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# The 5′ flanking sequence negatively modulates the *in vivo* expression and *in vitro* transcription of a human tRNA gene

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## ABSTRACT

**The consequences of altering the 5′ flanking region of a human amber suppressor tRNA$^{ser}$ gene on phenotypic expression *in vivo* and transcription *in vitro* was examined by constructing a series of upstream deletion and substitution mutants. The resulting tDNA variants were examined for functional tRNA expression *in vivo*, by measuring suppression of a nonsense mutation in the *Escherichia coli* chloramphenicol acetyltransferase (*cat*) gene in co-transfection assays, and for transcriptional activity *in vitro* using HeLa cell nuclear extracts. Mutant genes in which the 18 nucleotides 5′ proximal to the coding region were deleted and replaced with heterologous sequences were 2 to 5 fold more active *in vivo* in comparison to the wild type gene. There was a strong, but not exclusive, correlation between the levels of nonsense suppression observed *in vivo* and transcriptional activity *in vitro*. In certain cases, introduction of an oligonucleotide encompassing this 18 nucleotide element upstream of more active tRNA genes reduced both the levels of suppression and template activity. These results indicate that the immediate 5′ contiguous sequence of this tRNA gene negatively modulates expression both *in vivo* and *in vitro*.**

## INTRODUCTION

It is well established that accurate and specific transcription of tRNA genes by RNA polymerase III is contingent upon the recognition of two highly conserved, spatially separated, intragenic control regions by the transcription component TFIIIC (reviewed in 1,2). Binding of TFIIIC initiates the cooperative and step-wise assembly of a multi-component transcription complex that contains TFIIIB and which is recognized by RNA polymerase III. Recently, a number of other factors, including the TATA box binding protein which is required for transcription by RNA polymerase I and RNA polymerase II, have been shown to be involved in RNA polymerase III mediated transcription (reviewed in 3,4)

Despite an increasing understanding about factor requirements and mechanisms of transcription of tRNA genes, our knowledge concerning the regulation of this class of genes remains limited. tRNA genes comprise large reiterated families of independently transcribed genes whose members can be regulated in diverse ways. This serves to adapt the tRNA population to distinct codon frequencies and amino acid utilization particular to different cell types and different developmental stages (5,6,7). tRNA genes, even within an isoacceptor family, can exhibit considerable differences in transcriptional activity *in vitro*. These differences cannot be ascribed to the highly conserved nature of the internal control elements (8,9,10). It is well documented, however, that expression of tRNA genes can be dramatically influenced in a wide variety of species by extragenic flanking sequences. In particular, the 5′ flanking region can modulate transcription in both a positive and negative manner, in part, by influencing the assembly or stability of active transcription complexes (11–22). Transcription of tRNA genes have been examined predominantly *in vitro*, where the function of extragenic flanking sequences may be influenced by variables inherent to the *in vitro* transcription system or the source of the transcription extract itself (23). In addition, the synthesis of functional tRNA involves a number of complex post-transcriptional maturation steps and modifications which may not be faithfully executed in cell free systems. Thus, when considering the importance of extragenic sequences on modulation of tRNA gene expression *in vitro* it is important to obtain *in vivo* correlations in order to establish physiological relevance. Since monitoring the expression of a specific tRNA gene *in vivo* is hindered by the large number of endogenous isoacceptor tRNA genes, artificial or heterologous tRNA genes, whose transcription can be selectively analyzed over this background population, have generally been used for this purpose (10, 24).

An alternative approach has been to use nonsense suppressor tRNA genes, which afford a facile phenotypic assay for their functional expression *in vivo* through the direct analysis of translational readthrough of premature termination codons. Suppressor tRNA genes have been used in yeast to examine the role of both 5′ and 3′ extragenic sequences on expression *in vivo*

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(25,26,27). In this report, we describe a similar strategy using a human amber suppressor tRNA$^{ser}$ gene to examine the consequences of altering upstream flanking sequences on functional tRNA expression *in vivo* and transcriptional activity *in vitro*. Our results demonstrate that the 5′ proximal flanking sequence negatively modulates expression of this gene both *in vivo* and *in vitro*.

## MATERIALS AND METHODS

### Plasmids

pUCtS Su$^+$(am) contains a human amber suppressor tRNA$^{ser}$ gene and has been described previously (28,29). pRSV*cat* and pRSV*βgal* express the *E.coli* chloramphenicol acetyltransferase (*cat*) (30) and *E.coli* β-galactosidase genes (obtained from Dr R. Rosenberg), respectively. pRSV*cat*(am27) is a derivative of pRSV*cat* that contains a suppressible UAG nonsense codon in place of the serine codon at position 27 of the *cat* gene (31). pBRVA contains the Adenovirus VA1 gene cloned into pBR322 (obtained from Dr R. Bhat).

