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Effects of alterations in the 3′ flanking sequence on *in vivo* and *in vitro* expression of the yeast *SUP4*-o tRNA^{Tyr} gene

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The *SUP4*-o gene of *Saccharomyces cerevisiae* codes for an altered tRNA^{Tyr} capable of suppressing ochre mutations. We constructed mutant *SUP4*-o genes with deletions in the 3′-flanking sequence and tested each for its ability to suppress ochre mutations in transformed yeast cells. The effects of the different 3′ deletions on various aspects of *in vitro* transcription and RNA processing were also determined, using a yeast cell-free extract. Deletions that leave five or fewer consecutive T residues in the 3′-flanking sequence of *SUP4*-o were found to result in decreased efficiency of transcription termination, both *in vitro* and *in vivo*. Unexpectedly, the suppression strength of each mutant *SUP4*-o gene is highly correlated with the relative extent of transcription termination at the 3′ end of the gene. This result indicates that *SUP4*-o readthrough transcripts are not efficiently processed to functional suppressor tRNA in yeast cells. Deletions that extend into the T cluster in the 3′-flanking sequence also significantly decrease the ability of *SUP4*-o to compete for a transcription factor that is limiting in our extracts. This latter finding implies that the 3′-flanking sequence of *SUP4* plays a role in transcription factor binding.

Key words: yeast tRNA/transcription/RNA processing

Introduction

Transcription of eucaryotic tRNA genes by RNA polymerase III (PolIII) is usually dependent on the presence of two internal control regions, termed the A block (positions 8–19) and B block (positions 52–62) (Hofstetter *et al.*, 1981; Sharp *et al.*, 1981; Galli *et al.*, 1981; Ciliberto *et al.*, 1982; Allison *et al.*, 1983). In addition, the level of transcription of tRNA genes can be regulated by the 5′-flanking sequence (DeFranco *et al.*, 1981; Hipskind and Clarkson, 1983; Larson *et al.*, 1983; Shaw and Olson, 1984). This study is concerned with the role of 3′-flanking regions in the synthesis of eucaryotic tRNA. Transcription by PolIII has been shown to terminate within stretches of consecutive T residues located in the 3′-flanking sequences of class III genes (Koski *et al.*, 1980; Bogenhagen and Brown, 1981; Hipskind and Clarkson, 1983). A cluster of T residues is usually present within the first 20 bp following a tRNA gene, thereby giving rise to precursor molecules with relatively short 3′ trailer sequences that must be removed during processing. However, experiments with several different vertebrate tRNA genes lacking normal termination signals have shown that the long readthrough transcripts made are processed to tRNA-size molecules in *Xenopus* oocytes (Hofstetter *et al.*, 1981; Galli *et al.*, 1981; Adeniyi-Jones *et al.*, 1984). It is thus unclear to what extent synthesis of functional cytoplasmic tRNA is dependent on termination of transcription at a site near the 3′ terminus of the gene.

In addition to controlling termination, the 3′-flanking sequence of tRNA genes may also be involved in binding proteins required for transcription initiation. DNA footprinting experiments have shown that one or more yeast proteins protect regions containing the A and B blocks of tRNA genes (Klemenz *et al.*, 1982; Camier *et al.*, 1985). The 3′ border of the protected region was found in these cases to extend into or beyond the T cluster located next to each gene in the 3′-flanking sequence. Recently, dimethyl sulfate protection assays were used to show that a highly purified transcription factor from yeast (τ factor) makes contact with G residues at the extreme 3′ terminus of a yeast tRNA^{Glu} gene, as well as with G residues within the A and B blocks of this gene (Camier *et al.*, 1985).

The dominant phenotype of the yeast *SUP4*-o gene provides a functional *in vivo* assay for the effects of alterations in the flanking sequences of this tRNA gene. Previously, we found that the normal 5′-flanking sequence of *SUP4*-o is not essential for high levels of suppression *in vivo* or transcription *in vitro* (Koski *et al.*, 1982; Allison, 1984). Here, we describe experiments in which mutant *SUP4*-o genes with deletions in the 3′-flanking sequence were assayed semi-quantitatively for their ability to suppress various ochre mutations in transformed yeast cells. The altered *SUP4*-o genes were also tested in a yeast cell-free extract in order to determine transcription levels, termination efficiency, the ability to compete for transcription factor and the processing of readthrough transcripts.

