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In vivo modulation of yeast tRNA gene expression by 5′-flanking sequences

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A pentadecanucleotide sequence, TTTCAACAAATAAGT, contiguous with the 5′-end of *Saccharomyces cerevisiae* $tRNA_3^{Leu}$ coding sequence acts as a positive modulator of transcription in a homologous *in vitro* system. To determine whether modulation also takes place *in vivo*, the amber suppressor forms of $tRNA_3^{Leu}$ genes with different 5′-flanking sequences were generated by site-specific mutagenesis and cloned into YCp19, a yeast vector maintained at 1–2 copies per cell. These plasmids were transformed into *S. cerevisiae* strains marked with amber mutations lys2-801, met8-1, and tyr7-1. The ability of the $tRNA_3^{Leu}$ amber suppressor genes ($tDNA_{3A}^{Leu}$) to suppress functionally lys2-801 and tyr7-1 mutations in the yeast host strain correlated well with template activities measured *in vitro*. We conclude that the plasmid-borne tRNA gene acts as an effective suppressor from the plasmid and the conserved pentadecanucleotide sequence modulates the expression of yeast $tRNA_3^{Leu}$ *in vivo* as well as *in vitro*. This regulatory sequence is found associated with genes coding for a number of tRNAs which are abundant in yeast. We postulate that this sequence represents a mechanism by which production of specific tRNAs can be enhanced to match demand created by codon use preferences.

Key words: gene expression/*in vivo*/tRNA/yeast

Introduction

The 5′-flanking region of some tRNA genes (tDNA) influences the level of transcription *in vitro* and homologous systems are most sensitive to this regulation. The regions responsible for this modulation have been identified in three invertebrate cell types *Bombyx mori, Drosophila melanogaster* and *Saccharomyces cerevisiae* (DeFranco *et al.*, 1981; Larson *et al.*, 1983; Raymond and Johnson, 1983; Schaack *et al.*, 1984; Wilson *et al.*, 1985). No similarities are apparent between the insect species. The third cell type *S. cerevisiae*, shows little or no sequence homology to the insect sequences, although it is present in several yeast tRNA genes.

The $tDNA_3^{Leu}$ is present in ~9 copies in *S. cerevisiae* (Venegas *et al.*, 1979; Liebman *et al.*, 1984; Raymond and Johnson, unpublished results). The region which specifies the nucleotide sequence of mature $tRNA_3^{Leu}$ is identical in the four copies of these genes which have been studied (Kang *et al.*, 1979; Venegas *et al.*, 1979; Standring *et al.*, 1981; Andreadis *et al.*, 1982). These same clones also show extensive sequence homology in the immediate 5′- and 3′-flanking regions as well as the intervening sequence (IVS) (Raymond and Johnson, 1983; Fischhoff *et al.*, 1984). A pentadecanucleotide sequence, TTTCAACAAATAAGT, is almost perfectly conserved in all four genes even though the sequences just upstream from this region bear only slight similarities.

Replacement of yeast DNA up to position −22 in the 5′-flanking sequence with vector DNA (d5′-22) results in a moderate reduction in transcriptional activity of yeast $tDNA_3^{Leu}$ in a homologous cell-free extract (Raymond and Johnson, 1983). When replacement is extended to position −2 (d5′-2), the gene is nearly inert. However, when the same templates are transcribed in HeLa cell or *Xenopus laevis* oocyte extracts the influence of the 5′-flanking sequence is lost and both templates, d5′-22 and d5′-2, are transcribed with an efficiency near that of the unmodified form of the gene (Johnson and Raymond, 1984).

An *in vivo* assay was developed to analyze the role of the conserved pentadecanucleotide sequence in the production of a biologically active yeast $tRNA_3^{Leu}$. Three forms of the gene were studied, the unmodified form, pJD137, d5′-22 and d5′-2. This was accomplished by generating the amber suppressor forms of the genes, $tDNA_{3A}^{Leu}$, and cloning them into the yeast/*Escherichia coli* shuttle vector YCp19. Upon transformation into yeast, the plasmid is stably maintained at an average of one or two copies per cell (Clarke and Carbon, 1980; Bloom *et al.*, 1982; Koshland *et al.*, 1985). The activity of the $tRNA_{3A}^{Leu}$ genes was measured by their ability to suppress amber mutations present in yeast host strains causing the cells to become prototrophic for the appropriate nutrients.

The YCp19 clones were also transcribed in a homologous cell-free system to determine whether the anticodon mutation affected gene activity and to provide a quantitative measurement of template activity for the various forms of the gene. When these results were compared with the *in vivo* suppressor activity, correlation coefficients of 0.35–0.79 were observed.

Results

Conversion of yeast $tDNA_3^{Leu}$ to $tDNA_{3A}^{Leu}$ by site-directed mutagenesis

A 900-bp yeast fragment containing the $tRNA_3^{Leu}$ gene, 350 bp of 5′-flanking sequence and 440 bp of 3′-flanking sequence, isolated from the plasmid pJD137 (Johnson *et al.*, 1980) was cloned between the *Eco*RI and *Bam*HI sites of M13mp9, creating m9137. A 290-bp fragment, d5′-2, with 2 bp of 5′-flanking sequence, the yeast $tDNA_3^{Leu}$ and 170 bp of 3′-flanking sequence was obtained from an *Eco*RI plus *Bam*HI digestion of the plasmid d5′-2 (Raymond and Johnson, 1983) and cloned between the *Eco*RI and *Bam*HI sites of M13mp9, creating m9d5′-2.