### Construction of tRNA$^{ser}$ 5′ flanking region variants

The human serine tRNA gene, present in pUCtS-Su$^+$(am), contains 464 nucleotides of the natural 5′ flanking region. The deletion mutants ptS-66, ptS-18 and ptS-1 are missing the natural 5′ flanking sequences up to and including nucleotide −66, −18 and −1, respectively, and have been described previously (29). The numbering is with respect to the 5′ nucleotide of the mature tRNA which is taken as +1.

ptS-32X, ptS-4X, ptS-1XA and ptS-1XB were derived from ptS-66 by bidirectional *Bal*31 exonuclease digestion from a unique *Hind*III site (present upstream in the polylinker) followed by ligation of an *Xba*I linker (5′GTCTAGAC) to the ends, *Xba*I digestion, and recircularization. These derivatives are missing the natural 5′ flanking sequences up to nucleotide −32, −4, −1 and −1, respectively (see Figure 1). ptS-1, ptS-1XA, and ptS-1XB differ from each other in the vector sequences that replace the natural 5′ flanking sequences of the gene.

Nested *Bal*31 deletions from the unique *Sna*B I site at nucleotide −1 were also constructed and, using selected combinations with the above plasmids for reconstruction, were used to generate a number of internal deletions. In pdel-35/3, nucleotides −35 to −3 were substituted with the *Xba*I linker; similarly, for pdel-17/3 and pdel-17/4, the *Xba*I linker replaces nucleotides −17 to −3 and nucleotides −17 to −4, respectively. For pdel-35/3 and pdel-17/3 the actual 3′ deletion endpoint was −1, however, the sequence of the *Xba*I linker resulted in the restoration of the natural nucleotides to −2.

p*LacO*ts(am1) contains a synthetic double stranded 18 residue oligonucleotide (ATTGTGAGCGCTCACAAT; corresponding to the binding site for the *E.coli lac* repressor) cloned into the *Sna*B I site of pUCtS-Su+(am) (32). Insertion mutants pXBin6F, pXBin5R, p4Xin9F and p4Xin8R were created by inserting a double stranded synthetic oligonucleotide, corresponding to the flanking region between base pairs −18 to −1 of the tRNA$^{ser}$ gene (5′ctaGGTTGTTGAAGGAGGTAC and its complement 5′ctaGGTACCTCCTTCAACAAC), in both orientations, into the *Xba*I site of ptS-4X (creating p4Xin9F and p4Xin8R) or ptS-1XB (creating pXBin6F and pXBin5R). The designation F and R indicates whether the oligonucleotide was inserted in the wild type (wt) or reverse orientation, respectively.

Plasmid DNA was purified by cesium chloride gradient centrifugation, quantitated by fluorometry (33) and examined by agarose gel electrophoresis to ensure that the relative proportion of supercoiled DNA was the same for each plasmid. All of the above plasmids were sequenced to verify the accuracy of the constructs using a double stranded DNA sequencing kit (Sequenase, US Biochemicals). Oligonucleotides were synthesized and purified at the Central Facility of the Molecular Biology and Biotechnology Institute, McMaster University.

### Transfection and measurement of suppression activity

Transfections were carried out using 60 mm plates of BSC-40 cells by the DEAE dextran/DMSO shock procedure (34) with 20 μg of DNA per plate (normalized with sonicated salmon sperm DNA). All transfections were performed using varying amounts of each suppressor tRNA$^{ser}$ gene derivative (0.25 to 4 μg; four replicates for each DNA concentration) in the presence of 5 μg pRSV*cat*(am27) and 0.5 μg of pRSV*βgal*, the latter serving as an internal control. Cell extracts were prepared 48 hours post transfection; CAT activity was quantitated by the modified scintillation counting method (35,36) and β-galactosidase activity was determined as described (37). Normalizing CAT activity against β-galactosidase activity reduced the coefficient of variation from 40% to 20% which is an improvement also noted by others (38). Correction of CAT activity against protein concentration in the cell extracts had negligible effects on the coefficient of variation and as such was not routinely used for normalization.

The suppression activity of each tRNA gene was measured from the slope of a plot of normalized CAT activity versus the amount of tRNA gene transfected as determined by linear regression analysis (see inset of Figure 2). The values obtained were compared to the activity from the wt gene pUCtS-Su$^+$(am) which was transfected in parallel in each experiment and whose activity was normalized to 1. The statistical significance of the data was assessed using the one tailed t-test, $\varrho < 0.05$.