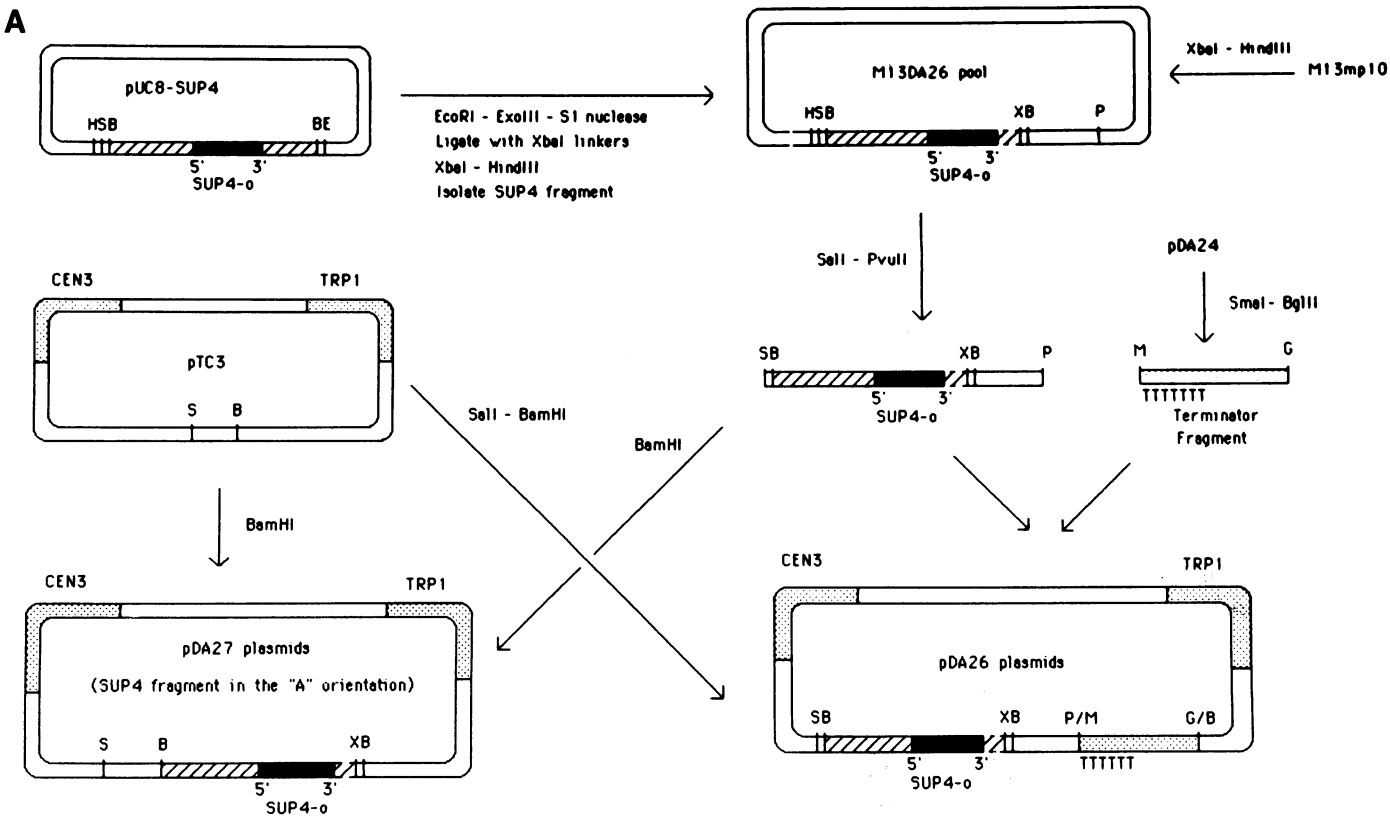
Results

Construction of 3′ deletion mutants of SUP4-o in a yeast vector

The construction of 3′ deletion mutants of *SUP4*-o is diagrammed in Figure 1. Mutant genes with deletions to +53, +7, +5, +4, +1 or 68, were chosen for study, where '+1' refers to the first base pair of 3′-flanking sequence (Figure 1B). A *SalI*/*PvuII* fragment containing each of the six mutant genes was ligated into the yeast vector pTC3 (Shaw and Olson, 1984), together with a *SmaI*/*BglII* yeast DNA fragment containing a PolIII termination sequence. The *SmaI*/*BglII* fragment was ligated on the 3′ side of each 3′-deleted *SUP4* gene to provide a back-up terminator for those mutant genes for which transcription fails to terminate at the 3′ terminus of *SUP4* due to deletion of the normal T cluster. The resulting six *SUP4*-o plasmids (the pDA26 series) contain the yeast sequences *TRP1*, *ARS1* and *CEN3*, which allow for stable maintenance of each plasmid in transformed yeast cells at a copy number of 1–2 per cell (Botstein and Davis, 1982; Carbon, 1984).

Expression in vivo

Because the 3′ deletion mutants were constructed using the ochre-suppressing allele of *SUP4*, their expression *in vivo* can be assayed by measuring the ability of each mutant *SUP4* gene to suppress various ochre alleles in transformed yeast cells. The *Saccharomyces cerevisiae* strain D12-18C was constructed for



B

SUP4 CODING SEQUENCE

B BLOCK

PLASMID	DELETION ENDPOINT	50 60 70 +1 +10 +20 PvuII/SmaI
pDA26-142	+53	...GGCGTTCGACTCGCCCCCGGGAGATTTTTTTGTTTTTTATGTCTCCATT...236 BP...CAGGGGAGATTTTTTT...
		XbaI BamHI
pDA26-96	+7	...GGCGTTCGACTCGCCCCCGGGAGATTTTTTTCTCTAGAGGATCCCCGGG...190 BP...CAGGGGAGATTTTTTT...
		XbaI BamHI
pDA26-94	+5	...GGCGTTCGACTCGCCCCCGGGAGATTTTTCTCTAGAGGATCCCCGGGCG...188 BP...CAGGGGAGATTTTTTT...
		XbaI BamHI
pDA26-93	+4	...GGCGTTCGACTCGCCCCCGGGAGATTTTCTCTAGAGGATCCCCGGGCGA...187 BP...CAGGGGAGATTTTTTT...
		XbaI BamHI
pDA26-90	+1	...GGCGTTCGACTCGCCCCCGGGAGATCTCTAGAGGATCCCCGGGCGAGCT...184 BP...CAGGGGAGATTTTTTT...
		XbaI BamHI
pDA26-84	68	...GGCGTTCGACTCGCCCCCGCTCTAGAGGATCCCCGGGCGAGCTCGAATT...178 BP...CAGGGGAGATTTTTTT...

POLYLINKER/LACZ DNA — TERMINATOR FRAGMENT

Fig. 1. Construction and structure of plasmids containing *SUP*4-o genes with 3′ deletions. **(A)** Plasmids are not drawn to scale. Solid areas represent *SUP*4-o coding sequence and diagonal lines indicate normal *SUP*4 flanking sequence. Stippled areas indicate other yeast DNA sequences and open regions show bacterial plasmid or M13 DNA. The *SUP*4 gene in pUC-SUP4 contains 114 bp of normal 5′-flanking sequence and 63 bp of normal 3′-flanking sequence. pUC8-SUP4 and pDA24 are described in Allison (1984). The yeast vector pTC3 is described in Shaw and Olson (1984). Each pDA27 plasmid is named in accordance with the corresponding 3′-deleted *SUP*4 gene in the pDA26 plasmid, and according to the orientation of the *SUP*4 gene in the pDA27 plasmid. For example, pDA27-90A contains the +1 deletion mutant of *SUP*4 (also present in pDA26-90) in the orientation such that transcription of *SUP*4 proceeds away from the unique *Sal*I site of this plasmid. pDA27-90B contains the +1 deletion mutant in the opposite orientation. Restriction sites are: H (*Hind*III), E (*Eco*RI), B (*Bam*HI), S (*Sal*I), X (*Xba*I), P (*Pvu*II), M (*Sma*I), and G (*Bgl*II). **(B)** The 3′-flanking sequence begins with +1. Arrows show the extent of each deletion, which were determined by DNA sequencing (Sanger *et al.*, 1977; Messing, 1983). The ~200-bp stretch of DNA between the deletion end point and the downstream T cluster is polylinker/*LacZ* sequence derived from the region between the *Xba*I and a *Pvu*II site in M13mp10 (Messing, 1983), as shown in **A**.