Viral DNA from m9137 and m9d5′-2 was used as a template for site-directed mutagenesis with a 47-bp primer from a *Hae*III plus *Hinc*II restriction endonuclease digestion of the plasmid pYLEU2SUP53 (Newman *et al.*, 1983). This fragment contains the anticodon region from a naturally occurring amber suppressor form of $tDNA_3^{Leu}$, SUP53. It spans the anticodon region of the gene and can introduce a T-A transversion in the central position of the anticodon of the m9137 and m9d5′-2 clones. This transversion generates an anticodon that recognizes the amber codon, UAG, and also gives rise to an *Xba*I site in the gene,

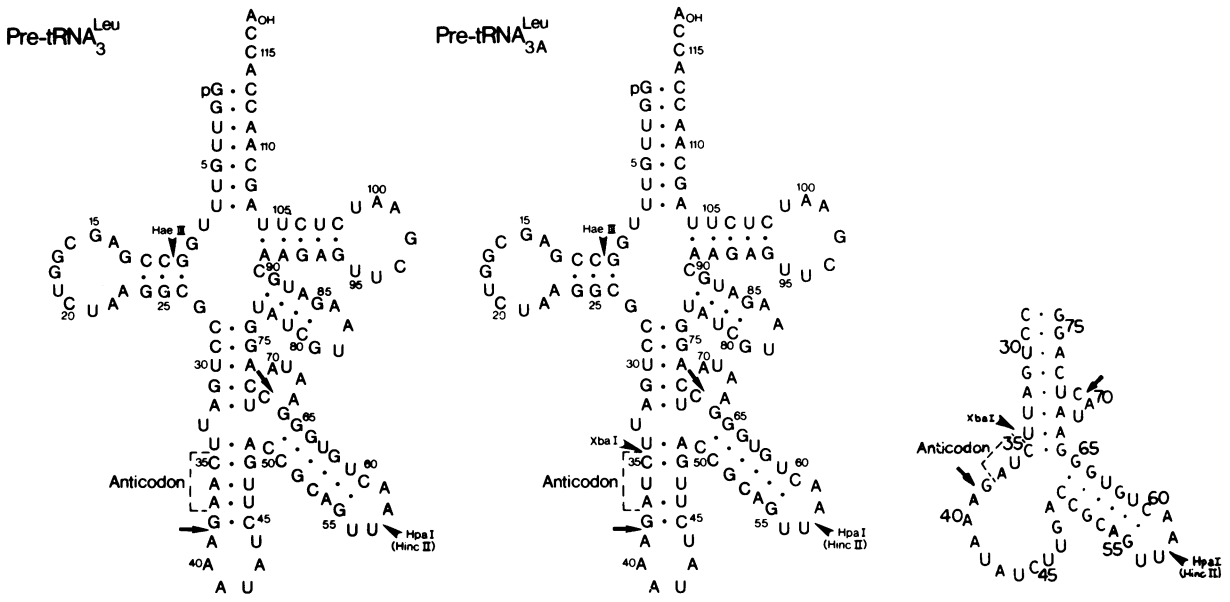

Fig. 1. The primary and putative secondary structures of the pre-tRNA$_3^{Leu}$ molecules. Pre-tRNA$_3^{Leu}$ represents the unmodified form of the gene and pre-tRNA$_{3A}^{Leu}$ is the amber suppressor form of the gene. The small arrows, in both cases, bracket the IVS. The locations of the *Hae*III and *Hinc*II restriction sites used to obtain the 47-bp primer from the plasmid pYLEU2SUP53 are shown. The base change in the anticodon of the pre-tRNA$_{3A}^{Leu}$ and the new *Xba*I restriction endonuclease site are indicated. The insert shows the anticodon stem and loop of pre-tRNA$_{3A}^{Leu}$ as its structure is predicted by computer analysis (Zuker and Stiegler, 1981).

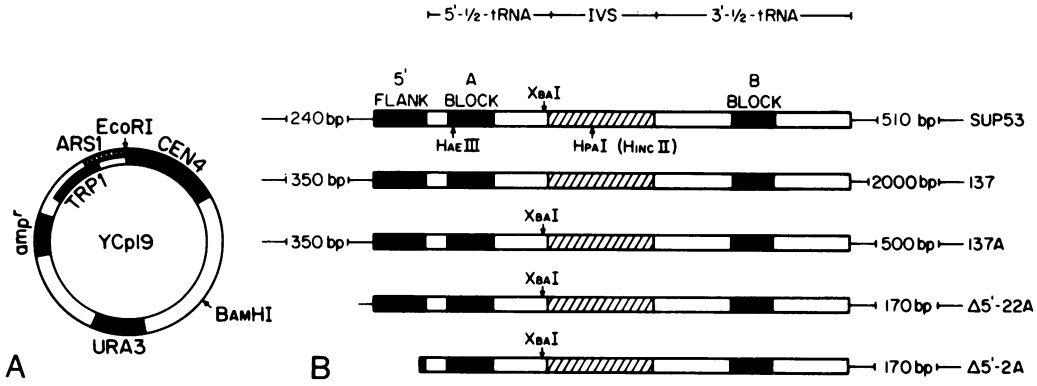

Fig. 2. Subcloning into the yeast vector YCp19. **(A)** YCp19 is a 10.6-kb yeast/*E. coli* shuttle vector containing a complete copy of pBR322 and some yeast DNA. The yeast portion includes a centromere sequence from chromosome IV, CEN4, an autonomous replication sequence, ARS1, as well as the *URA3* and *TRP1* genes, all of which are indicated. The positions of the *Eco*RI and *Bam*HI cloning sites are shown. **(B)** The yeast DNA fragments depicted, except 137 and SUP53, were each cloned into YCp19 in both orientations at each of the two cloning sites. The exceptions are explained in the text. The tDNA^{Leu} and control regions are boxed with the IVS being hatched and regions essential for efficient transcription shown in black.

tDNA$_{3A}^{Leu}$ (Figure 1). Clones carrying the amber suppressor form were identified by examining phage replicative form (RF) DNA for the presence of a new *Xba*I restriction site and, for m9d5'-2, confirmed by DNA sequence analysis. The amber suppressor forms of m9137 and m9d5'-2 are referred to as m9137A and m9d5'-2A, respectively.

The d5'-22A form of the tDNA$_{3A}^{Leu}$ was derived from m9137A RF DNA by subcloning a *Fnu*DII restriction fragment which includes tDNA$_{3A}^{Leu}$ plus 22 bp of 5'-flanking sequence and 170 bp of 3'-flanking sequence.

Cloning into the yeast vector YCp19

YCp19 is a 10.6-kb yeast/*E. coli* shuttle vector, containing a complete copy of pBR322 plus yeast CEN4, ARS1, URA3 and TRP1 DNA. Cloning sites for *Eco*RI and *Bam*HI fragments are present in non-essential regions of the yeast DNA (Figure 2A) (Clancy *et al.*, 1984).