### *In vitro* transcription

Nuclear extracts from suspension cultures of HeLa cells were prepared as described (39). A standard 20 μl transcription reaction mixture contained 80−100 mg/ml of extract, 10 mM hydroxyethyl piperazine-N′2-ethanesulphonic acid buffer pH 7.9, 10% glycerol (v/v), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.4 mM each ATP, CTP, UTP, and 0.04 mM GTP, 2 μCi [α-$^{32}$P]GTP (400 Ci/mmole), 8 mM creatine phosphate, 0.25 mM dithiothreitol, and 0.25 mM phenylmethylsulphonylfluoride. Transcription reactions were optimized for the amount of extract, cation concentration, template concentration and total amount of DNA in preliminary experiments. Reaction mixtures were incubated at 30° for 90 minutes and were terminated by the addition of 10 vol of 300 mM sodium acetate (pH 5.0) containing 0.5% sodium dodecylsulphate. After phenol extraction and ethanol precipitation, products were analyzed on 10% acrylamide/7 M urea gels, visualized by autoradiography, and quantitated by densitometry or phosphoimager analysis.

Competitive strength of the tRNA mutants was determined in direct competition assays as described (40) using pBRVA as the reference plasmid. The amount of pBRVA plasmid required for just saturating levels of VA1 transcription was first determined using established methods (41). Increasing amounts of the wild type tRNA gene or mutant template was added along with a constant amount of pBRVA (0.4 μg pBRVA; total DNA kept optimal at 1 μg with pBR322) prior to the addition of extract

and nucleoside triphosphates (NTPs). The amount of VA1 transcripts synthesized was quantitated in each lane by phosphorimager analysis and expressed as a percentage of VA1 made in the absence of competitor tDNA (taken as 100%). The reciprocal of this value was plotted against the amount of tDNA added, and the slope, which is a measure of competitive ability (42), was determined by linear regression analysis. Slopes were normalized to the wild type tRNA$^{ser}$ gene whose competitive ability was taken as 1.

## RESULTS

### The 5′ proximal region of a human tRNA$^{ser}$ gene modulates functional expression *in vivo*

We have demonstrated previously that functional expression of mammalian nonsense suppressor tRNA genes can be quantitated *in vivo* in cells by measuring suppression of nonsense mutations in the *E.coli cat* gene, and that the level of suppression activity is a linear function of the amount of tDNA transfected (29,31). Using this suppression assay we had previously demonstrated that expression of a human serine amber suppressor tRNA gene was apparently influenced by sequences in the 5′ flanking region (29), although the role of this region on transcriptional activity *in vitro* was not addressed. Subsequent analyses determined that the relative and absolute levels of suppression among different tRNA gene derivatives can deviate significantly between experiments due to variation in transfection efficiencies and the amount of tDNA plasmid used (not presented). In the present report, we investigate the influence of the 5′ flanking sequence of the tRNA$^{ser}$ gene using quantitative transfection procedures to directly compare *in vivo* suppression activity to *in vitro* transcription efficiency.

A series of upstream deletion and substitution mutants of the human amber suppressor tRNA gene were constructed (Fig. 1) and tested for suppression activity by co-transfection with pRSV*cat*(am27). To control for variations in transfection efficiencies, pRSV*βgal* was included as an internal standard and transfections were repeated several times with varying amounts of tDNA to ensure that measurements were within the linear range of activity for each mutant. The parental plasmid for all of the constructs was ptS-66, which contains the natural 5′ flanking region of the tRNA$^{ser}$ deleted up to nucleotide −66 (numbering is relative to the first nucleotide of the coding region which is taken as +1). Deletion up to this nucleotide has no effect on functional expression of the tRNA gene *in vivo* (29; present work). As shown in Fig. 2, and summarized in Table 1, deletion of the natural sequences up to nucleotide −18 had little effect on suppression activity; ptS-66, ptS-32X and ptS-18 all exhibited levels of suppression activity which were similar to the wild type gene. This relationship held over a wide range of tDNA concentrations (0.25 to 4 μg DNA transfected). In contrast, deletion up to −4 (ptS-4X) or to −1 (ptS-1) resulted in a 2.5 to 5 fold increase in the level of suppression activity compared with the wild type gene. This increase was statistically significant as determined by the one tailed t-test ($\varrho > 0.05$). To ensure that this increase in activity was not related to the fortuitous placement of stimulatory vector sequences upstream of the coding region, ptS-1XA and ptS-1XB, which are similar to ptS-1 but differ in the nature of the substituting vector sequences, were constructed and tested. Both of these plasmids also showed a significant (2 to 4 fold) increase in suppression activity compared with the wild type gene. Suppression activity of internal deletion mutants missing nucleotides −35 to −3 (pdel-35/3), −17 to −4 (pdel-17/4) and −17 to −3 (pdel-17/3) were also statistically greater than that of the wt gene (Table 1). These results suggest