Table I. Suppression phenotypes of 3′ deletion mutants of *SUP4*-o in pDA26 plasmids

Plasmid	3′ Deletion end point	Growth of transformants on media lacking:			Color on YEPD	Suppression Index
		Ade	Lys	Met		
pBSU4	W.T.	+	+	+	white	5
pDA26-142	+53	+	+	+	white	5
pDA26-96	+7	+	+	+	white	5
pDA26-94	+5	+	+	+	light pink	4
pDA26-93	+4	−	−	+	red	1
pDA26-90	+1	−	−	−	red	0
pDA26-84	68	−	−	−	red	0

Eight yeast transformants of each plasmid were grown on media lacking tryptophan and replicated to plates lacking adenine, lysine or methionine, or to the rich media YEPD for color determination. Growth was scored after 2 days at 30°C. In most cases all eight transformants exhibited the same growth phenotype. The Suppression Index is determined in the following way: Ade^+, Lys^+, Met^+, white color (Suppression Index is 5); Ade^+, Lys^+, Met^+, light pink color (SI is 4); Ade^+, Lys^+, Met^+, dark pink color (SI is 3); Ade^-, Lys^+, Met^+ (SI is 2); Ade^-, Lys^-, Met^+ (SI is 1); Ade^-, Lys^-, Met^- (SI is 0).

this purpose and carries the three ochre alleles *ade*2-1, *lys*2-1 and *met*4-1, as well as a *trp*1 allele to permit selection of transformants. The *met*4-1 mutation is the most easily suppressed of the three ochre mutations, while *ade*2-1 requires the highest level of ochre-suppressing tRNA (Hawthorne and Leupold, 1974). The *ade*2-1 mutation is especially useful since a completely suppressed *ade*2-1 mutation will result in white colony color on YEPD medium, while decreasing levels of suppression will result in an increasingly darker red colony due to accumulation of a colored intermediate in the adenine biosynthetic pathway. The suppressor strength of each mutant *SUP*4 gene can be summarized by the Suppression Index, a number ranging from 0 to 5. A Suppression Index of '5' indicates the highest level of suppression, and '0' indicates no suppression by the plasmid (see legend to Table I).

Table I shows the results of testing each 3′-deleted *SUP*4 gene for its ability to suppress ochre alleles in yeast transformants. Deletions extending to position +53 (the '+53 deletion mutant') or to +7 do not noticeably alter the ability of *SUP*4-o to suppress, relative to an undeleted *SUP*4-o gene (Table I and legend). The +5 deletion mutant of *SUP*4 is slightly reduced in suppression strength, as indicated by a light pink color of the transformants. Deletion of one additional base pair, to position +4, results in a drastic reduction of suppression by *SUP*4 (Suppression Index is 1). Further deletion to position +1 completely abolishes suppression as measured by this assay, even though the +1 mutant contains the complete coding sequence for *SUP*4-o $tRNA^{Tyr}$ (Figure 1B and Table I). The weaker suppression by the +1 and +4 deletion mutants most likely results from a lowered *in vivo* level of suppressor $tRNA^{Tyr}$. To determine whether this decrease is brought about by defective transcription or lack of processing, several *in vitro* tests were carried out using the deleted *SUP*4 genes.

In vitro assays

In all of the *in vitro* transcription experiments described below, a whole-cell extract from *S. cerevisiae* was used as the source of RNA polymerase III and co-factors (Klekamp and Weil, 1982; Koski *et al.*, 1982). All of the 3′-deleted *SUP*4 genes tested are transcriptionally active (Figure 2) and give rise either to the normal 106 − 108 nucleotide precursor (pre-tRNA) or to an ~320 nucleotide transcript (RT in Figure 2). The 320-nucleotide RNA probably results from readthrough past the normal site of termination and subsequent termination within the T cluster in the back-up terminator (Figure 1). Termination at the normal site of *SUP*4 occurs efficiently upon transcription of either the +53 (100%) or the +7 deletion mutant (98%), and very inefficiently when using either the +4 deletion mutant (1%) or the +1 mutant (<1%) as template (Table II). The +5 deletion mutant is intermediate, with 38% of its transcripts being of normal size. We conclude from these results that either six or seven of the T residues in the T cluster which abut the *SUP*4 gene are necessary for efficient termination.

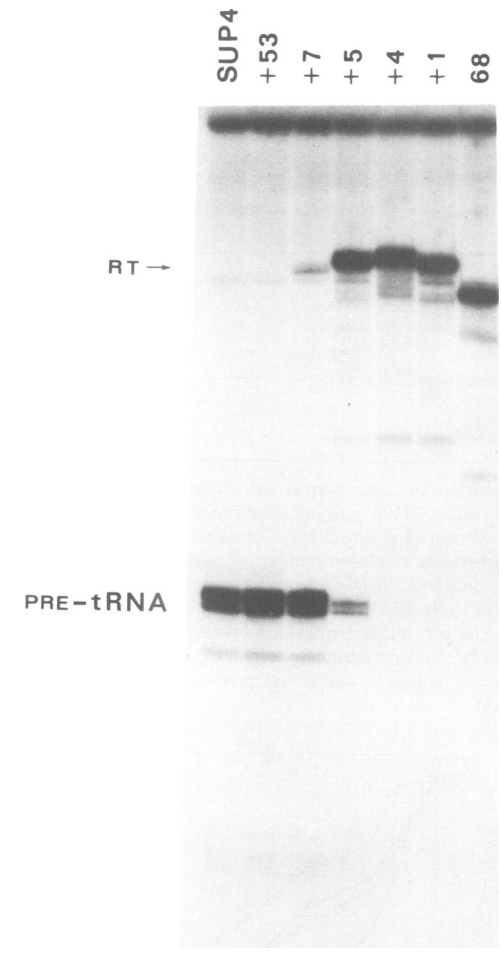


Fig. 2. *In vitro* transcription of *SUP*4 genes with 3′ deletions. Standard template activity assays were carried out as previously described (Allison *et al.*, 1983). Plasmid DNAs were at 1 μg/ml and [α-^{32}P]UTP was used to label the RNAs, which were electrophoresed through a 7 M urea, 10% polyacrylamide gel. The lane labeled *SUP*4 corresponds to the wild-type *SUP*4-o gene on pSU4-A (Goodman *et al.*, 1977). The remaining lanes are labeled according to the deletion end point of the *SUP*4 gene used in the reaction. RT indicates readthrough transcripts which probably result from termination within the T cluster shown on the right side of Figure 1B.

