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Analysis of the Role of 5′ and 3′ Flanking Sequence Elements upon in Vivo Expression of the Plant tRNA^{Trp} Genes

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We have isolated the majority (seven) of the tRNA^{Trp} genes of Arabidopsis and have studied the 5′ and 3′ flanking sequence requirements for their efficient expression in vivo by using an assay requiring translational suppression of the luciferase reporter gene. The expressed tRNA^{Trp} genes contain no highly conserved 5′ flanking sequences; however, these sequences are distinctly AT rich, contain several possible TATA elements, and are bound in vitro by recombinant plant TATA binding protein. Replacement of the natural 5′ flanking sequences with three different sequences lacking TATA elements reduced expression in vivo up to 10-fold; the same effect was observed when the TATA elements of the natural 5′ sequences were inactivated by point mutations. Introduction of a single TATA element from the adenovirus major late promoter into an artificial 5′ flanking region of the tRNA^{Trp} gene enhanced expression in vivo when the TATA element was placed at position −32 relative to the first nucleotide of the mature tRNA sequence, but not when it was placed at position −24. Primer extension analyses of in vitro transcripts revealed that the position of the TATA element helps dictate the start site of transcription. Efficient expression of the tRNA genes in vivo also required 3′ flanking sequences capable of terminating transcription.

INTRODUCTION

The DNA sequences and proteins directing transcription of tRNA genes by RNA polymerase III in lower eukaryotes and in animal cells have been defined through biochemical and genetic studies (reviewed in Willis, 1993; White, 1994; Sprague, 1995). Two multicomponent transcription factors bind to the internal control region and to the 5′ flanking DNA sequences of tRNA genes. The first, TFIIIC, helps directs the second, TFIIIB, to an upstream position, where it in turn helps position RNA polymerase III at the site of transcription initiation. Superimposed upon these general features may be additional species-specific requirements.

In contrast with our knowledge of tRNA gene expression in lower eukaryotes and in animal cells, we have limited knowledge of the synthesis of cytoplasmic tRNAs in plant cells. In large part, this is due to the intransigence of plant gene in vitro transcription systems. To circumvent this limitation, we developed an assay to study tRNA gene expression in vivo (Franklin et al., 1992). Our approach relies on detecting the expression of plant tRNA genes capable of translational suppression of reporter genes having nonsense codons; subsequently, others reported a similar approach (Carneiro et al., 1993).

In this study, we define the members of the tRNA^{Trp} nuclear gene family of Arabidopsis and demonstrate a requirement for 5′ and 3′ flanking sequences for the efficient in vivo expression of these genes by using an assay for translational suppression that is more sensitive and reliable than the assay we reported previously (Franklin et al., 1992). We relate these observations to the properties of genes of other organisms whose transcription by RNA polymerase III has been characterized intensively.

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RESULTS

Isolation and Sequence Analysis of Arabidopsis Nuclear tRNA^{Trp} Genes

The few plant tRNA genes that have been isolated are organized either as single interspersed copies or in repetitive clusters (Waldron et al., 1985; Palmer and Folk, 1987; reviewed in Marechal-Drouard et al., 1993). To define the tRNA^{Trp} gene family, we isolated seven tRNA^{Trp} genes from Arabidopsis (Lin et al., 1992; this work). Hybridization analyses with genomic gel blots suggest there might be only one or two additional members of the tRNA^{Trp} gene family (data not shown). All but one of the genes have identical coding regions, with the tRNA^{Trp2} gene containing a single transition (C → T in position +5, where +1 is the position of the first nucleotide of the mature tRNA; Figure 1).

The tRNA^{Trp} gene organization may be the result of an ancient gene dispersal event. All seven tRNA^{Trp} genes have unique flanking sequences, indicating they are not organized in a repetitive cluster, as are the plant tRNA^{Tyr} genes (Beier et al., 1991). Apart from the presence of an RNA polymerase III terminator element in the 3′ flank of each gene (Bogenhagen and Brown, 1981; Wang and Folk, 1994), there are no other recognizable conserved flanking sequence elements that suggest a sequence element required for expression. To determine whether these genes are expressed and to enable studies of their transcription in vivo, we mutated several to encode tRNAs having the anticodon CUA and thereby made them able to suppress reporter genes with an amber nonsense codon.