PLASMID:

| | −60 | −50 | −40 | −30 | −20 | −10 | +1 | |
|---|---|---|---|---|---|---|---|---|
| ptS-66 (WT) | AAAGATGTCTGTGAAAAGAAACATATATTCCTCATGGGAATATATCCA | | | | | | TAC | GTAG |
| ptS-32X | cagtcacgacgttgtaaaacgacggccgtctagacGGGAATATATCCA | | | | | | TAC | GTAG |
| ptS-18 | cacgacgttgtaaaacgacggccagtgccaagcttgggctgcaggtca | | | | | | TAC | GTAG |
| ptS-4X | gaaaggggatgtgctgcaaggcgattaagttgggtaacgccagggtttt | | | | | | TAC | GTAG |
| ptS-1 | agcggataacaatttcacacaggaaacagctatgaccatgattacgccaagcttgggctgcaggtC | | | | | | | GTAG |
| ptS-1XA | gcgccattcgccattcaggctgcgcaactgttgggaagggcgatcggtgcgggcctcgtctagAC | | | | | | | GTAG |
| ptS-1XB | gctggcgaaaggggatgtctcgaaggcgattaagttgggtaacgccagggttttcctctagAC | | | | | | | GTAG |
| pdel-35/3 | GCCAGTGCCAAGCTTGGGCTGCAGGTCAAACATGTCTGTGAAAACAAAGATATATTCCgtctagAC | | | | | | | GTAG |
| pdel-17/3 | GCTGCAGGTCAAAGATGTCTGTGAAAAGAAACATATATTCCTCATGGGAATATATCCAGtctagAC | | | | | | | GTAG |
| pdel-17/4 | GCAGGTCAAAGATGTCTGTGAAAAGAAACATATATTCCTCATGGGAATATATCCAGtctagacTAC | | | | | | | GTAG |
| pIacOtS(am1) | AAACATATATTCCTCATGGGAATATATCCA | | | | | | attgtgagcgctcacaat | GTAG |
| pXB(in6F) | tgcaaggcgattaagttgggtaacgccagggttttccgtctag | | | | | | ctagAC | GTAG |
| pXB(in5R) | tgcaaggcgattaagttgggtaacgccagggttttccgtctag | | | | | | tagAC | GTAG |
| p4X(in9F) | gcgattaagttgggtaacgccagggttttcccacgtctag | | | | | | ctagacTAC | GTAG |
| p4X(in8R) | gcgattaagttgggtaacgccagggttttcccacgtctag | | | | | | tagacTAC | GTAG |

**Figure 1.** 5′ Flanking sequences of the wild type and deletion/insertion mutants of the tRNA$^{ser}$ gene. The first line shows the sequence of the 5′ upstream region of the wt gene. Numbering is with respect to the first nucleotide present in the mature transcript which is taken as +1. Upper case letters denote sequences corresponding to the natural 5′ flanking region while lower case letters are sequences derived from the pUC vector (the *Xba*I linker is underlined). The sequence shown in bold and double underlined represents the −1 to −18 element, with the arrows indicating its orientation with respect to the wt gene.