Yeast DNA fragments containing tDNA$_3^{Leu}$, tDNA$_{3A}^{Leu}$, 5'-flanking sequence modifications of tDNA$_{3A}^{Leu}$ and SUP53 were subcloned into YCp19 in both orientations at each of the two cloning sites (Figure 2B).

Abbreviations for the recombinant plasmids follow the convention that Y denotes they are YCp19 clones and B or R indicate the fragments are in the *Bam*HI or *Eco*RI recognition sites, respectively. Orientation of the fragments such that transcription is left- or rightward within the vector as depicted in Figure 2A is designated by L or R. Isolates were obtained with the various fragments in both orientations at each of the cloning sites with two exceptions. The fragment 137, having two *Eco*RI ends, was cloned only into the *Eco*RI site and one clone was generated, YR137L. The SUP53 clones, YBSUP53R and YRSUP53L, were generated in one orientation only, at each of the two cloning sites.

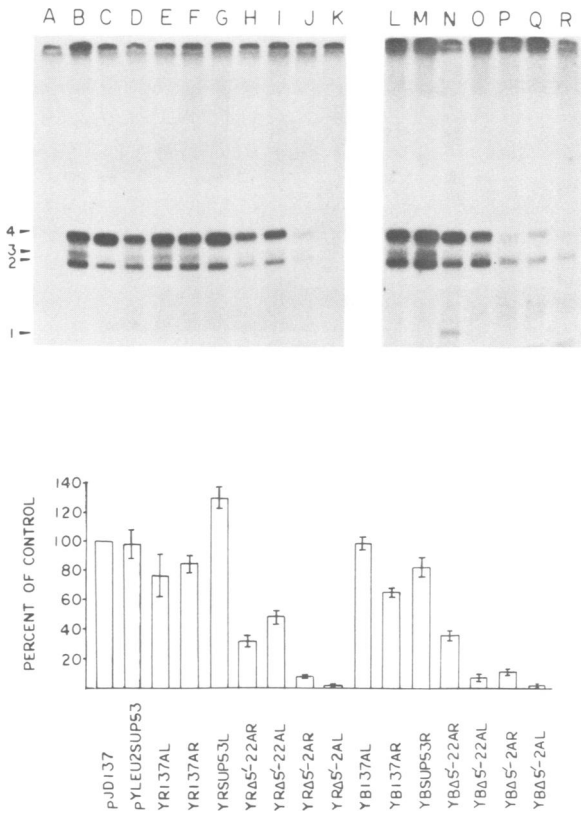

Fig. 3. *In vitro* transcription of YCp19 subclones in yeast cell-free extracts. **(A)** The plasmids were transcribed in 25 μl reactions containing 22 fmol of the plasmid as described in Materials and methods. The ^{32}P-labeled reaction products were electrophoresed on a 10% polyacrylamide gel containing 4 M urea and the radioactive bands visualized by fluorography. The templates for each lane are: A=YCp19, B=pJD137, C=pYLEU2SUP53, D=YR137L, E=YR137AL, F=YR137AR, G=YRSUP53L, H=YRd5′-22AR, I=YRd5′-22AL, J=YRd5′-2AR, K=YRd5′-2AL, L=YB137AL, M=YB137AR, N=YBSUP53R, O=YBd5′-22AR, P=YBd5′-22AL, Q=YBd5′-2AR, and R=YBd5′-2AL. The identities of the RNA species present in the numbered bands are: band 1=mature $tRNA^{Leu}$; band 2=pre-$tRNA^{Leu}$ containing the IVS, 5′ and 3′ end processed without $-CCA_{OH}$ addition; band 3=as band 2, but with the $-CCA_{OH}$ addition (as depicted in Figure 1); and band 4=as band 2, but 5′ and 3′ end extended. The band just below band 1 is not dependent upon added template and is thought to result from guanylylation of $tRNA^{His}$ present in the yeast extract (Cooley *et al.*, 1982). **(B)** Radioactive bands were cut from the gel, immersed in Biofluor and counted in a liquid scintillation spectrometer. The radioactivity incorporated into RNA from pJD137 **(lane B)** was used as a reference standard to compare transcription from each template. The bars represent the percent of RNA produced from each plasmid relative to the reference. Each value is the average of three transcriptions with the standard deviation shown.

In vitro transcription

The fragments 137, d5′-22, and d5′-2 cloned in pBR322 have been transcribed *in vitro* (Raymond and Johnson, 1983). The removal of the conserved 5′-flanking sequence between positions −22 and −2 greatly decreased the efficiency of transcription when compared with either a deletion to position −22 or unmodified pJD137. The position of the gene within pBR322 also influenced the efficiency of transcription for the d5′-22 and d5′-2 fragments. It was for this reason that each of these fragments was examined in different plasmid positions and orientations.

The various YCp19 subclones were used as templates for *in vitro* transcription to determine whether their activities were similar to the comparable pBR322 clones and for comparison with subsequent *in vivo* studies. The efficiency of transcription was standardized by comparing the radioactivity incorporated into RNA from each template with that from the plasmid pJD137. The data is presented as a percent value and error bars are shown representing the standard deviation for the three experiments performed (Figure 3B).

The YCp19 clones behave similarly to their pBR322 counterparts. Transcription of the gene is relatively unaffected by the T-A transversion in the anticodon of the gene. The d5′-22A clones are transcribed with 30−50% of the efficiency of clones containing >22 bp of yeast 5′-flanking sequence. This indicates that bases further upstream from −22 are also involved in the modulation of transcription. Removal of the yeast $tDNA^{Leu}_{3A}$ 5′-flanking sequence to −2, causes a drastic decrease in the level of transcription. This 10- to 20-fold transcriptional decrease as compared with the standard, pJD137, is thought to occur due to the removal of most of the conserved pentadecanucleotide sequence.