Refinement of the Translational Suppression Assay To Measure tRNA Gene Expression

Previously, we described a method for measuring plant tRNA gene expression in vivo that relies upon translational suppression of nonsense codons in the chloramphenicol acetyltransferase (*CAT*) reporter gene (Franklin et al., 1992). The recent development of the firefly luciferase (*LUC*) gene as an even more sensitive reporter for gene expression in plants encouraged us to use it in place of the *CAT* gene as a measure of nonsense suppression (Schultz and Yarus, 1990; Luehrsen et al., 1992). To that end, the *LUC* reporter gene was placed downstream of the cauliflower mosaic virus (CaMV) 35S promoter and the tobacco mosaic virus (TMV) translation enhancer, and the fourth (alanine) codon of the *LUC* sequence was altered to encode a Trp (UGG) codon or to encode amber (am; UAG), ochre (oc; UAA), or opal (op; UGA) stop codons (Figure 2A). The fourth codon of the *LUC* gene was chosen as the site for these changes because it has been demonstrated previously that N-terminal residues of LUC are dispensable for activity (Barnes, 1990). When DNAs encoding LUC$_{am}$, LUC$_{oc}$, or LUC$_{op}$ were introduced into carrot protoplasts, no LUC activity was detected. However, when plasmids containing the tRNA$^{Trp1}_{am}$ gene mutated to express a tRNA with a CUA anticodon were introduced together with the *LUC*$_{am}$ DNA, the expressed LUC activity reached 2 to 5% of that of the wild-type LUC control, or ~140-fold above the background luminescence observed with the *LUC*$_{am}$ DNA alone (Figure 2B).

In comparison with the previously reported assay for translational suppression using the *CAT* reporter gene, the LUC assay proved to be ~40-fold more sensitive; furthermore, polyethylene glycol (PEG)–mediated transformation (rather than electroporation, as previously described) required lower amounts of DNA. These modifications greatly improved our ability to study tRNA gene expression in plant cells.

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trp1  taaagatgcgaatctgtagtttgtttgatgttctgcaaaattcgcatgtataaaattaat 60
trp2  ttctcatgctaatggaaatggaccagcaaattccaatgttttattcaaccaaccaatgta
trp3  ..........................................................
trp4  agatgagagattgttgataacccttgagaaggcggcggtttgtaataaaacgatatgtg
trp5  tttggtcaccggccgtgttgggaacgcgtcctcaaaatgttttgtttaatttgcgtatac
trp6  ..............................tacagaaacataattatgtgactatg
trp7  ttacacaacaaatactaaactcatatcaatgggagagataaactactttagcaaagacat

trp1  taaacgtttagaaatatatagatgaactttatagtacaaaGGATCCGTGGCGCAATGGTA 120
trp2  ttttggtataaagaatattaaatgtaacagataagcaaggGGATTCGTGGCGCAATGGTA
trp3  ....................................GGATCCGTGGCGCAATGGTA
trp4  ttttactatataaatatcctttggtgcttgtaatcacaagGGATCCGTGGCGCAATGGTA
trp5  tacggtattatatattttataaatgcaacataataacatccGGATCCGTGGCGCAATGGTA
trp6  attagcgttatgattagggttaaggttaagagtattatccGGATCCGTGGCGCAATGGTA
trp7  cattgaaatatatgtaaggttgagtaatcattgtatatccGGATCCGTGGCGCAATGGTA