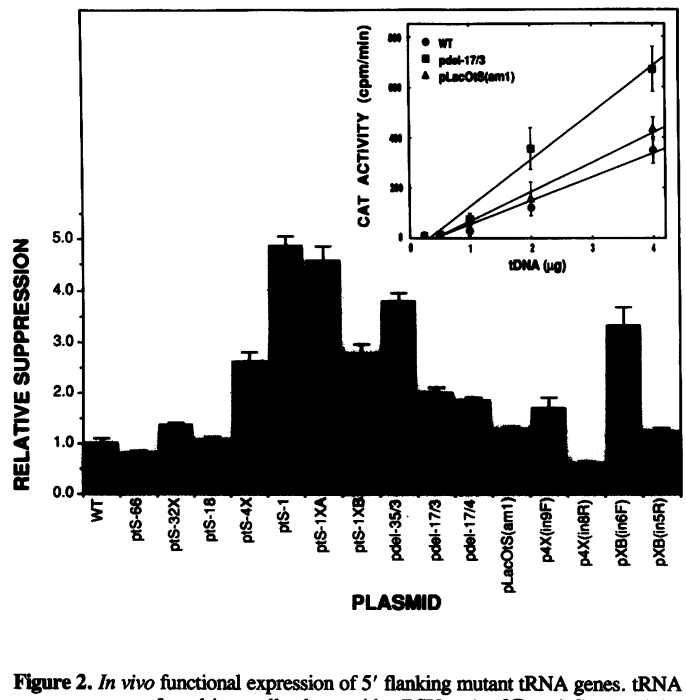


**Figure 2.** *In vivo* functional expression of 5′ flanking mutant tRNA genes. tRNA genes were transfected into cells along with pRSV*cat(am27)* and CAT activity was determined. Each tRNA gene was transfected in quadruplicate at each of 5 different DNA concentrations (0.25, 0.5, 1, 2, and 4 μg). The suppression activity, normalized to the internal transfection control, represents the slope (+/− SD) of the CAT activity versus the amount of transfected DNA as determined by linear regression analysis. The values are shown relative to the activity of the wt gene (pUCts Su$^+$(am)) which was normalized to 1. The inset shows representative data for pUCts Su$^+$(am), pdel-17/3, and p*LacOts*(am1) which was used to calculate the relative suppression activity.

**Table 1.** Summary of the *in vivo* suppression, *in vitro* transcription and competition data

| Plasmid | Suppression[a] | *In Vitro* Transcription[b] | Competitive Strength[c] |
|---|---|---|---|
| WT (pUCts $Su^+$(am)) | 1.00 (.11) | 1.00 (.26) | 1.00 (.50) |
| ptS-66 | 0.80 (.04) | 1.04 (.25) | 1.51 (.58) |
| ptS-32X | 1.34 (.06)* | 1.20 (.19) | 1.81 (.38) |
| ptS-18 | 1.05 (.08) | 1.70 (.81) | 0.69 (.36) |
| ptS-4X | 2.58 (.20)* | 1.42 (.40) | 1.05 (.54) |
| ptS-1 | 4.85 (.22)* | 4.58 (.37)* | 2.86 (1.3)* |
| ptS-1XA | 4.57 (.30)* | 3.55 (.72)* | 2.26 (1.2)* |
| ptS-1XB | 2.73 (.20)* | 6.73 (2.1)* | 2.01 (.57)* |
| pdel-35/3 | 3.77 (.17)* | 7.08 (1.5)* | 3.77 (1.2)* |
| pdel-17/3 | 1.98 (.12)* | 3.96 (.90)* | 2.57 (1.1)* |
| pdel-17/4 | 1.81 (.09)* | 3.02 (.55)* | 1.64 (.91) |
| p*LacO*ts(am1) | 1.25 (.07) | 0.64 (.30) | 1.43 (1.1) |
| pXBin6F | 3.28 (.38)* | 0.68 (.13) | 3.92 (1.3)* |
| pXBin5R | 1.22 (.08) | 0.24 (.05) | 3.14 (.97)* |
| p4Xin9F | 1.68 (.21)* | 1.24 (.13) | 3.04 (.54)* |
| p4Xin8R | 0.58 (.05) | 0.23 (.08) | 1.03 (.58) |

[a] Data from Fig. 2 (± SD).
[b] The transcription assay using HeLa cell nuclear extracts represents the average of 3 separate *in vitro* transcription reactions (± SD) using 0.1 μg of template DNA in each reaction. Quantitation was carried out by densitometric scanning and normalized against the activity of the wt gene which was taken as 1.
[c]Values represent the slope (± SD) determined as in Fig. 5 using 8 different tDNA concentrations and normalized to the slope of the wt gene which was taken as 1.
*These values are statistically higher than wild type as determined by one tailed t-tests ($\varrho < 0.05$).

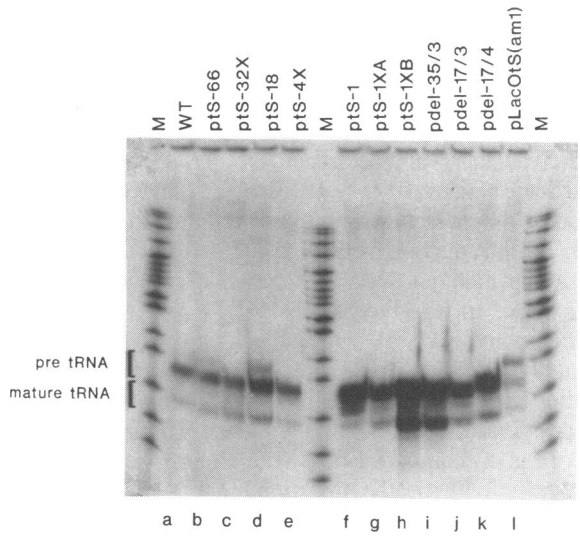


**Figure 3.** *In vitro* transcription of 5′ flanking mutant tRNA genes. The various tRNA genes, as indicated at the top of the figure, were transcribed in HeLa cell nuclear extracts and analyzed by gel electrophoresis. M represents size markers derived from *Hpa*II digested pBR322. The positions of the precursor and mature sized transcripts are indicated.

