo hybridized to the same bands as the end-labeled oligo (complementary to 21 bases of the 3′ end of the gene) used to determine the copy number of the *tRNA^{Trp}* gene (results not shown). Thus, all of the *tRNA^{Trp}* genes in *S. cerevisiae* appear to contain an intron.

The Southern blots were also probed with an end-labeled oligo complementary to sequences 5′ to the start of the mature coding sequences of the gene cloned by Kang et al. (1980). A single band was observed in each genomic digest for each strain (fig. 1B), suggesting that there is very little sequence homology in the 5′ flanking sequences between the original member to be cloned (Kang et al., 1980) and the other members of this family of genes. The pattern of bands observed for RJ293-13C (fig. 1B) was identical to the patterns of bands observed for the Southern blots of genomic digests of DNA isolated from the three other strains. Thus, no RFLPs appear to be associated with this member of the *tRNA^{Trp}* gene family, at least among the four strains studied herein.

(b) Chromosomal location of the* tRNA^{Trp} *genes

The genomic locations of the *tRNA^{Trp}* genes were examined by probing Southern blots of intact yeast chromosomes separated by pulsed-field gel electrophoresis (fig. 2). The Southern blot of the yeast chromosomes was first probed with the end-labeled oligo complementary to the 3′ end of a *tRNA^{Trp}* gene, as in figure 1. Five bands were observed (fig. 2A), four of equal intensity and one with approximately twice the intensity. The more intense band correlated with the expected positions of chromosomes XV and VII. Thus, there may be two genes on one of these chromosomes, or these genes may be located on separate chromosomes. The same Southern blot was hybridized with a 0.41-kb *tRNA^{Trp}* fragment subcloned from the 3.4-kb fragment cloned by Kang et al. (1980) to determine to which chromosome this gene maps. This probe hybridizes to chromosome XV or VII (fig. 2B). Thus, the first member of this gene family to be cloned maps to chromosome XV or VII, and the six *tRNA^{Trp}* genes in the haploid genome of *S. cerevisiae* are dispersed on five or six chromosomes.

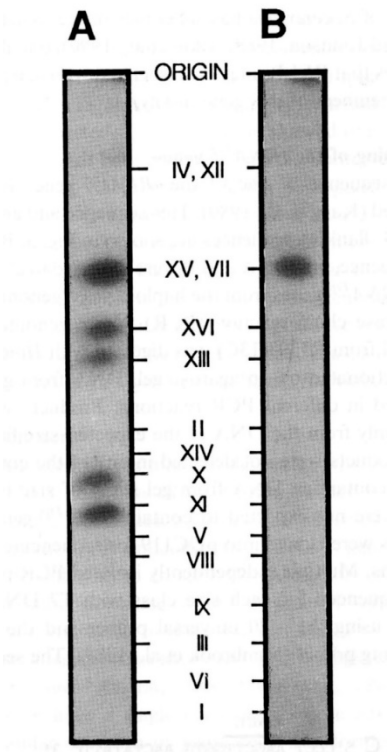


Figure 2. Southern hybridization of yeast chromosomes separated by pulsed-field gel electrophoresis. (Panel A) The Southern transfer was hybridized with an end-labeled oligo complementary to 21 nt of the antisense strand at the 3′ end of the *tRNA*^{Trp} gene, as in figure 1. (Panel B) The same Southern blot was hybridized to a random primer-labeled 0.41-kb fragment of yeast DNA containing a *tRNA*^{Trp} gene. The location of the yeast chromosomes is indicated. The position that the individual yeast chromosomes migrated to was determined from the ethidium-bromide-stained 1% agarose gel before the Southern transfer. The isolation of intact yeast DNA for pulsed-field gel electrophoresis was performed as described in Sambrook et al. (1989). Intact yeast chromosomes were resolved in a CHEF gel system, and DNA transferred to GeneScreen Plus was hybridized to end-labeled oligos, as in figure 1. The protocol of Klessig and Berry (1983) was used for hybridizing the random primer-labeled probe to these Southern transfers.