trp1  GCGCGTCTGACTCCAGATCAGAAGGTTGCGTGTTCGATTCACGTCGGGTTCAaatatccc 180
trp2  GCGCGTCTGACTCCAGATCAGAAGGTTGCGTGTTCGATTCACGTCGGGTTCAaaaaatcc
trp3  GCGCGTCTGACTCCAGATCAGAAGGTTGCGTGTTCGATTCACGTCGGGTTCAatcccccg
trp4  GCGCGTCTGACTCCAGATCAGAAGGTTGCGTGTTCGATTCACGTCGGGTTCAaaccccccg
trp5  GCGCGTCTGACTCCAGATCAGAAGGTTGCGTGTTCGATTCACGTCGGGTTCAatcccccg
trp6  GCGCGTCTGACTCCAGATCAGAAGGTTGCGTGTTCGATTCACGTCGGGTTCAaatccccc
trp7  GCGCGTCTGACTCCAGATCAGAAGGTTGCGTGTTCGATTCACGTCGGGTTCAaatccccc

trp1  gaatcttgatccttaatttttttttcctcttgggcttactacgactacataaggatc   237
trp2  cgaaacattattcggaattccccaacttttttttcctcgaggccgttaagagaatcc
trp3  aactttatccggatatctttgtttacctgcttattccaatttttgccttttgatgca
trp4  gtttaaacatgatccttaacttttattttaacttttaaaaaatatcctagattcaa
trp5  aacaatttccggttttttttttttttcttctcctgaagtctatcccatacggcccaa
trp6  gaacaataaatttccggaatatttttttacgtagtgtcggtcggcacattcaacatag
trp7  gaactatatattccggaatatctttttttgacatgtttttagtcaatggagtcaac
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Figure 1. Nucleotide Sequences of Seven tRNA^{Trp} Genes from the Nuclear Genome of Arabidopsis (Ecotype Columbia).

Each gene is given at left, with nucleotide numbers at right. The mature tRNA coding regions are shown in bold capital letters. The putative transcription termination signals in the 3′ flanking regions are indicated by bold lowercase letters. The sequences of three Arabidopsis tRNA^{Trp} genes (*trp1*, *trp2*, and *trp3*) have been published previously (Lin et al., 1992). EMBO/GenBank/DDBJ accession numbers are as follows: X57592 for tRNA^{Trp1}, X57593 for tRNA^{Trp2}, X57594 for tRNA^{Trp3}, L34745 for tRNA^{Trp4}, L35907 for tRNA^{Trp5}, L35908 for tRNA^{Trp6}, and L35909 for tRNA^{Trp7}. Dots were introduced to optimize alignment.

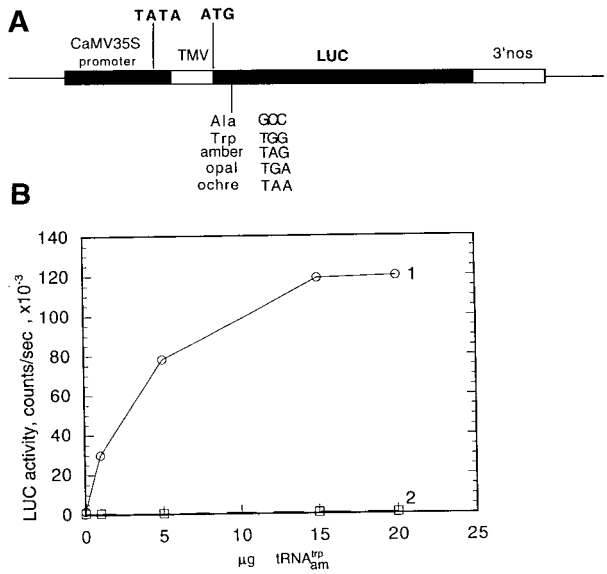

Figure 2. Assay for Translational Suppression Using Reporter Genes.

(A) Principal components of the *LUC* gene constructs used for transient expression experiments. The wild-type *LUC* gene sequence was altered to create Trp, amber, ochre, and opal codons in place of the fourth codon encoding alanine.

(B) LUC activity as a function of the quantity of suppressor tRNA gene. Twenty micrograms of the plasmid containing a 35S–*LUC*$_{am}$–*NOS* reporter gene was transfected into carrot protoplasts along with varying amounts of the suppressor tRNA$^{Trp1}_{am}$ gene (open circles, line 1) or with the wild-type tRNA^{Trp1} gene (open squares, line 2) cloned in pBluescriptII KS+. Each point represents the average of three independent experiments.