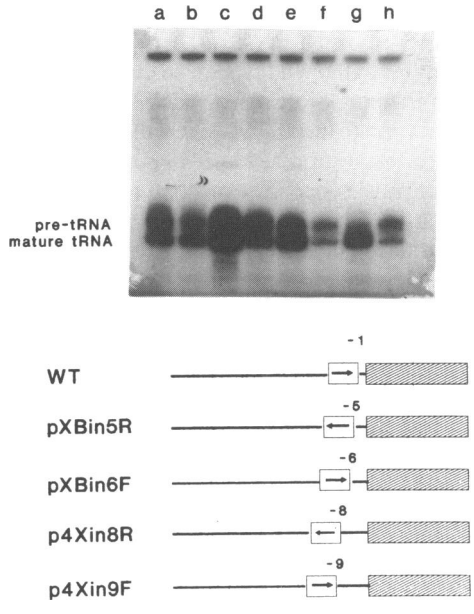


**Figure 4.** The 5′ proximal 18 nucleotides of the $tRNA^{ser}$ gene negatively modulates *in vitro* transcription depending on position and orientation. Mutant tRNA genes were transcribed as in Fig. 3. Lanes a and b, wt gene; lane c, ptS-1XB; lane d, ptS-4X; lane e, p4Xin9F; lane f, p4Xin8R; lane g, pXBin6F; lane h, pXBin5R. The schematic at the bottom indicates the relative position and orientation of the 18 nucleotide long proximal element for the various insertion mutants with respect to the wt gene.

that the natural sequence between −18 and −4, while not essential for biological activity, negatively modulates expression of the $tRNA^{ser}$ gene *in vivo*.

The effect of this upstream region on suppression activity was further explored by re-introducing, in both orientations, a synthetic oligonucleotide corresponding to nucleotides −18 to −1 back into ptS-4X, to generate p4Xin9F and p4Xin8R, or into ptS-1XB, to generate pXBin6F and pXBin5R. In general, the higher levels of suppression activity observed with the parental plasmids ptS-4X and ptS-1XB were negated by the re-insertion of this element, irrespective of orientation, resulting in constructs which exhibited suppression levels similar to that obtained with the wild type gene (Fig. 2). One exception to this was pXBin6F, whose suppression activity remained elevated at a level similar to it's parental plasmid ptS-1XB. p*LacO*ts(am1) which contains an 18 residue long insertion of heterologous DNA at position −1, effectively displacing the 5′ flanking region 18 nucleotides further upstream of the coding region, exhibited suppression activity indistinguishable from the wild type gene.

The above results, taken together, indicates that the natural 5′ proximal sequence between −1 to −18 exerts a dominant negative effect on functional expression *in vivo*.

## The 5′ flanking sequence influences *in vitro* transcription activity

The variation in functional tRNA expression observed with different 5′ flanking tRNA gene derivatives could be a consequence of a number of post-transcriptional modification and maturation events that are distinct from transcription. This is of particular significance in the present study since the region between −18 and −1 encompasses the transcription start site. Thus, it is possible that some of the observed differences in suppression activity are due to increased stability or more efficient processing of the primary RNA transcript rather than to differences in transcriptional rates. To ascertain if the differences observed *in vivo* were related to transcriptional activity *in vitro*, we compared the *in vitro* transcriptional efficiency of each mutant tRNA gene.

A typical transcription reaction is shown in Figure 3 and the results from the average of several experiments are summarized in Table 1. All of the deletion mutants up to base pair −18 exhibited levels of *in vitro* template activity which were comparable to the wild type gene (lanes a−d). In contrast, the deletion mutants (with the exception of ptS-4X, see below) which omitted nucleotides −1 to −18 displayed 3 to 7 fold higher levels of transcriptional activity compared to the wild type gene (lanes f−k). Re-introduction of the −1/−18 element into ptS-1XB in either orientation (pXBin6F and pXBin5R) reduced transcriptional activity back to wild type levels (Fig. 4, lanes c,g, and h). Re-insertion of the −1/−18 element back into ptS-4X in the forward orientation (p4Xin9F) did not affect transcription, however, insertion in the opposite orientation (p4Xin8R) reduced transcription to levels below that of the parental and wild type genes (Fig. 4, compare lane d with e and f, respectively). The template activity of the insertion mutant p*LacO*ts(am1) was similar to the wild type tRNA gene.

The above data indicates that the 5′ proximal 18 nucleotide region of this gene can exert a dominant negative effect on transcription *in vitro*, and can do so even if placed at different positions and orientations with respect to the coding region.