The 5′ deletion clones at the *Bam*HI site are transcribed more efficiently when oriented rightward in the vector *versus* leftward. The same fragments in the *Eco*RI site behave somewhat differently. YRd5′-2AR is transcribed more efficiently than YRd5′-2AL, but YRd5′-22AL is more efficient than YRd5′-22AR. Since the gene is not always transcribed more efficiently in one orientation than the other, this indicates that it is not orientation itself that is affecting the level of transcription. It has been proposed that the vector sequence, which replaces the yeast sequence, could influence transcription either positively or negatively (Hall *et al.*, 1982). Raymond and Johnson (1983) also suggested that this might be causing the difference in transcriptional efficiencies of d5′-22R and d5′-22L. The vector sequence flanking the d5′-22L clone used by Raymond and Johnson (1983), is the same as that in YBd5′-22AL, that of pBR322 from the *Bam*HI site at position 375 rightward, as pBR322 is usually represented (Sutcliffe, 1978). This sequence is much richer in G-C base pairs than the sequence in the unmodified form, pJD137. It would appear that the vector sequence replacing yeast DNA does indeed influence transcriptional efficiency since this data corroborates previous findings in this laboratory. Schaack *et al.* (1984) have also observed a negative effect on transcription from this sequence. Vertebrate systems, however, are much less sensitive to 5′-flanking sequence deletions and replacement by the aforementioned DNA (Sharp *et al.*, 1981; Dingermann *et al.*, 1982; Sharp *et al.*, 1982; Johnson and Raymond, 1984).

In vivo analysis of $tRNA^{Leu}_{3A}$ synthesis

Effective suppression is determined by several factors, including (i) the position of the suppressible codon, (ii) the ability of the amino acid inserted to produce a functional polypeptide, (iii) the level of expression of the suppressor tRNA, and (iv) the cellular demand for the polypeptide. To determine the acceptability of leucine insertion for functional suppression at each of the three different amber loci, transformations were performed with clones containing the naturally occurring suppressor, SUP53. The acceptability is different at each locus (Tables I and II), but SUP53 is able to suppress, at least partially, each mutation.

The yeast strains contain the amber mutations lys2-801, met8-1 and tyr7-1. Upon transformation with the yeast-vector clones containing SUP53, suppression is much more effective at the met8-1 locus than at either of the other two (Tables I and II). This elevated efficiency of suppression is probably responsible for the complete suppression of the met8-1 mutation by all forms of the $tRNA^{Leu}_{3A}$ gene. Suppression of the lys2-801 and tyr7-1 mutations in the yeast host strain parallels the template activities *in*

Table I. Transformation of the yeast strain YNN 217

DNA used for transformation	No. of colonies appearing on selective SD plates		Suppressor activity
	−URA	−LYS	(lys2-801)
0 DNA	0	2	–
YCp19	620	10	–
YR137L	2000	9	–
YR137AL	1200	237	19.8
YR137AR	860	85	9.9
YRSUP53L	800	309	38.6
YRd5′-22AR	1000	112	11.2
YRd5′-22AL	700	98	13
YRd5′-2AR	67	35	4.5
YRd5′-2AL	1100	24	1.5
YB137AL	1400	87	5.5
YB137AR	1100	105	9
YBSUP53R	400	59	14
YBd5′-22AR	1860	172	8.5
YBd5′-22AL	1370	11	0
YBd5′-2AR	970	26	2
YBd5′-2AL	860	24	2

Transformations were performed as described in Materials and methods and the number of colonies appearing on each plate was counted after incubation at 30°C for 48 h.
Suppressor activity = No. of colonies on −LYS plates/No. of colonies on −URA plates (100).

Table II. Transformation of the yeast strain SL797-1A

DNA used for transformation	No. of colonies appearing on selective SD plates			Suppressor activity	
				(met8-1)	(tyr7-1)
	−URA	−MET	−TP		
0 DNA	0	22	5	–	–
YCp19	260	11	3	–	–
YR137L	247	14	3	–	–
YR137AL	540	570	260	100	47
YR137AR	421	520	95	119	22
YRSUP53L	500	454	350	86	69
YRd5′-22AR	525	570	200	104	37
YRd5′-22AL	470	550	220	112	46
YRd5′-2AR	515	525	70	97	13
YRd5′-2AL	480	418	44	82	8
YB137AL	365	452	145	119	39
YB137AR	320	255	110	75	33
YBSUP53R	285	334	188	112	65
YBd5′-22AR	505	545	235	103	46
YBd5′-22AL	395	340	13	81	2
YBd5′-2AR	250	274	32	105	12
YBd5′-2AL	432	465	26	103	5

Transformations were performed as described in Materials and methods and the number of colonies appearing on each plate was counted after incubation at 30°C for 48 h.

vitro. The correlation between *in vitro* template activity and suppressor activity *in vivo* is 0.79 at these loci (Figure 4). The suppression is specific for amber codons and tightly linked to a plasmid-borne URA3 gene. We conclude that the suppression is plasmid borne and that the pentadecanucleotide sequence modulates the expression of yeast $tDNA_3^{Leu}$ *in vivo* as well as *in vitro*.

Yeast strains transformed with subclones of $tDNA_3^{Leu}$ in YCp19 were also selected for URA3 gene complementation. Those clones containing the amber suppressor form of $tDNA_3^{Leu}$ should concomitantly acquire complementation. To show that

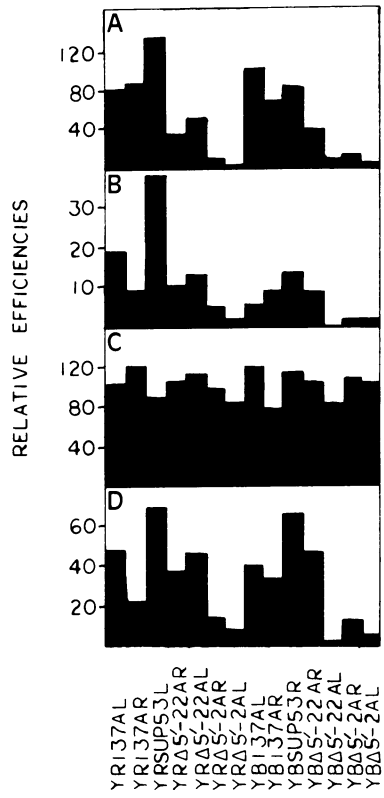

Fig. 4. Relative amounts of RNA transcript produced *in vitro* compared with suppressor activity *in vivo*. **Panel A** represents the relative amounts of RNA produced from each plasmid *in vitro* as shown in Figure 3B. The suppressor activity of the tDNA clones with each locus tested was calculated as described in Materials and methods. **Panels B−D** are graphical representations of the numberical data in Tables II and III. **Panel B** represents suppressor activity at the lys2-801 locus in the yeast strain YNN 217. **Panels C** and **D** represent suppression at the met8-1 and tyr7-1 loci, respectively, both in the yeast strain SL797-1A. The plasmid being examined is indicated on the abscissa. Correlation coefficients for the *in vitro* transcription and suppression are 0.79 for lys2-801, 0.35 for met8-1 and 0.79 for tyr7-1.