The results of the Southern analyses for gene enumeration and chromosomal locations are substantially in accordance with those previously reported (Yesland et al., 1991). Using similar methodology (and including strain RJ293-13C), their analysis suggests there are seven copies of the *tRNA*^{Trp} gene in *S. cerevisiae*. This conclusion was based on the observation of a seventh very faint band in addition to six prominent bands of approximately equal intensity in the lane with the *Hin*dIII digest of RJ293-13C genomic DNA probed with an end-labeled oligo. Figure lA shows a *Hin*dIII digest of RJ293-13C genomic DNA, also probed with an end-labeled oligo. Only six bands of equal intensity were observed. We feel that the fainter band observed by Yesland et al. (1991) is artefactual or the result of a

polymorphism within their culture of the strain, although the authors reported no RFLPs within this family of genes for any of the three yeast strains that they examined. The present work supports the original estimate that the haploid genome of S. *cerevisiae* has six copies of the *tRNA*^{Trp} gene (Kim and Johnson, 1988; Atkin et al., 1990) but also demonstrates that RFLPs may be associated with at least some of the members of this gene family.

(c) Cloning of the* tRNA^{Trp} *genes

The sequence of one of the *tRNA*^{Trp} genes has been published (Kang et al., 1980). This sequence and additional 5′ and 3′ flanking sequences are shown in figure 3. Based on this sequence, primers A and B were designed to clone each of the *tRNA*^{Trp} genes from the haploid yeast genome by the polymerase chain reaction (PCR). Yeast genomic DNA (isolated from RJ293-13C) was digested with *Hin*dIII and size-fractionated on an agarose gel. DNA from gel slices was used in different PCR reactions. Products were obtained only from the DNA of the expected size fractions. PCR products were not detected in any of the control reactions containing DNA from gel slices of size fractions which were not expected to contain *tRNA*^{Trp} genes. The products were cloned into pUC119 for sequencing in both directions. Multiple independently isolated PCR products were sequenced for each size class with T7 DNA polymerase, using the –20 universal primer and the reverse sequencing primer (Sambrook et al., 1989). The sequences of the mature coding regions and the introns of the cloned *tRNA*^{Trp} genes were identical; however, the PCR technology used herein could have missed encoded polymorphisms that were included within sequences covered by the primers.

***(d) Replacement of a copy of a* tRNATrp *gene with* URA3**

To determine whether six copies of the *tRNA*^{Trp} gene are necessary for viability of haploid cells, a copy of this gene was replaced by the *URA3* gene. The first member of the *tRNA*^{Trp} gene family to be cloned was chosen for gene replacement. No restriction length polymorphisms are associated with this *tRNA*^{Trp} gene (see section a). This raises the possibility that it is tightly flanked by essential sequences. To ensure that only the *tRNA*^{Trp} gene was replaced by the *URA3* gene, PCR was used to clone the 5′ and 3′ flanking DNA from this *tRNA*^{Trp} gene and create restriction sites for insertion of the *URA3* gene between these sequences. Primers C and D (fig. 3) were used to clone a 155-bp fragment from the 5′ flank. These primers were designed to create *Hin*dIII and *Eco*RI restriction sites, respectively. The 149-bp fragment from the 3′ flank was cloned using primers E and F (fig. 3). These primers were designed to create *Pst*I and *Kpn*I restriction sites, respectively. A 0.9-kb *Sma*I-*Pst*I fragment (with an *Eco*RI linker added to the *Sma*I end) capable of complementing the *ura3*-52 mutation was cloned between the 5′ and 3′ *tRNA*^{Trp} flanking sequences. The resultant 1.2-kb *Hin*dIII-*Kpn*I fragment, with the *URA3* gene cloned between the 5′ and 3′ *tRNA*^{Trp} flanking sequences, was used to transform a diploid strain, ALA11, constructed for this study (see table I).