The molar amount of transcripts, including both pre-tRNA and readthrough RNA, decreases with increasing size of the deletion (Table II). These differences in transcript yield are not due to greater instability of the readthrough transcript relative to the normal *SUP*4 transcript, because *in vitro* pulse-chase experiments show that these two species of transcript are about equally stable *in vitro* (data not shown). The 2- to 3-fold decrease in transcript yield caused by the deletions to +4 or +1 is probably not suffi-

Table II. *In vitro* transcript yield and termination efficiency of 3′ deletion mutants of *SUP4*

Plasmid	3′ Deletion end point	Percent of transcript yield relative to that from undeleted *SUP4*-o			Percent termination within the
		Normal size transcript (pre-tRNA)	Readthrough transcript	Total	*SUP4* T cluster
pBSU4	W.T.	100	0	100	100
pDA26-142	+53	103	0	103	100
pDA26-96	+7	89	2	91	98
pDA26-94	+5	18	29	47	38
pDA26-93	+4	<1	40	40	1
pDA26-90	+1	0	31	31	0
pDA26-84	68	0	27	27	0

The relative number of either normal size or readthrough transcripts produced from each mutant *SUP4* gene was determined by first excising the bands from a gel as shown in Figure 2 and Cerenkov counting. The resultant c.p.m. is converted to a relative number of transcripts compared with that deriving from undeleted *SUP4*-o, by taking into account the number of uridine residues present in each type of RNA. Transcripts are labeled using [α-^{32}P]UTP. The percentage termination events within the *SUP4* T cluster was calculated by dividing the relative number of normal size transcripts made by the total number of transcripts produced (normal *SUP4* and readthrough RNAs).

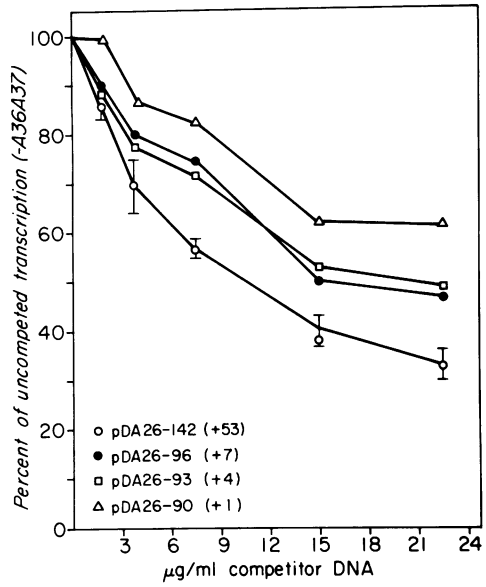


Fig. 3. Competition of -A36A37 transcription by *SUP4* plasmids with 3′ deletions. These competition assays were carried out exactly as previously described (Allison *et al.*, 1983). A given set of competition reactions contain a constant amount of the prematurely terminating *SUP4* mutant -A36A37 and variable amounts of competitor DNA (a given 3′-deleted *SUP4* gene). After electrophoresis of the reaction products on a polyacrylamide gel, the labeled -A36A37 RNAs are excised from each lane and Cerenkov counted. These values are then plotted as a function of the concentration of competitor DNA. The various competitor DNAs are indicated in the lower left corner. The error bars represent the wild-type *SUP4*-o competition curve, and are derived from three independent competition experiments using undeleted *SUP4* as a competitor against -A36A37 (Allison *et al.*, 1983).

cient to account for the severe reduction in suppression strength from these deletions. By way of comparison, we found previously that a 5′ deletion to a position 15 bp upstream from *SUP4*-o results in a 10-fold decrease in transcription *in vitro*, yet this mutant can suppress the *met*4-1 ochre mutation *in vivo* (Allison, 1984).

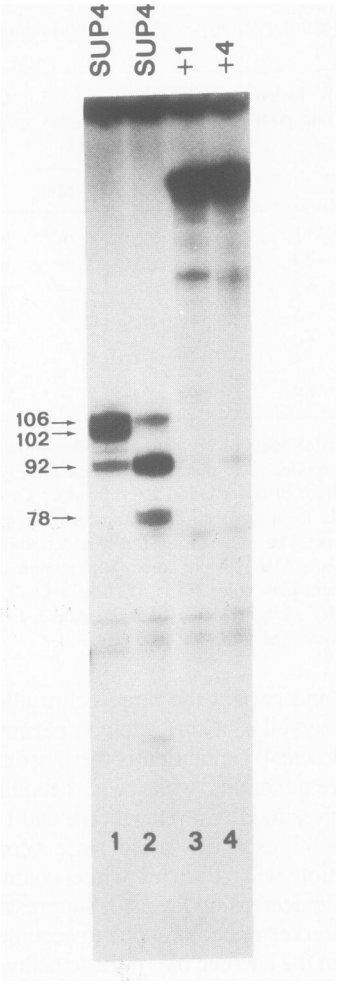


Fig. 4. *In vitro* transcription and processing of RNAs derived from deletion mutants which produce readthrough transcripts. The transcription reaction in **lane 1** was a standard template assay as in Figure 2 (in 70 mM KCl). In **lanes 2–4**, the reactions were identical to that of **lane 1** except that after 20 min the KCl concentration was increased from 70 mM to 140 mM and the reactions incubated an additional 20 min at 30°C. The DNAs used in **lanes 1** and **2** were wild-type *SUP4*-o. **Lanes 3** and **4** correspond to the +1 and +4 deletion mutants of *SUP4*, respectively.