gene complementation and amber suppression were acquired coordinately, cells were selected for either URA^+ or SUP^+ phenotypes then replated onto media requiring both gene complementation and suppression. The number of colonies capable of expressing the unselected character was determined. Table III presents the results of replating transformed YNN 217 cells. When colonies were transferred from the gene complementation (SD-ura) plates, in all but one case, 98 − 100% were capable of growth on the SD-ura/lys plates. YBd5′-22AL produced only 82% capable of expressing both traits. When cells were first selected for suppression (SD-lys plates), both traits were not always expressed in a second screening. This can be explained by the number of colonies (2 − 10) that appear as apparent revertants on lysine selective plates when cells were transformed with either YCp19 or YR137L, neither of which are capable of suppression (Table I). The number of apparent revertants becomes significant only when a small number of colonies could be tested from the SD-lys plates (Table III). Overall, the results indicate suppression is tightly linked to gene complementation and therefore is due to transformation.

The ochre mutation in YNN 217 cells, ade2-101, was used to show that suppression by $tRNA_{3A}^{Leu}$ was specific for amber codons. No transformants capable of suppressing the ochre muta-

Table III. Characteristics of transformed YNN 217 cells

DNA used for transformation	No. of colonies on SD −URA/LYS plates[a]		No. of colonies on SD −URA/ADE plates[b]	
	From −URA plates	From −LYS plates	From −URA plates	From −LYS plates
YCp19	0/50	0/10	0/10	0/10
YR137L	0/50	0/10	0/10	0/10
YR137AL	50/50	48/50	0/10	0/10
YR137AR	50/50	45/50	0/10	0/10
YRSUP53L	50/50	46/50	0/10	0/10
YRd5′-22AR	49/50	46/50	0/10	0/10
YRd5′-22AL	50/50	45/50	0/10	0/10
YRd5′-2AR	50/50	32/35	0/10	0/10
YRd5′-2AL	50/50	22/26	0/10	0/10
YB137AL	50/50	30/50	0/10	0/10
YB137AR	50/50	46/50	0/10	0/10
YBSUP53R	50/50	49/50	0/10	0/10
YBd5′-22AR	50/50	50/50	0/10	0/10
YBd5′-22AL	41/50	6/10	0/10	0/10
YBd5′-2AR	49/50	22/26	0/10	0/10
YBd5′-2AL	50/50	20/24	0/10	0/10

Colonies were transferred from primary selection plates onto media requiring both gene complementation and suppression. Scoring occurred after incubation at 30°C for 72 h.
[a]Those colonies showing gene complementation and suppression at the amber locus (lys2-801).
[b]Those colonies showing gene complementation and suppression at the ochre locus (ade2-101).

tion were found (Table III). The $tRNA^{Leu}_{3A}$ is therefore specific for the amber locus.

Transformed SL797-1A cells were also replated to test for linkage of the URA3 gene and amber suppression. The analysis shows (Table IV) that 95 − 100% of the colonies plated on SD-ura/met plates from either complementation (SD-ura) or suppression (SD-met) plates were capable of expressing both traits. The coupling of complementation and suppression is not as apparent when the results of replating from uracil selection to SD-ura/tp plates are examined. Although 95 − 100% of the colonies from suppression selection plates were capable of simultaneously expressing gene complementation, the reciprocal test showed only 15 − 50% of cells selected on SD-ura plates were able to suppress tyr7-1. The apparent reduction in suppressor activity is not due to any loss of $tDNA^{Leu}_{3A}$, as these same transformants were completely competent in suppressing the met8-1 mutation. Because the SUP53 fragment, subcloned at the *Eco*RI or *Bam*HI site, is more efficient at suppressing the methionine than the tyrosine locus (Table II), it seems most probable that the $tRNA^{Leu}_{3A}$ is not as efficient at suppressing the tyr7-1 locus as the met8-1 locus.

The transformed SL797-1A colonies were also tested for suppression of an ochre mutation, leu2-1 (Table IV). As with the ade2-101 locus in YNN 217, no suppression at the ochre locus was observed. This result, in conjunction with the cells ability to simultaneously express gene complementation and amber suppression, can again be attributed to the level of expression of the various forms of $tDNA^{Leu}_{3A}$ present in the plasmid.

The *in vitro* transcription of these same clones (Figure 3B) yielded a direct measurement of relative levels of expression. When the *in vitro* activities were compared with suppressor capacity observed *in vivo*, correlation coefficients of 0.79 were observed for lys2-801 and tyr7-1 and 0.35 for met8-1 (Figure 4).

Table IV. Characteristics of transformed SL797-1A cells

DNA used for transformation	No. of colonies on selective SD plates					
	−URA/MET[a]		−URA/TP[b]		−URA/LEU[c]	
	From −URA	From −MET	From −URA	From −TP	From −MET	From −TP
YCp19	0/20	0/20	0/20	0/3	0/10	0/3
YR137AR	20/20	20/20	10/20	19/20	0/10	0/10
YRSUP53L	20/20	20/20	5/20	20/20	0/10	0/10
YRd5′-22AR	20/20	19/20	6/20	20/20	0/10	0/10
YRd5′-2AR	20/20	19/20	3/20	20/20	0/10	0/10

Colonies were transferred from primary selection plates onto media requiring both gene complementation and suppression. Scoring occurred after incubation at 30°C for 72 h.
[a]Those colonies showing gene complementation and suppression at the amber locus (met8-1).
[b]Those colonies showing gene complementation and suppression at the amber locus (tyr7-1).
[c]Those colonies showing gene complementation and suppression at the ochre locus (leu2-1).