Expression of tRNA^{Trp} Genes in Vivo Is Dependent upon 5′ Flanking Sequences

The seven tRNA^{Trp} genes from Arabidopsis have no obvious sequence homologies in either the 5′ or 3′ flanks, apart from the putative terminator elements. To determine whether these genes are expressed in vivo, and whether significantly altered expression results because of the different 5′ and 3′ sequences flanking these genes, four of the tRNA^{Trp} genes (tRNA^{Trp1}, tRNA^{Trp2}, tRNA^{Trp4}, and tRNA^{Trp5}) were mutagenized to encode amber suppressors and were introduced into carrot protoplasts together with the *LUC*$_{am}$ gene. Measuring the extent of LUC suppression indicated they were all expressed, with only a threefold difference between them (Figure 3A). This small variation suggests it is unlikely that strictly conserved flanking sequence elements are required for transcription of plant tRNA genes.

To determine whether the sequences sufficient for tRNA$^{Trp2}_{am}$ expression were wholly contained within the fragment of genomic DNA present in the plasmid vector (210 bp of 5′ sequences and 70 bp of 3′ sequences), constructs were prepared containing seven or 24 copies of the tRNA and immediate flanking sequences. The observed levels of suppression increased proportionally with increasing molar amounts of the tRNA$^{Trp2}_{am}$ gene. A plasmid with seven copies, representing a fourfold molar excess of the tRNA^{Trp2} gene over the single-copy construct, suppressed LUC activity approximately fourfold more efficiently than did the single-copy construct (Figure 3B). This indicates that each tRNA$^{Trp2}_{am}$ is expressed independently. In the construct containing 24 copies, only a small increase in expression was observed over that of the construct containing seven copies, suggesting that some component of the expression system is saturable. This might occur at the level of transcription, processing, aminoacylation, or translation.

Comparison of the 5′ upstream regions of the four tRNA^{Trp} genes we have shown to be expressed in vivo, together with that of the tRNA^{Leu} gene shown to be expressed by Carneiro et al. (1993) and that of an Arabidopsis tRNA^{Lys} gene we have recently isolated and shown to be expressed (data not shown), indicates that all contain TATA-like sequences in the 5′ flanking region (Figure 4). Because the TATA binding protein (TBP) is required for expression of genes by RNA polymerase III in animal cells and yeast (Margottin et al., 1991; White et al., 1992; reviewed in Willis, 1993; White, 1994; Sprague, 1995), we decided to determine whether TBP binds to these 5′ sequences upstream of the plant tRNA coding sequences. To assess this, we performed DNase I footprint analyses of the noncoding and the coding DNA strands of the tRNA^{Trp1} gene both before and after incubation with recombinant TBP-2 from Arabidopsis. These analyses demonstrated binding by TBP to the 5′ flanking sequence of the tRNA^{Trp1} between nucleotides +1 and −52 (centered around −26) (Figure 5).

The observed binding of TBP to the 5′ flanking sequences prompted us to test whether the binding sites for TBP were important for transcription in vivo. When the natural 5′ flanking