Overall, the increased functional expression observed with the tDNA mutants *in vivo* correlated well with increased transcriptional efficiency *in vitro*. The coefficient of correlation between the data sets, excluding the values for pXBin6F (see below), is 0.724. However, this correlation did not hold for every tDNA mutant. For instance, the *in vitro* template activity of ptS-4X was similar to the wild type gene even though it exhibited higher levels of suppression. Also, pdel-17/4 was significantly more active *in vitro* than ptS-4X, even though these templates are identical up to position −11. It is possible that the nature of the heterologous sequences present upstream of −11 in ptS-4X, which do not affect biological expression, may nevertheless influence *in vitro* activity. Alternatively, the natural upstream sequences retained in pdel-17/4, which are absent in ptS-4X, may have some positive effect on transcriptional efficiency *in vitro*.

Some of the discrepancies between the *in vivo* measurements and the *in vitro* measurements may be due to differences in processing or stability of the gene products since the sequences altered in each mutant likely include the transcription start site. However, there was no apparent difference in the sizes of the primary and mature transcripts between the mutant templates compared with the wild type gene (as judged using high resolution sequencing gels; data not presented) which would indicate alterations in start sites or aberrant processing. Indeed, with the exception of ptS-1 and pXBin6F, all the transcripts from the various templates were processed with comparable efficiencies. Interestingly, transcripts from pXBin6F appeared to be almost completely processed *in vitro* and the ratio of mature tRNA to primary transcript was consistently much greater than that observed for any other template (Fig. 4, lane g). This may explain the 3 fold higher suppression activity observed with this construct *in vivo* even though overall transcriptional activity *in vitro* was similar to the wild type gene.

### Influence of the 5′ flanking region on the assembly of transcription complexes

In certain studies upstream sequences of tRNA genes have been shown to influence the assembly or stability of transcription complexes. The various tRNA genes were thus tested for their ability to stably interact with transcription factors by performing template competition assays using the adenovirus VA1 gene as the reference template.

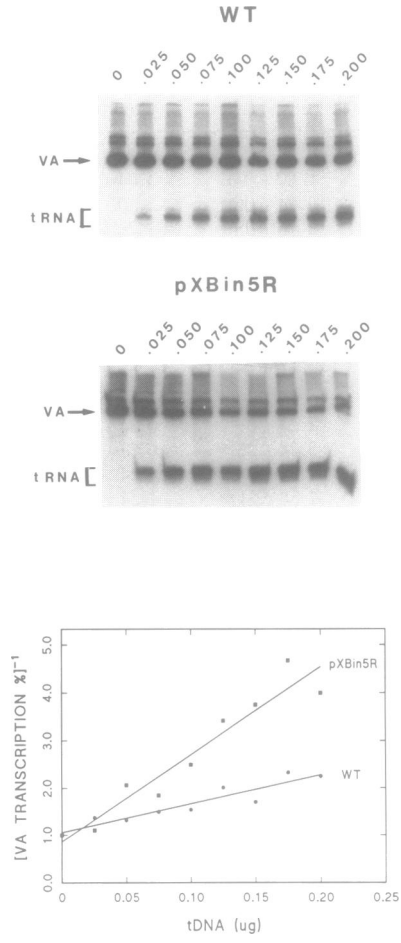


**Figure 5.** Competitive ability of 5′ mutant tRNA genes. A constant amount of pBRVA (0.4 μg) was incubated in a transcription reaction in the absence of competitor DNA or in the presence of varying amounts (shown in μgs) of wild type (WT) tDNA or pXBin5R tDNA, as indicated at the top of each gel. Total DNA was normalized to 1 μg in each case with pBR322. The amount of VA1 transcription was quantitated by phosphoimager analysis and expressed as a percentage of the amount of VA1 made in the absence of competitor tDNA. The reciprocal of this value was plotted against the amount of competitor tDNA (see bottom panel) and the competitive strength was determined from the slope of the line by linear regression analysis. Similar analyses were performed for each tRNA mutant and the results are summarized in Table 1.

A representative competition experiment is shown in Figure 5 and the results obtained with all the tRNA gene variants are summarized in Table 1. There was at best only a weak correlation between the *in vitro* transcriptional activity of the tDNA mutants and their *in vitro* competitive strength (coefficient of correlation between the data sets was less than 0.4). While the 5′ deletion derivatives that displayed higher suppression and transcriptional activity tended to be better competitors, the most active templates were not necessarily the most effective at sequestering transcription factors (Table 1). More importantly, while *in vitro* transcriptional activity and *in vivo* suppression levels of p4Xin9F and pXBin5R were the same or lower than that of the wild type gene their competitive abilities were significantly higher (Table 1). Thus, while the immediate upstream region may indeed influence the interaction or stability of limiting transcription

factors with the tRNA gene *in vitro*, this is not sufficient to explain the negative effect that this element exerts on functional expression *in vivo*.