Discussion

Suppression has been observed previously from plasmid-borne tDNAs. Johnson and Abelson (1983) reported suppression from plasmids carrying the $tRNA^{Tyr}$ (SUP6) gene. Two types of vectors were used, a high copy number plasmid and an integrating plasmid. They observed that a reduced suppressor efficiency was compensated by the effect of high gene dosage. Fischhoff *et al.* (1984) used the yeast vector YEp13 which contains a yeast $tDNA^{Leu}_{3}$ and characterized a variant designated YEp13-a. This variant was capable of suppressing a variety of amber mutations *in vivo*. When this isolate was characterized by DNA sequence analysis and compared with YEp13, a single base change had occurred in the anticodon region creating the amber suppressor form of the gene, $tDNA^{Leu}_{3A}$. Hottinger *et al.* (1984) observed ochre suppression by the *Schizosaccharomyces pombe sup3-i* suppressor both *in vivo* and *in vitro*. *S. cerevisiae* strains transformed with plasmid DNA carrying the *S. pombe sup3-i* gene, exhibited ochre, but not amber or UGA suppression *in vivo*.

Regulation or modulation of gene expression is often conveniently measured in cell-free extracts or in oocyte injection experiments. These systems often suffer from perturbations of the normal relationships between gene copy number and any putative regulatory macromolecules. One can estimate that injection of a *Xenopus* oocyte with as little as 1 ng of a plasmid-borne gene introduces $10^7 - 10^8$ copies of a specific DNA fragment into a single cell. Clearly, specific regulatory systems could easily be overwhelmed. In cell-free extracts, equivalence relationships are difficult to ascertain quantitatively.

Biosynthesis of a functional tRNA also requires a number of additional processes including IVS splicing, end-maturation, 3′-CCA_{OH} addition, and base modifications. *In vitro* systems do not always address all of these processes, any of which could be affected *in vivo* by alterations in the transcription unit. For these reasons, a sensitive *in vivo* assay was developed, initially to examine the role of the 5′-flanking sequence of yeast $tDNA^{Leu}_{3A}$ in the production of a biologically active tRNA.

The system reported here measures the biological activity of the various forms of the gene, but does not determine which aspect of tRNA biosynthesis is affected. Since the *in vivo* and *in vitro* results correlate strongly, it appears that the pentadecanucleotide sequence is active *in vivo* in modulating the level of $tRNA^{Leu}_{3}$. The sequence is not required for biological activi-

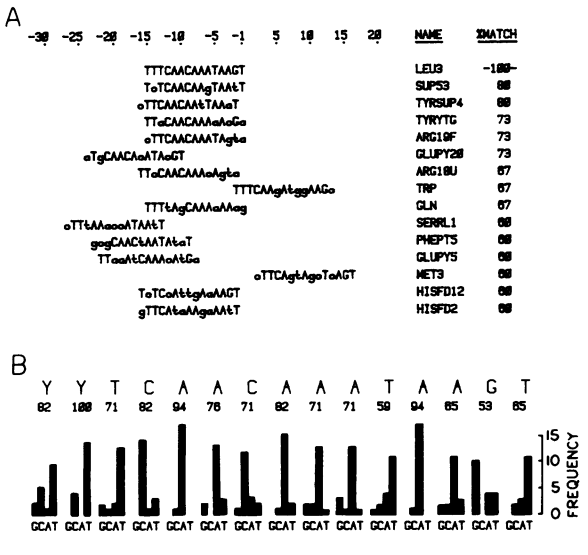

Fig. 5. 5′-Flanking sequence homology among different yeast tRNA genes. DNA sequences of different tRNA genes cloned from *S. cerevisiae* genomic DNA (Sprinzl and Gaus, 1984) were searched to position −50 in the 5′-flanking region, when possible, for the presence of the conserved sequence identified in $tDNA_3^{Leu}$ genes, TTTCAACAAATAAGT. **Panel A** shows, for each gene, the pentadecanucleotide sequence with greatest homology identified using the search program of Larson and Messing (1983). The bases which correspond to the $tDNA_3^{Leu}$ sequence are capitalized, mismatches are in lower case. The position of the sequence relative to the coding sequence is depicted and the genes ordered by percent match. The base sequence of all the sequences identified in **panel A** including three different $tDNA^{Leu}$ clones, was aligned and the frequency of appearance of each base at each position was plotted. **Panel B** represents that information and identifies positions where the DNA sequence is most highly conserved. The two 5′ positions are most frequently occupied by either T or C. The bases adjacent to the 3′-distal base are not as highly conserved as the remainder of the sequence. The number under each base in **panel B** is the percent occurrence of that base at that position.

ty, as seen in the d5′-2A clones, but increases the level of expression when present, as seen in the d5′-22A clones. Residual activity in genes with altered 5′-flanking sequence may be the reason Kurjan *et al.* (1980) failed to isolate any SUP^- mutants mapping in the flanking sequences of a $tRNA^{TYR}_{SUP4}$ gene. The d5′-22A clones never reach the level of expression of clones containing additional yeast 5′-flanking sequence. This suggests that bases further upstream from −22 are also involved in the modulation of transcription. This is similar to the finding of Schaack *et al.* (1984) that the 5′-flanking sequence dependence of a *Drosophila* $tDNA^{Arg}$ extends to position −33. Larson *et al.* (1983) have identified a control region extending to position −34 in the *B. mori* $tDNA^{Ala}$ which is required for transcription.

DNA sequences referred to as sigma and delta have been found adjacent to tRNA genes of yeast (Del Ray *et al.*, 1982; Eigel and Feldmann, 1982). The 340-bp sigma element is repeated many times in yeast and, when found adjacent to a tDNA, is always located either 16 or 18 bp to the 5′ side of the mature tRNA coding sequence. The position of the 340-bp delta sequence relative to tDNA is not as precise. Both of these elements are hypothesized to have a role in transcriptional regulation of the adjacent tDNA and share a common sequence, -CAACA-, found very near their ends. This same sequence is part of the conserved $tDNA_3^{Leu}$ pentadecanucleotide.