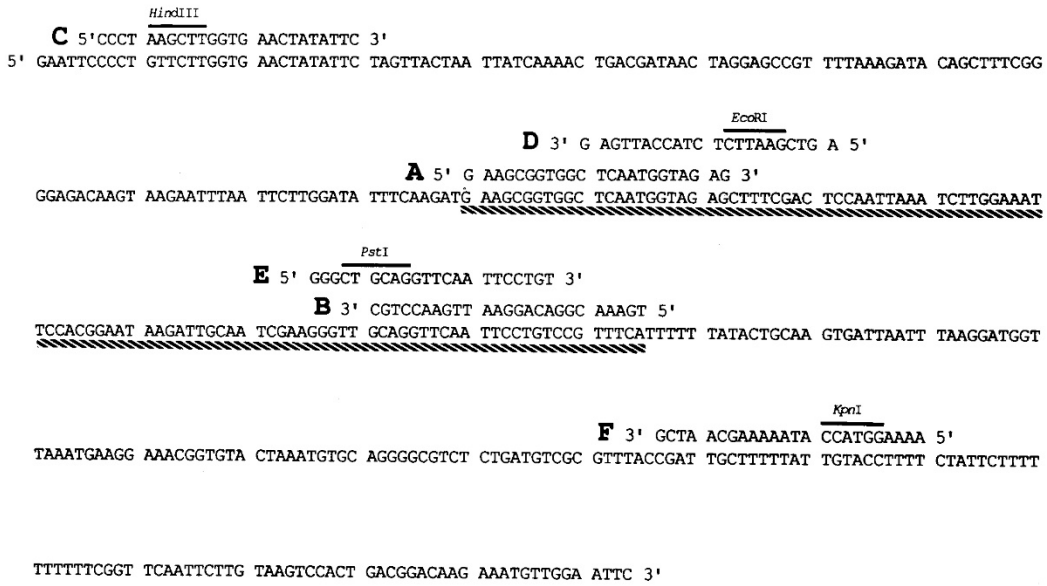
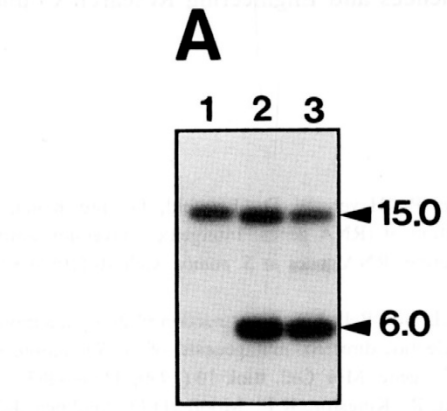


Figure 3. Sequence of a cloned *tRNA*^{Trp} gene (modified from Kang et al., 1980). A 0.41-kb fragment of yeast DNA in M13mp19 was sequenced using dideoxy-mediated sequencing reactions with the Klenow fragment of *E.coli* DNA polymerase 1 (Sambrook et al., 1989). The location of the *tRNA*^{Trp} gene is indicated by the stippled line below the sequence. Primers A, B, C, D, E, and F were used for PCR according to published methods (Saiki et al., 1988). Yeast genomic DNA, isolated from yeast strain RJ293-13C, served as the PCR template for isolation of the *tRNA*^{Trp} genes with primers A and B. Plasmid DNA with the 0.41-kb fragment of yeast DNA encoding a *tRNA*^{Trp} gene was used to clone 5′ and 3′ flanking sequences. Primers C and D were used to clone a 155-bp fragment from the 5′ flank of the *tRNA*^{Trp} gene. A 149-bp fragment was cloned from the 3′ flank of the *tRNA*^{Trp} gene with primers E and F. These DNA fragments were incorporated, respectively, into a construct flanking a copy of the *URA3* gene for the replacement experiment.