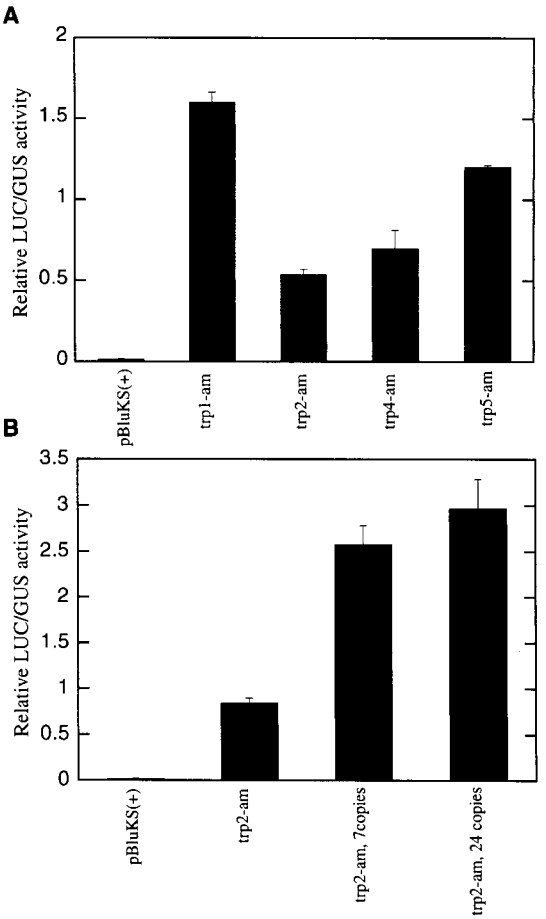

Figure 3. Relative Efficiencies of Nonsense Suppression among Different tRNA$^{Trp}_{am}$ Constructs.

(A) Relative efficiencies of nonsense suppression among four different tRNA$^{Trp}_{am}$ genes. Carrot protoplasts were transfected with 20 μg of *LUC*$_{am}$ reporter plasmid together with 5 μg of tRNA$^{Trp1}_{am}$, tRNA$^{Trp2}_{am}$, tRNA$^{Trp4}_{am}$, and tRNA$^{Trp5}_{am}$ plasmids, respectively. pBluescript KS+ (pBlu KS(+)) was used as a carrier DNA to keep the total amount of DNA (40 μg) constant in each experiment. All values are represented as the mean percentage of activity relative to the wild-type LUC activity. A β-glucuronidase (*GUS*) reporter construct was used as an internal standard to correct for transfection efficiency variability. Error bars represent the standard deviation (n = 3).