DNA sequences from other *S. cerevisiae* tRNA genes have been searched for the presence of the conserved 5′-flanking sequences identified in $tDNA_3^{Leu}$, TTTCAACAAATAAGT (Figure 5A). The pentadecanucleotide sequence with the greatest homology was identified and its position relative to the coding sequence is depicted. The bases which match the sequence from $tDNA_3^{Leu}$ are capitalized and the mismatches are shown in small case. Several different *S. cerevisiae* tRNA genes have 5′-flanking regions very similar to that in $tDNA_3^{Leu}$, in both position and sequence. The percent match with the $tDNA_3^{Leu}$ flanking sequence was calculated for each gene. The base sequences from all tRNA genes having >60% match with the pentadecanucleotide in yeast $tDNA_3^{Leu}$, were aligned and the frequency of each base at each position plotted (Figure 5B). The base appearing most frequently at each position was used to derive a consensus sequence, YYTCAACAAATAAGT (Y= Pyrimidine).

Whether this sequence is involved in modulating transcription in other tRNA genes is not known. The $tRNA^{Leu}$, $tRNA^{Tyr}$, $tRNA^{Arg}$ and $tRNA^{Glu}$ genes which show the best fit to the consensus sequence all code for tRNAs which are abundant in *S. cerevisiae* (Ikemura and Ozeki, 1983). It is possible that this sequence may be one mechanism by which yeast cells adjust tRNA biosynthesis to match demand created by codon use preferences. This could be further tested by fusing this conserved pentadecanucleotide sequence to tRNA genes that do not have it to determine whether any activation takes place. The $tRNA^{Arg}$-$tRNA^{Asp}$ gene pair from *S. cerevisiae* is transcribed as a dimeric precursor in a homologous extract (Kjellin-Straby *et al.*, 1983). Synthesis of this precursor is not dependent on the internal promoter of the second gene and the second gene is not independently transcribed when the normal upstream initiation site is removed. The conserved pentadecanucleotide sequence is found immediately adjacent to the $tRNA^{Arg}$ gene, but not in front of the second gene, $tDNA^{Asp}$. Both genes in this case appear to be modulated by the one pentadecanucleotide sequence.

The 3′-flanking region of a *B. mori* $tRNA^{Ala}$ gene has been shown to be required for full transcriptional activity (Wilson *et al.*, 1985). Use of high template DNA concentrations to overcome the effects of an inhibitor present in extracts was found to be partly responsible for masking the contribution of this region. They suggest that this inhibitory effect is present in extracts from many organisms and that the 3′-flanking requirements can be generalized to tRNA genes in other organisms as well. The template concentration used in this study, 1.1×10^{-9} M, is in the range reported by these authors for high sensitivity. We have not investigated the effect of 3′-flanking sequence on the transcription of this gene.

The *in vivo* assay system reported here can be used to further study DNA signals necessary for transcription and processing of biologically active tRNA by introducing other alterations and observing the effects on suppression or accumulation of transcripts. We are presently mapping point mutants in the 5′-flanking sequence to identify positions responsible for the positive modulation of transcription. An *in vitro* system is an excellent tool for rapidly assaying the results of gene alterations and purifying components. It is encouraging that, in this study, *in vitro* results accurately reflect *in vivo* processes.

Materials and methods

Plasmids

The yeast $tDNA_3^{Leu}$ clones pJD137 and d5′-2 used in this study have been previously described (Johnson *et al.*, 1980; Raymond and Johnson, 1983). The plasmid pYLEU2SUP53 (Newman *et al.*, 1983) containing the naturally occurring amber suppressor form of yeast $tDNA_3^{Leu}$, SUP53, was generously provided by A. Newman. R. Davis kindly provided YCp19.

Phage and bacterial strains

Bacteriophage M13mp9 replicative form (RF) DNA, *E. coli* JM103, and the M13 Cloning/Sequencing Kit were from Pharmacia P-L Biochemicals. m9137 is bacteriophage M13mp9 with a 900-bp yeast fragment containing 350 bp of yeast 5'-flanking sequence, $tDNA_3^{Leu}$, and 440 bp of 3'-flanking sequence cloned between the *Eco*RI and *Bam*HI sites. m9d5'-2 is a comparable clone containing only 2 bp of 5'-flanking sequence, $tDNA_3^{Leu}$, and 170 bp of 3'-flanking sequence.

Yeast strains

The yeast strain YNN 217 [*a*, his3-200, lys2-801 (amber), ade2-101 (ochre), ura3-52, suc-2] was a generous gift from R. Gesteland. The strain SL797-1A [*a*, met8-1 (amber), leu2-1 (ochre), tyr7-1 (amber), ilv1-1, lys2-1, ura3-52, his (his5-2, 3-1, or 4-300)] was a generous gift from S. Liebman.

Enzymes and chemicals

Restriction endonucleases, *E. coli* DNA polymerase I (Klenow fragment), T4 DNA ligase and T4 polynucleotide kinase were purchased from either New England Biolabs or Bethesda Research Laboratories (BRL) and were used as recommended by the supplier. [γ-^{32}P]ATP (5000 Ci/mmol) was from ICN Radiochemicals. [α-^{32}P]GTP (700 Ci/mmol) and [^{35}S]dATPαS (500 Ci/mmol) were from New England Nuclear. *Bam*HI/*Eco*RI linker-adapters were purchased from Collaborative Research, Inc.

Site-directed mutagenesis

The primer, a 47-bp fragment corresponding to the central region of $tRNA_{3A}^{Leu}$, was purified from a *Hae*III/*Hin*cII digestion of the plasmid pYLEU2SUP53 by polyacrylamide gel electrophoresis then dissolved in H_2O at ~0.25 pmol/μl. Single-stranded DNA isolated from viral stocks of m9137 and m9d5'-2 was purified by alkaline sucrose-gradient sedimentation (Goulian *et al.*, 1967) and used as template. The procedure used was a modification of that described by Wallace *et al.* (1980). 1 pmol of primer was denatured by heating in boiling water for 5 min then immediately put into an ice-water bath. Template (0.2 pmol) was added and the solution brought to 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), in a total volume of 10 μl. This was incubated at 55°C for 10 min then put at 22°C for 30 min to allow the template and primer to anneal. Primer extension was done in a 50 μl reaction mixture containing template-primer, 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM ATP, 250 μM each dGTP, dATP, dTTP and dCTP, 4 U of *E. coli* DNA polymerase I (Klenow fragment), and 2 U of T4 DNA ligase. The reaction was allowed to proceed for 12 h at 12°C.