The single-step gene replacement of a chromosomal copy of the *tRNA*^{Trp} gene with the 1.2-kb *Hin*dIII-*Kpn*I fragment was performed essentially as described by Rothstein (1983). A diploid recipient, ALA11, was used in case the disruption was a lethal event in a haploid strain. Transformants that were prototrophic for uracil were selected and examined for a *tRNA*^{Trp}::*URA3* disruption by Southern analysis. Genomic DNA from untransformed and transformed diploids was digested with *Bam*HI and hybridized with a *URA3*-specific probe. The same blots were stripped and rehybridized with the 0.41-kb *tRNA*^{Trp} probe. DNA from the parental diploid (ALA11) produced a 15-kb *Bam*HI band when hybridized to the *URA3* probe. DNA from the selected transformants produced two bands of homology to the *URA3* probe, a 15-kb band representative of the endogenous mutant *ura3*-52 gene and a 6-kb band (fig. 4A). The faster migrating 6-kb band also hybridizes to the 0.41-kb *tRNA*^{Trp} probe (data not shown), indicating that this band represents the targeted replacement event.



B

YEAST STRAIN	NUMBER OF TETRADS	SEGREGATION PATTERNS OBSERVED FOR		
		ADE+/ade-	URA+/ura-	
ALA11	3	2+/2-	0+/4-	In all tetrads
ALA12	9	2+/2-	2+/2-	"
ALA13	16	2+/2-	2+/2-	"
ALA14	7	2+/2-	2+/2-	"

Figure 4. Single-step gene replacement of a chromosomal copy of a *tRNA*^{Trp} gene with the *URA3* gene. (Panel A) An autoradiograph of a Southern hybridization of genomic DNA from the parental diploid, ALA11 (lane 1), and two uracil-prototrophic transformants, ALA12 (lane 2) and ALA13 (lane 3), restricted with *Bam*HI and hybridized with an oligo-labeled *URA3* probe, as described in figure 2. The sizes of the bands observed were determined by comparison to a λ *Hin*dIII size marker in kb. Yeast genomic DNAs were isolated, restricted, electrophoresed, and blotted as in figure 1. (B) Tetrad analysis of the parental diploid, ALA11, and three uracil-prototrophic transformants, ALA12, ALA13, and ALA14. Segregation patterns were determined for all tetrads.

The parental diploid (ALA11) and the disrupted diploids (ALA12, ALA13, and ALA14) were sporulated, and the asci were dissected. Tetrad analysis showed that, in all 32 asci from diploids containing the gene disruption and which produced four viable spores, URA segregated 2 + :2 – (fig. 4B). This is the expected result if one copy of the *tRNA*^{Trp} gene has been replaced by *URA3* in an otherwise *ura3*-52 background. This demonstrates that all six copies of the *tRNA*^{Trp} gene in the haploid yeast genome are not essential for growth, since the Ura$^+$ spores were viable but had only five copies of the *tRNA*^{Trp} gene. Thus, a maximum of five copies of this gene are sufficient for viability.

(e) Conclusions

(1) An attempt was made to determine why suppressor alleles of a S. *cerevisiae tRNA*^{Trp} gene, isolated by conventional genetic techniques, have not been reported. A single change in the CCA anticodon of a *tRNA*^{Trp} gene to CTA should be sufficient to create a tRNA capable of interacting with amber nonsense codons. A gene with this change has been constructed in vitro and was found to be an efficient suppressor when expressed from a single copy plasmid (Kim and Johnson, 1988; Atkin et al., 1990). Yeast cells which have a single copy of this gene have growth rates similar to those of yeast cells transformed with a single

copy of the wild-type gene, although this amber suppressor is toxic when present on a multicopy plasmid (Kim et al., 1988; Atkin et al., 1990). The inability to recover amber suppressor alleles of this gene by conventional genetic techniques suggested that there may be only a few copies of the *tRNA*^{Trp} gene in the yeast genome and that all may be essential. This possibility was addressed herein by identification and partial characterization of all of the *tRNA*^{Trp} gene. Data are presented which show there are six copies of this gene in the haploid genome of S. *cerevisiae*. All copies were cloned and shown to contain identical introns. The possibility that all six copies are required was tested by replacing a copy with the *URA3* gene. The results of this experiment clearly show that a maximum of five *tRNA*^{Trp} genes is sufficient for viability of haploid yeast cells. Although this result could be extended by systematically replacing additional and/or specific *tRNA*^{Trp} genes to see what minimum number of *tRNA*^{Trp} genes is essential, it is important to note that at least one gene can be replaced. This eliminates simple dosage constraints as the reason spontaneous tRNA^{Trp} suppressors have never been reported.