(B) Effect of the multimerization of the tRNA$^{Trp2}_{am}$ gene on expression. Carrot protoplasts were cotransfected with 20 μg of the *LUC*$_{am}$ reporter plasmid along with 5 μg of plasmid DNA containing one, seven, and 24 copies of tRNA$^{Trp2}_{am}$, respectively. pBluescriptII KS+ was used as a carrier DNA to keep the total amount of DNA (40 μg) constant in each experiment. All values are represented as the mean percentage of activity relative to the wild-type LUC activity. A β-glucuronidase reporter construct was included in each transformation as an internal standard to correct for transfection efficiency variability. Error bars represent the standard deviation (n = 3).

```
      -50                                                    -1
trp2    AACCAATGTA TTTTGGtata aaGAATATTA AATGTAACAG ATAAGCAAGG
leu     CAACTAACTA ATATATttat ataaCTTTTA TAGTAAAAAC TAACAAATGT
trp5    TTGCGTATAC TACGGtatta taatTTTATA AATGCAACAT AATAACATCC
trp1    TAAAATTAAT TAAACGttta GaaaTATATA GATGAACTTT ATAGTACAAA
trp4    ACGATATGTG TTTTACtata taaaTATCCT TTGGTGCTTG TAATCACAAG
lys1    TAGGTTAACG GTAATAttat ataCTGGTTG TCTAACTTAC AAATCACCAA
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Figure 4. Alignment of the 5′ Noncoding Sequences of Plant tRNA Genes That Have Been Shown To Be Expressed in Vivo.

The identity of each gene is indicated at left. The most proximal nucleotide of the noncoding 5′ region of tRNA genes is −1. TATA or TATA-like sequences are represented by lowercase letters.

sequences of $tRNA^{Trp1}_{am}$ were replaced with heterologous sequences derived from the cloning vector pBluescript KS+ that contained no TATA motifs (yielding construct Δ−1; Figure 6A), a 10-fold reduction in expression of LUC was observed (compare Trp1 with the Δ−1 construct; Figure 6B). Because the sequences immediately upstream of tRNA coding sequences have been shown to be important for efficient transcription in yeast and animal cells (Raymond and Johnson, 1983; Shaw and Olson, 1984; reviewed in White, 1994; and in Sprague, 1995), we tested the importance of the 10 bases most proximal to the 5′ end of the $tRNA^{Trp}_{am}$ gene; this includes a sequence (CAA) present in many of the known plant tRNA genes (B. Ulmasov and W. Folk, unpublished observations). However, only a small effect upon expression was observed when this sequence was introduced into the Δ−1 construct (yielding construct Δ−10 in Figure 6).

A general requirement for transcription is that the RNA polymerase open the DNA surrounding the site of initiation, and therefore, the effect of 5′ flanking sequences on expression might be attributable to differences in the thermodynamic stability of the sequences surrounding the transcription initiation site. Consequently, we empirically assessed the stability of these sequences, using the algorithm developed by Natale et al. (1992). The natural 5′ flanking sequences of the expressed $tRNA^{Trp}$ genes displayed one or several minima in DNA helical stability (ΔG) between −5 and −35 (Figure 7), and the average ΔG for the four $tRNA^{Trp}$ genes in this region was ~9 kilocalories per mol (Table 1). Replacement of these 5′ flanking sequences with plasmid sequences in the Δ−1 and Δ−10 mutants dramatically increased the stability of the sequences proximal to the tRNA gene (Table 1), because the natural AT-rich flanking sequences were replaced with highly GC-rich vector sequences. To address the possibility that the reduction in expression observed for the Δ−1 and Δ−10 constructs could be due to the changed thermodynamic stability of the 5′ flanking region, we substituted the flanking sequences in the Δ−1 construct with sequences from the early palindrome of the simian virus (SV40) replication origin. Sequences within the SV40 early palindrome do not possess a high AT content but have the low helical stability required to facilitate T antigen–induced melting and the initiation of DNA replication (Lin and Kowalski, 1994). Constructs containing the early palindrome in either orientation (Δ−1EP or $\Delta-1EP_{rev}$, the forward and reverse orientations, respectively) did not restore $tRNA^{Trp}$ expression (as measured by LUC activity), despite their 5′ flanking sequences having a ΔG similar to that of the natural $tRNA^{Trp}$ (Figure 6). From these data, it seems unlikely that the thermodynamic stability of the 5′ flanking sequences is the overriding factor governing tRNA expression.

Because the only common sequences among the 5′ flanking regions of the $tRNA^{Trp}$ genes are TATA-like elements and because TBP binds to the 5′ flanks of the $tRNA^{Trp1}_{am}$ genes in vitro, we sought to establish the importance for expression of the TATA elements in the 5′ region of the $tRNA^{Trp1}_{am}$ gene. The DNA sequence requirements of the Arabidopsis TBP for transcription in vitro have been published by Mukumoto et al. (1993), and based on this work, we introduced five point

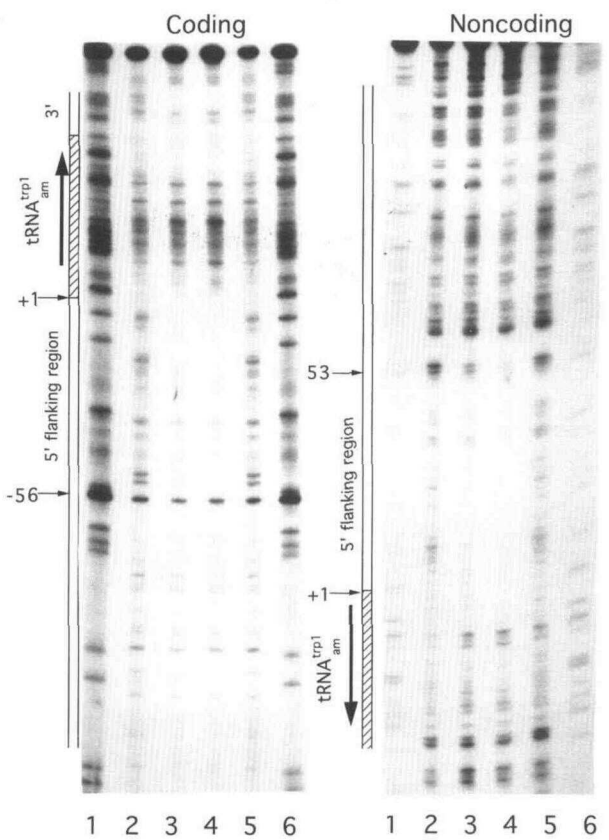

Figure 5. DNase I Footprint Analysis of Arabidopsis TBP Binding to $tRNA^{Trp1}_{am}$ 5′ Flanking Sequences.

The coding and noncoding DNA strands spanning base pair 200 to +128 (+1 is the first nucleotide of the mature $tRNA^{Trp1}$ sequence) were prepared as described in Methods. Reaction with dimethylsulfate (Sambrook et al., 1989) was used to prepare the G-track marker (lanes 1 and 6). DNA was incubated without protein (lanes 2 and 5) or with increasing amounts of recombinant Arabidopsis TBP (lanes 3, 100 ng; lanes 4, 300 ng). The +1 indicates the first nucleotide of the coding region of the $tRNA^{Trp1}$ gene, and −53 and −56 indicate the positions of nucleotides in 5′ flanking sequences with respect to the first nucleotide of the coding region.

A