Previously, we used competition assays to show that certain point mutations within the A or B blocks of *SUP4* alter the affinity of the gene for a limiting transcription factor (Allison *et al.*, 1983), even though in some cases transcript yield *in vitro* remains the same as that of wild-type *SUP4*. To test for altered promoter activity, a similar competition analysis was carried out with the 3′ deletion mutants. As shown in Figure 3, the +53 deletion mutant does not differ from wild-type *SUP4* in competitive strength. In contrast, all three mutants tested with deletions up to or beyond +7 have reduced competitive ability. The +1 deletion mutant is the weakest competitor, with a 3-fold reduced ability to compete for transcription factor. These results suggest that the 3′-flanking sequence of *SUP4* plays a significant role in binding a transcription factor. However, the equal competitive strengths of the +7 deletion mutant (a strong suppressor *in vivo*) and the +4 mutant (weak suppressor) argues against the possibility that a decreased ability to bind transcription factor is responsible for the weak suppression by the +4 mutant.

Given the above data, a reasonable explanation for the suppression phenotypes of the 3′ deletion mutants would be that the readthrough transcripts derived from the +4 and +1 mutants are not correctly or efficiently processed. This possibility was first investigated *in vitro*. Transcription of the wild-type *SUP4* gene under standard conditions (70 mM KCl) results in accumulation of two major transcripts, ~106 and 102 nucleotides in length (Figure 4, lane 1). Increasing the KCl concentration of the transcription mixture to 140 mM results in processing of the 102 – 106 nucleotide transcripts to a predominant RNA of ~92 nucleotides, together with an additional band of ~78 nucleotides (Figure 4, lane 2). The 92-nucleotide RNA is likely to be a $tRNA^{Tyr}$ transcript with processed ends but an unspliced intervening sequence, while the 78-nucleotide band is probably mature sized $tRNA^{Tyr}$. The size and order of appearance of these bands are consistent with processing studies of yeast $tRNA^{Tyr}$ precursors after injection of cloned genes into *Xenopus* oocytes (Melton *et al.*, 1980). In contrast, the readthrough transcripts produced from the +1 and +4 mutants are not efficiently processed to the 92-nucleotide RNA (Figure 4). Because both of these mutant genes contain the complete $tRNA^{Tyr}$ sequence, their transcripts should be capable of forming the tRNA cloverleaf structure. Nevertheless, the readthrough RNAs do not undergo 3′ processing.

Northern analysis of RNA from yeast transformants

To determine whether the *in vitro* 3′ processing defect of the +1 and +4 mutant RNAs also occurs *in vivo*, an RNA blotting experiment was carried out to test for the presence of readthrough transcripts *in vivo*. RNA from yeast cells transformed with the 3′ deletion mutant plasmids was isolated, electrophoresed through a polyacrylamide gel, transferred to APT (aminophenylthioether) paper, and the blot probed with labeled DNA containing *SUP4* sequences. The bulk of the $tRNA^{Tyr}$ and its two major precursors (the 108- and 92-nucleotide RNAs) which appear in the resulting autoradiogram (Figure 5) are chromosomally derived, since yeast contains a total of eight $tRNA^{Tyr}$ genes. Therefore, only the readthrough transcripts are of relevance here.

RNAs in the size range of the expected readthrough transcripts are detected by the *SUP4* sequence probe in transformants of the 68, +1, +4 and +5 deletion mutants (Figure 5, bands labeled RT), but not in transformants of the +7 or +53 mutant genes. This result further supports the conclusion that weak suppression by the +1 and +4 deletion mutants of *SUP4* is due to inefficient processing of the readthrough transcript rather than an absence of transcription. Although the rates of processing or degradation *in vivo* of the readthrough transcript relative to the normal $tRNA^{Tyr}$ precursors is not known, the amounts of readthrough transcript we detect (except from the +5 mutant) are comparable with the amounts of 108 and 92 nucleotide precursors detected from the eight chromosomal genes. There is significantly less readthrough transcript detected in the lane with +5 mutant RNA compared with those lanes with RNA from deletion mutants 68, +1 or +4 (Figure 5), which is consistent with the relatively strong suppression phenotype of the +5 mutant (Suppression Index is 4).

Failure to process the *SUP4* readthrough transcripts to suppressor tRNA could result either because yeast cells lack an enzymatic activity capable of removing long 3′ trailer sequences from tRNA precursors, or because some specific sequence in the readthrough RNA (polylinker/*LacZ* sequence) inhibits processing. To distinguish between these two possibilities, we constructed two additional sets of 3′ deletion mutants of *SUP4*-o each with a different 3′-flanking sequence.

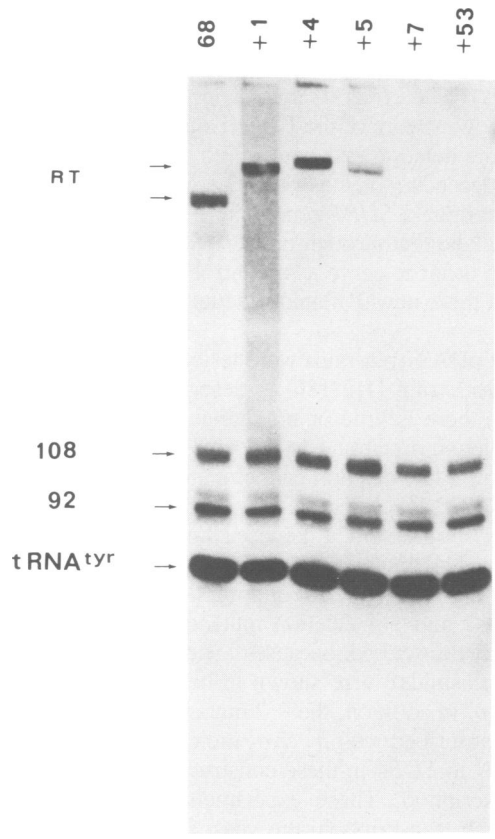


Fig. 5. Northern blot analysis of RNA from yeast transformants containing 3′-deleted *SUP4*-o genes. The yeast strain D12-18C was transformed with the pDA26 plasmids shown in Figure 1B. An aliquot of small RNA extracted from these cells was electrophoresed through a polyacrylamide gel, transferred to APT paper, which was then probed with labeled DNA containing *SUP4* sequence. Each lane is labeled according to the deletion end point of the *SUP4* gene that the transformant carries. RT indicates readthrough transcripts.