## DISCUSSION

A large number of *in vitro* studies have demonstrated that 5′ flanking sequences can modulate transcription of mammalian tRNA genes, however, in only a few cases has the importance of extragenic sequences for *in vivo* expression been examined. The nonsense suppression assay described in this paper, coupled with *in vitro* analyses, provides a quantitative strategy to examine the relationship between transcriptional activity *in vitro* and functional expression *in vivo* of mammalian tRNA genes. This provides an opportunity to address various aspects of mammalian tRNA gene regulation *in vivo* in a manner which has been used successfully in lower eukaryotic systems.

We have determined that functional tRNA expression *in vivo* and transcriptional efficiency *in vitro* of a human $tRNA^{ser}$ gene are both negatively modulated by the proximal 5′ flanking 18 nucleotides. Deletion of this upstream element, and replacement with heterologous sequences, resulted in a 2 to 5 fold increase in suppression activity and a 2 to 7 fold increase in transcriptional efficiency in comparison to the wild type gene. Results obtained from the insertion mutants indicate that this modulatory element may act in a dominant negative manner *in vivo* and *in vitro*.

A direct comparison between the *in vivo* and *in vitro* measurements must be tempered by the fact that the flanking element that we have identified includes the transcription start site. Thus, negative modulation via this region may reflect differences in RNA stability or tRNA maturation and modification, rather than transcriptional activity. This seems to be the case with pXBin6F where elevated suppression activity, but normal transcriptional activity, appears to be due to differences in rates of processing to mature tRNA. Despite this exception, the strong correlation between the *in vivo* and *in vitro* results suggests that the flanking sequence negatively modulates transcription *in vivo*. Most 5′ modulatory elements identified thus far exert a positive effect on expression, however, negative acting elements have been described for *Drosophila* $tRNA^{lys}$, $tRNA^{arg}$, and Xenopus $tRNA^{met}$ genes (12,13,15). There is no obvious similarity in the sequence of these negative modulatory elements with the proximal region of the serine tRNA gene.

For the mouse $tRNA^{asp}$ and human $tRNA^{val}$ genes, distinct extragenic sequences can differentially affect transcriptional activity by influencing the formation or stability of active transcription complexes (20,42). The flanking region of the $tRNA^{ser}$ did have some negative effect on competitive strength since in most cases, deletion of the element resulted in templates with higher competitive abilities. However, competitive ability did not correlate well with the *in vivo* suppression or *in vitro* transcriptional activity. For instance, while suppression and transcriptional activity with pXBin9F and pXBin5R was lower compared with their corresponding parental plasmids, their competitive strength remained elevated. This suggests that, for these plasmids at least, the natural flanking region compromises transcriptional efficiency and suppression activity at a step subsequent to assembly of the transcription complex.

The natural position of this negative element, immediately upstream of the coding region of the gene, suggests several plausible explanations for the observed effects on transcriptional efficiency and expression *in vivo*. The multi-subunit complex TFIIIB is the pivotal component in RNA polymerase III transcription and, for yeast tRNA genes at least, has been shown to directly interact with the 5′ flanking region and the transcription start site (43,44). This interaction, which is dependent upon cooperative interactions with TFIIIC, is sequence independent, which is consistent with the position and the non-conserved nature of 5′ flanking modulatory elements. Although there is no apparent specific sequence requirement for this extended TFIIIB interaction, it is likely that particular sequences may affect the function of TFIIIB or some other factor that is required for initiation and transcription. Recently, a novel RNA polymerase III transcription factor, called TFIIIE, has been described (45). This factor is necessary for single and multiple rounds of transcription but is not required for the formation of stable pre-initiation complexes. It is not known whether the function of this factor is influenced by flanking sequences.

The diversity of upstream modulatory sequences may reflect the existence of gene specific factors which may attenuate the interaction of TFIIIB, or other transcription factors, with the upstream region. Indeed, there is evidence that sequence or gene specific DNA binding proteins can bind upstream of tRNA genes and affect their expression (18,20, 21). In this regard, we have recently demonstrated that the *E.coli lac* repressor, bound to its cognate operator site appropriately placed upstream of the $tRNA^{ser}$ gene, can stringently and reversibly inhibit expression *in vivo* and transcription *in vitro* (32). Whether there exists a specific inhibitory cellular factor that recognizes the proximal element of the serine tRNA gene remains to be determined.

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