Phage from plaques generated by transfection of *E. coli* JM103 with aliquots of the reaction mixture were used to infect 5 ml cultures of exponentially growing *E. coli* JM103 cells. RF DNA was isolated from these cultures (Birnboim and Doly, 1979) and screened by digestion with the restriction endonuclease *Xba*I. The T-A transversion fortuitously gives rise to an *Xba*I restriction site in the anticodon region of the gene. Phage from clones containing the new *Xba*I restriction site were plaque purified, RF DNA re-isolated and screened as described to ensure homogeneity of the clones. Dideoxynucleotide DNA sequence analysis of m9d5'-2 and m9d5'-2A using [^{35}S]dATPαS (Biggin *et al.*, 1983) was done to confirm the mutation and verify that no other changes had been introduced.

Cloning into the yeast vector YCp19

pJD137 was the source of an *Eco*RI fragment, 137, with 2.5 kbp of yeast DNA, containing an unmodified $tDNA_3^{Leu}$. An *Eco*RI plus *Bam*HI digestion of m9137A RF DNA released the 900-bp fragment, 137A. A 900-bp fragment SUP53, containing a naturally occurring yeast $tRNA_{3A}^{Leu}$, was obtained from an *Alu*I digestion of the plasmid pYLEU2SUP53. A 310-bp *Fnu*DII fragment, d5'-22A, with 22 bp of 5'-flanking sequence, was from m9137A RF DNA. A 290-bp fragment, d5'-2A, with 2 bp of 5'-flanking sequence, was from an *Eco*RI plus *Bam*HI digestion of m9d5'-2A RF DNA. The restriction endonuclease fragments were isolated by electrophoresis on 7% polyacrylamide gels in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3), except the 2.5-kbp fragment which was electrophoresed on a 1% agarose gel in E buffer (40 mM Tris, 20 mM NaOAc, pH 7.9). The ends of the fragments 137A and d5'-2A were modified by ligation of linker-adapters as recommended by the supplier (Collaborative Research, Inc.) to produce compatible ends for cloning. The fragments with linker-adapters were then digested with either *Bam*HI or *Eco*RI to produce fragments with identical ends compatible with either the *Bam*HI or *Eco*RI cloning sites. These fragments were then inserted into YCp19.

The plasmid (20 μg) was digested with 30 U of either *Bam*HI or *Eco*RI and 10 μg of DNA from each digestion was removed and the 3'-recessed ends extended to produce blunt-ends. The fill-in reaction [10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 10 mM BME, 2.5 mM each of the dNTPs, with 0.5 U of *E. coli* DNA polymerase I (Klenow fragment)] was incubated at 22°C for 30 min.

Fragments were subcloned into YCp19 at each of the two cloning sites, with the exception of the 2.5-kbp fragment, 137, which was inserted only into the *Eco*RI site. The SUP53 and d5'-22A fragments which have flush ends from restriction endonuclease cleavage were ligated to vector molecules with ends that had been filled in. All ligation reactions had 0.1 pmol vector and 0.5 pmol fragment, in 20 mM Tris-HCl (pH 7.4), 7.5 mM $MgCl_2$, 0.1 mM EDTA, 500 μM ATP with 2 U of T4 DNA ligase in a volume of 20 μl. The reactions were incubated at 22°C for 2 h to facilitate formation of linear fragment-vector dimers, then were diluted to 100 μl maintaining the same buffer conditions and incubated for four more hours to enhance circularization of the chimeras. Half of each ligation reaction was used to transform *E. coli* C600SF8. Ampicillin-resistant colonies were screened by colony hybridization (Grunstein and Hogness, 1975) using the ^{32}P-phosphorylated *Hae*III/*Hin*cII 47-bp fragment from pYLEU2SUP53 to identify clones containing $tDNA_{3A}^{Leu}$. Plasmid DNA from hybridization-positive colonies was analyzed with the restriction endonuclease *Xba*I to verify the identity of clones and determine the orientation of the gene within the plasmid.

In vitro transcription

Whole-cell extracts from *S. cerevisiae* strain 20B-12 were prepared according to the method of D. Engelke (personal communication). Transcriptions, performed with 22 fmol of plasmid DNA in a 25 μl reaction at 140 mM NaCl and electrophoretic separation of products were performed as described (Raymond and Johnson, 1983). Radioactive bands were visualized by fluorography for 16 h using Dupont Cronex film and Dupont Cronex Lightning-Plus CD intensifier screens.

The amount of radioactivity incorporated into RNA was determined by cutting the radioactive bands from the gel, adding 2 ml of Biofluor, and counting in a Beckman liquid scintillation counter for 10 min. The data was then normalized by comparing the level of transcription of mutants of $tDNA_3^{Leu}$ with that of the unmodified gene and reporting the data as a percent value.

In vivo analysis of $tRNA_{3A}^{Leu}$ synthesis

The two different *S. cerevisiae* strains used for the transformations, YNN 217 and SL797-1A were grown in YPD media (1% bacto-yeast extract, 2% each bacto-peptone and dextrose) until they reached an optical density of 2.0 at 590 nm. Transformations were performed following the method of Ito *et al.* (1983) using 5 μg of recombinant plasmid DNA (in 20 μl volume), 10 μg of sonicated calf thymus DNA and 100 μl of competent cells. The final cell pellets were resuspended in 500 μl YPD broth and incubated at 30°C with agitation for 30 min. Aliquots of the transformation mixtures were plated onto synthetic dextrose (SD) plates (0.67% bacto-yeast nitrogen base without amino acids, 2% dextrose, and 2% bacto-agar) supplemented with appropriate nutrients to screen for complementation by the *URA3* gene or amber suppression at one of the three genetic loci. The plates were incubated at 30°C for 48 – 72 h before colony counts were done.

Suppressor activity was measured by comparing the number of colonies that showed suppression with the number that showed complementation. To show that suppression and gene complementation were occurring together when the plasmid used for transformation carried a form of $tDNA_{3A}^{Leu}$, colonies selected from complementation plates (SD-ura) were transferred to SD plates supplemented such that a cell could only grow if it expressed both the *URA3* gene product and the $tDNA_{3A}^{Leu}$. Colonies were also moved from transformation plates where selection was for suppression of an amber mutation onto plates requiring both traits for growth.

To determine whether suppression was specific for amber codons, the transformed cells were transferred onto plates which selected for complementation of the URA3 mutation and suppression of an ochre mutation.

Acknowledgements

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