The ability to construct functional amber tRNA^{Trp} suppressors in vitro by a single bp change to the gene and the requirement for a maximum of only five of the six *tRNA*^{Trp} genes for viability of haploid yeast cells suggest that it should be possible to isolate tRNA^{Trp} amber suppressors by conventional genetic techniques. A possible explanation for this apparent contradiction may be that the *tRNA*^{Trp} genes are not as active at their native loci as they are when present on a plasmid. Thus, suppression would not be detected when one of them is appropriately mutated. A direct test of this suggestion would be to replace a copy of a *tRNA*^{Trp} gene in its natural chromosomal context with an in vitro–constructed amber suppressor allele. These experiments will be attempted.

(2) The absolute sequence identity observed for the introns of the yeast *tRNA*^{Trp} genes is unusual. Polymorphisms have been observed in the sequences of the introns encoded by members of other tRNA gene families in yeast. There appears to be little selective pressure on the sequence of introns in tRNA genes. Profound changes can be made to these sequences without affecting their removal from pre-tRNA. Only mutations which affect the secondary structure of the precursor or prevent the formation of the single-stranded loop at the 3′-splice junction have an effect on intron removal (reviewed in Atkin et al., 1990). Thus, the absolute sequence identity observed amongst the introns of the *tRNA*^{Trp} genes, despite the seemingly limited selective pressure and the dispersed locations of these genes, implies that ectopic conversion likely occurs among the members of this gene family.

Ectopic conversion has been shown to occur among three genes encoding two serine tRNA isoacceptors located on different chromosomes or chromosome arms in *Schizosaccharomyces pombe* (Kohli et al., 1984; Amstutz et al., 1985). All three of these genes are closely associated with methionine initiator tRNA genes and give rise to dimeric primary transcripts. Conversion events among these genes were found to be limited to the sequences within the dimeric tRNA genes. Changes were never seen within the flanking DNA. In addition, the transfer of information was undirected. Ectopic conversion is also thought to occur between the tRNA$^{Ser}_4$ and tRNA$^{Ser}_7$ genes of *Drosophila melanogaster* (Cribbs et al., 1987). The sequences of these genes are 96% identical, differing only at the first position of the anticodon and two other sites. This unusual degree of homology suggests these genes are undergoing concerted evolution.

The homology observed for the family of *tRNA^{Trp}* genes in *S. cerevisiae* and their dispersed locations suggest that they could be an excellent model system for studying ectopic conversion events among members of a family of genes in their native chromosomal context. Most studies on ectopic recombination in yeast involve examination of events between a gene at its natural position and a copy of the gene which has been inserted into a different locus by gene replacement (reviewed in Petes and Hill, 1988). Although these studies have provided valuable information, it is still necessary to show that these results are representative of the recombination events that occur among genes at their normal loci. There is some evidence that the rate of conversion is lower among genes at their native chromosomal loci than expected from studies involving artificial duplications. Meiotic rates of conversion among Ty elements at different chromosomal locations in diploid yeast strains is considerably less than the rates detected in studies using artificial duplications (Pete and Hill, 1988). Key to studies on ectopic recombination among *tRNA^{Trp}* genes will be the development of a sensitive, quantitative assay for suppression by amber alleles of the *tRNA^{Trp}* genes at their native loci. With such an assay system it would be possible to monitor conversion at *tRNA^{Trp}* loci which have been replaced with amber suppressor alleles.

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