Table III. Suppression phenotypes of 3′ deletion mutants of *SUP4*-o in pDA27 plasmids

Plasmid	3′ Deletion end point	Growth of transformants on media lacking:			Color on YEPD	Suppression Index
		Ade	Lys	Met		
pDA27-96A	+7	+	+	+	light pink	4
pDA27-96B	+7	+	+	+	white	5
pDA27-93A	+4	–	–	+	red	1
pDA27-93B	+4	–	–	+	red	1
pDA27-90A	+1	–	–	–	red	0
pDA27-90B	+1	–	–	–	red	0
pDA26-96	+7	+	+	+	white	5
pDA26-93	+4	–	–	+	red	1
pDA26-90	+1	–	–	–	red	0

The pDA27 plasmids were analyzed for suppression as in Table I. For comparison, the phenotypes of some of the pDA26 plasmids are also shown.

SUP4 mutants with different 3′-flanking sequences

In these experiments most of the 3′-flanking sequence of *SUP4* was substituted with either of two different sequences from pBR322 DNA. These substitutions were made next to the +1 deletion mutant of *SUP4*-o (pDA26-90), the +4 mutant (pDA26-93) and the +7 mutant (pDA26-96). These three pDA26

plasmids were cleaved with *Bam*HI, and the small fragments containing the 3′-deleted *SUP*4 genes were ligated into the *Bam*HI site of the yeast vector pTC3 to create the pDA27 plasmids (see Figure 1A). The effect of these constructions is to replace sequences downstream of the *Bam*HI site shown in Figure 1B (13 bp 3′ to the deletion end point) with two different pBR322 sequences, depending on the orientation of a particular *Bam*HI fragment containing a *SUP*4 gene. The +7 deletion mutant, which does not make readthrough transcript, was reconstructed in this fashion in order to serve as a control for any possible negative effect that these new 3′-flanking sequences might have on transcription.

The six pDA27 plasmids were tested for suppression strength in the yeast strain D12-18C as described above. As shown in Table III, there is little or no change in suppression by a particular deletion mutant (+1, +4 or +7) when sequences 13 bp downstream of its deletion endpoint are substituted with two different pBR322 DNA sequences. All three +7 deletion mutants are strong suppressors. Likewise, the +4 mutants all suppress only the *met*4-1 mutation, while the three +1 deletion mutants are completely negative for suppression (Table III). It is likely that the +1 and +4 deletion mutants in these new constructs are actually transcribed, because the original +1 and +4 mutants (pDA26 plasmids) were shown to be transcribed both *in vitro* and *in vivo*. In addition, the +7 mutants in pDA27 plasmids are clearly transcribed well *in vivo*, indicating that the pBR322 sequences 3′ to *SUP*4 in these constructs do not dramatically affect transcription. These experiments indicate that $tRNA^{Tyr}$ readthrough transcripts in the yeast nucleus are not efficiently processed to functional $tRNA^{Tyr}$ molecules.

Discussion

To study the role of the 3′-flanking sequence in expression of the *SUP*4-o gene, we constructed 3′ deletion mutants of the gene and measured their ochre suppressor activity in transformed yeast cells. In addition, each mutant *SUP*4 gene was tested *in vitro* for transcript yield, termination efficiency, competition for transcription factors and the ability of its RNA product to undergo 3′ processing. The results of these analyses are summarized in Figure 6.

In vivo effects of 3′ deletions

A deletion removing all but seven T residues of the normal 3′-flanking sequence of *SUP*4-o (+7 mutant) was found to have little or no effect on suppression strength. In contrast, deletion of three additional base pairs (to +4) drastically reduces the ability of this mutant gene to suppress, while a 3′ deletion to +1 completely eliminates detectable suppression. Correlation of the *in vivo* effects of these deletion mutations with the *in vitro* tests (Figure 6) shows that neither transcript yield nor competitive strength closely correlates with suppression data. However, a good correlation exists between strength of suppression (Suppression Index) and termination at the 3′ terminus of *SUP*4, which produces normal transcripts rather than readthrough RNAs. Transcription of the +7 deletion mutant terminates normally *in vitro* and this mutant is a strong suppressor. Conversely the +1 deletion abolishes termination at the 3′ terminus of *SUP*4 and eliminates any suppression by the mutant gene. The deletions to +5 and +4 produce an intermediate phenotype with respect both to termination *in vitro* and suppression *in vivo*. The inference can therefore be drawn that expression of *SUP*4 in this case is dependent on termination at the 3′ terminus of *SUP*4, because the readthrough transcripts are not efficiently processed *in vivo* to suppressor tRNA. This idea is consistent with *in vitro* processing experiments showing that the readthrough transcript, unlike the normal 106-nucleotide precursor, is not efficiently processed to the presumed 92-nucleotide intermediate with mature ends.

Readthrough transcripts are also made in *Xenopus* oocytes after injection of tRNA genes which lack their normal 3′-flanking sequences. However, at least in some cases these readthrough RNAs in *Xenopus* oocytes can be processed to mature tRNA (Hofstetter *et al.*, 1981; Galli *et al.*, 1981; Adeniyi-Jones *et al.*, 1984). Correct 3′ processing in these cases appears only to require an intact tRNA coding sequence, and may be due to the action of an endonuclease (Adeniyi-Jones *et al.*, 1984). Transcription of a *Bombyx mori* $tRNA^{Ala}$ gene in nuclear extracts from *Xenopus* oocytes results in a precursor molecule with 22 extra nucleotides at the 3′ end, which are subsequently removed as an intact fragment by an endonuclease activity in the extracts (Garber and Gage, 1979; Hagenbüchle *et al.*, 1979). Process-

Plasmid	3′ Deletion	Suppression Index	Relative Amount of Readthrough Transcript in vivo	Fraction of Readthrough Transcripts in vitro	Relative Total Transcript Yield in vitro	Relative Competitive Strength
	+1					
pBSU4 (WT)	...GGGAGATTTTTTGTTTTTT...	5	0	0	1.00	1.00
pDA26-142	...GGGAGATTTTTTGTTTTTT...	5	0	0	1.03	1.00
pDA26-96	...GGGAGATTTTTTT	5	0	0.02	0.91	0.45
pDA26-94	...GGGAGATTTTT	4	0.3	0.62	0.47	ND
pDA26-93	...GGGAGATTTT	1	1.2	0.99	0.40	0.45
pDA26-90	...GGGAGAT	0	1.0	1.0	0.31	0.33
pDA26-84	...G	0	1.0	1.0	0.27	ND

Fig. 6. Summary of *in vivo* and *in vitro* effects of 3′ deletions at the *SUP*4 locus. The Suppression Index is from Table I. The relative amount of readthrough transcript detected *in vivo* was derived from densitometry tracings of the autoradiogram shown in Figure 5; the amount of readthrough transcript in each lane was determined relative to the amount of 108-nucleotide precursor to $tRNA^{Tyr}$, which derives primarily from the eight chromosomal $tRNA^{Tyr}$ genes and thus serves as an internal standard in each lane. The fraction of readthrough transcripts and transcript yield is from Table II. The relative competitive strength derives from Figure 3 and is determined by dividing the amount of wild-type *SUP*4-o DNA needed for a 35% reduction of -A36A37 transcription by the amount of 3′-deleted *SUP*4 DNA required for the same effect. ND: not determined.

ing of 3′ termini has also been observed following long incubations of tRNA readthrough transcripts in extracts from HeLa cells (Adeniyi-Jones *et al.*, 1984).

It is not known whether an exonuclease or endonuclease is responsible for processing the 3′ ends of normal tRNA precursors in yeast nuclei. The failure to process *SUP4* readthrough transcripts to functional suppressor tRNA could result from the inability of the 3′ processing enzyme to remove long 3′ trailer sequences from tRNA precursors. An alternative explanation is that the yeast 3′ processing nuclease is unable to remove the extra 3′ nucleotides in these particular cases, possibly because some specific sequence in the 3′ trailer inhibits processing. The latter possibility seems less likely since all three of the +1 deletion mutants with different 3′-flanking sequences (in plasmids pDA26-90, pDA27-90A and pDA27-90B) were found not to produce a detectable level of suppression. In yeast tRNA genes, a cluster of >5 consecutive T residues is usually located within 10 bp of the 3′ terminus, and we are not aware of any with a T cluster further than 25 bp downstream from the gene. This means that relatively few extra nucleotides normally must be processed from the 3′ ends of cytoplasmic tRNA precursors in yeast. The yeast nucleus may therefore lack the enzymatic machinery necessary for efficient removal of long 3′ trailer sequences simply because such an activity is normally unnecessary.

Effects of 3′ deletions on transcription competition

Deletions that extend into the T cluster of *SUP4* affect not only termination but also decrease the ability of the gene to compete for limiting transcription factor *in vitro*. Both of the deletions to +7 and +4 reduce competitive strength by 2-fold, and further deletion to +1 decreases competitive ability 3-fold (Figure 6). A deletion leaving 53 bp of normal flanking sequence did not alter competition from that of wild-type *SUP4*. An effect on competition of this magnitude was not expected from deletions confined to the 3′-flanking sequence. Previously, we found that base substitutions within the B block of *SUP4*, but not between the B block and the 3′-flanking sequence, could dramatically affect competition (Allison *et al.*, 1983). The present results, however, suggest that sequences in the 3′-flanking region can have a significant effect on transcription factor binding. A similar but less severe effect on competition was observed with deletions in the 3′-flanking sequence of a *Drosophila* $tRNA^{Arg}$ gene (Sharp *et al.*, 1983).

DNaseI protection assays have demonstrated the presence in yeast extracts of one or more proteins that specifically bind to the internal regions of tRNA genes (Klemenz *et al.*, 1982; Newman *et al.*, 1983; Stillman and Geiduschek, 1984). Recent experiments indicate that a single transcription factor from yeast (τ, or factor C) interacts with both the 5′ and 3′ halves of a tRNA gene (Baker and Hall, 1984; Camier *et al.*, 1985). Using a dimethyl sulfate (DMS) protection assay, Camier *et al.* (1985) showed that highly purified τ factor contacts G residues located within the A block, the B block and just upstream from it, and at the extreme 3′ end of a yeast $tRNA^{Glu}$ gene. These positions correlate reasonably well with the regions and base pairs in the *SUP4* locus that this study and our earlier work implicate in factor binding. Previously, we showed that point mutations within *SUP4* resulting in an altered competitive strength are located exclusively with the A block (positions 8−19), the B block (52−62), or the extra-loop region just upstream of the B block (Allison *et al.*, 1983). In addition, deletions in the 5′-flanking sequence of *SUP4* do not affect competitive ability (Allison, 1984), while those extending into the T cluster near the 3′ end of *SUP4* are shown here to decrease competitive strength. It cannot be determined from DMS protection studies whether the transcription factor actually makes contact with the T cluster, although this region in several tRNA genes is protected from digestion by nucleases (Klemenz *et al.*, 1982; Camier *et al.*, 1985). Alternatively, the T cluster may facilitate factor binding by virtue of its high A-T content and consequent ease of DNA strand separation. In this regard, stable association between yeast tRNA genes and a transcription factor(s) has been shown to be temperature dependent (Ruet *et al.*, 1984; Stillman *et al.*, 1985).

Transcription termination

The results of this study indicate that, both *in vitro* and *in vivo*, five or more consecutive T residues in the 3′-flanking sequence of *SUP4* are required for efficient termination of transcription. Transcription was found to terminate efficiently within the T_7 cluster of the +7 deletion mutant, and inefficiently within the T_4 sequence of the +4 mutant (Figure 6). Both *in vitro* and *in vivo* five consecutive T residues in the 3′-flanking sequence were partially effective in terminating transcription. These conclusions are consistent with the results of *in vitro* transcription of *SUP4* genes with mutations in the regions encoding mature $tRNA^{Tyr}$. While a mutation that creates a T4 sequence (U21) does not cause premature termination (Koski *et al.*, 1982), mutations within *SUP4* that create either six consecutive T residues (-A36A37) or five T residues [U(IV)] result in efficient premature termination at frequencies of 99% and 85%, respectively (D. Allison, unpublished). Polymerase III in yeast therefore appears to require a somewhat longer T stretch for termination than does *Xenopus* PolIII, which can terminate with high efficiency at a sequence with as few as four consecutive T residues (Bogenhagen and Brown, 1981).

The above idea is consistent with two facts regarding yeast tRNA sequences. First, there are at least three species of tRNA from yeast (a lysine tRNA and two different serine tRNAs) which contain four consecutive U residues (Gauss and Sprinzl, 1981). Thus synthesis of these tRNAs requires that transcription does not terminate efficiently at T_4 clusters. Second, all known yeast tRNA gene sequences contain, within 34 bp of 3′ ends, a cluster of at least six consecutive T residues. This is in contrast to *Xenopus* class III genes, where clusters of four consecutive T residues in the 3′-flanking sequence are common, and transcription is known to terminate within a cluster of only three consecutive T residues (Bogenhagen and Brown, 1981; Galli *et al.*, 1981).

In conclusion, these deletion experiments have uncovered two functions for the extensive T clusters present in the 3′-flanking regions of yeast tRNA genes. First, they facilitate transcription factor binding, possibly because of the poly(T) sequence *per se*, or because of their high A-T content. Second, the long T clusters adjacent to yeast tRNA genes efficiently terminate transcription by RNA polymerase III, which otherwise would produce tRNA precursors incapable of being correctly processed in the yeast nucleus. Both of these aspects of transcriptional function of 3′-linked oligo(T) sequences underscore the self-contained and autonomous nature of yeast tRNA genes. Because transcription is initiated under the direction of control sequences within the gene and terminated immediately after it, expression of each yeast tRNA gene normally has little dependence upon DNA sequences outside the transcribed region.

Materials and methods

Yeast strains and media

The *S. cerevisiae* strain D12-18C (α, *ade*2-1, *lys*2-1, *met*4-1, *leu*1, *trp*1, *can*1-100, *ura*3) was used in transformations. The *ade*2-1, *lys*2-1, *met*4-1, *can*1-100 and

leu2 markers are all ochre alleles. YEPD and dropout media were prepared as described (Kurjan and Hall, 1982).

Yeast transformations

The spheroplast method of Beggs (1978) was used to transform the yeast strain D12-18C. Selection for growth on media lacking tryptophan allowed isolation of transformants.

Isolation of RNA

Small RNA from transformed yeast cells was prepared by phenol extraction of whole cells, essentially as described by Hopper *et al.* (1980). Transformants were grown at 30°C in media lacking tryptophan (100 ml) to 2 × 10^7 cells/ml. The cells were pelleted and resuspended in 3 ml ice-cold TSE (0.01 M Tris-HCl, pH 7.5; 0.01 M EDTA; 0.1 M NaCl)/0.1% SDS. An equal volume of phenol saturated with TSE was added and the mixture shaken in a small flask at 42°C for 1 h, followed by a 2-h incubation on ice. The cells were then vortexed and centrifuged to separate the phases. The aqueous phase was re-extracted with phenol, and extracted once with chloroform. The RNA was then precipitated with ethanol at −20°C and resuspended in water.

Northern blot analysis

20 μg of small RNA isolated as described above was first precipitated with ethanol and resuspended in 90% formamide, 10 mM EDTA (pH 8.0) and 0.04% each of Bromphenol Blue and Xylene Cyanol FF. The RNA was electrophoresed through a 10% polyacrylamide-4M urea gel (0.15 cm × 13 cm × 23 cm) at 4 W for 3 h. The gel was then soaked twice in 50 mM Na-PO_4 (pH 5.5) for 30 min. APT (aminophenylthioether) paper was prepared according to a procedure described by Maniatis *et al.* (1982). Transfer of RNA to activated APT paper was achieved using a Bio-Rad 'Trans-Blot' apparatus (model 250/2.5), using a protocol provided by the manufacturer. Following transfer the paper was soaked in 1% glycine for 2 h in order to inactivate the residual active groups. The blot was pre-hybridized in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0), 4× Denhardts (1× Denhardts is 0.02% each of bovine serum albumin, Ficoll (mol. wt. 400 000), and polyvinyl pyrolidone), 25 mM Na-PO_4 (pH 6.6) and 300 μg/ml sonicated, denatured calf thymus DNA. Pre-hybridization was at 42°C for 24 h. The hybridization solution was the same as the pre-hybridization solution except that 2× Denhardts solution was used and 7 × 10^6 c.p.m. of nick-translated probe was included. The nick-translated probe was prepared using pRB1, a plasmid which contains multiple inserts of the *SUP*4 266-bp fragment cloned into the *Bam*HI site of pBR322 (constructed by Richard Baker). Hybridization was at 42−44°C for 24 h. The blot was washed three times with 2× SSC, 0.1% SDS for 30 min at room temperature, and once with 0.1× SSC, 0.1% SDS for 30 min at room temperature. The blot was used to expose Kodak XRP film. Bands were quantitated using a Helana Laboratories 'Quick Scan' densitometer.

In vitro transcription reactions

Cell-free extracts from *S. cerevisiae* were prepared as previously described (Allison *et al.*, 1983). Unless otherwise noted, all transcription reactions were in a final volume of 20 μl, were incubated at 25°C for 30 min and included the following components: 20 mM Hepes-KOH, pH 7.9, 70 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM EDTA, 5% v/v glycerol, 2 μl of cell-free extract, 0.5 mM each of ATP, CTP and GTP, 0.05 mM [α-^{32}P]UTP (5 Ci/mmol; Amersham), pBR322 at 7 μg/ml and other DNAs as specified below. In template activity experiments, the mutant *SUP*4 DNAs were present at a concentration of 1 μg/ml. For competition reactions, -A36A37 plasmid was at 3.7 μg/ml and point mutant *SUP*4 DNAs were at 1.8−22 μg/ml. In all reactions, the DNAs were mixed and cooled on ice followed by addition of buffer and cell-free extract. The DNA/buffer/extract mixture was incubated on ice for 10 min, after which nucleotide triphosphates were added and the reaction transferred to 25°C. Reactions were terminated and RNA isolated as previously described (Allison *et al.*, 1983). The RNA was electrophoresed in 10% polyacrylamide, 7 M urea gels as described (Koski *et al.*, 1982). Using an autoradiogram as a template, the specific RNAs were excised from the gel and Cerenkov counted to determine the amount of RNA in each band.

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