```
                    -50                                          -1
Trp1                TAAAATTAATTAAACGTTTAGAAATATATAGATGAACTTTATAGTACAAA
Δ-1                 tatagggcgaattggagctccaccgcggtggcggccgctctagaactagt
Δ-10                tatagggcgaattggagctccaccgcggtggcggccgctcATAGTACAAA
Δ-1EP               cgctctagtaadcctcctcactacttctggaatagctcactagaactagt
Δ-1EPrev            cgctctagtgagctatttccagaagtagtgaggaggcttactagaactagt
Trp1TATAmut         TAAAATTAATTAAACGTTgAGAAAgAgATAtcTGAACTTTATAGTACAAA
Δ-1TATA-32          gcggtggcggcccggtgcTATAAAAggacgcgggccgctctagaactagt
Δ-10TATA-32         gcggtggcggcccggtgcTATAAAAggacgcgggccgctcATAGTACAAA
Δ-1TATA-24          gcggtggcggccgctctagcgcgtgcTATAAAAggacgcgtagaactagt
Δ-10TATA-24         gcggtggcggccgctctagcgcgtgcTATAAAAggacgcgATAGTACAAA
Δ-1TATA-24mut       gcggtggcggccgctctagcgcgtgcaAgAAAAggacgcgtagaactagt
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B

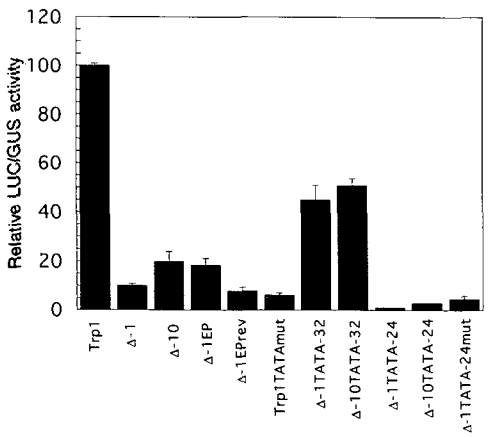

Figure 6. Effect of 5′ Flanking Sequences on the Expression of the tRNA$^{Trp1}_{am}$ Gene.

(A) Nucleotide sequences of 5′ flanking regions of tRNA$^{Trp1}_{am}$ mutants. The sequence of the native 5′ flanking region is shown on top in bold letters. Δ−1 and Δ−10 are deletion mutants of the 5′ flanking region to positions −1 and −10, respectively. The native 5′ region of tRNA^{Trp1} in these mutants was replaced with the sequence of the vector pBluescript KS+. Δ−1EP contains the sequence of the early palindrome of the SV40 replication origin from position −10 to −50 of the Δ−1 mutant. The Δ−1EPrev construct contains the same sequence in opposite orientation. Trp1TATA$_{mut}$ resulted from five point mutations (lowercase letters) of TATA elements in the natural 5′ region of the tRNA^{Trp1} gene. An oligonucleotide (stippled boxes) containing the TATA box from the adenovirus 2 major late promoter was inserted in two different positions (−32 and −24). Constructs resulting from these insertions are Δ−1TATA$_{-32}$, Δ−10TATA$_{-32}$, Δ−1TATA$_{-24}$, and Δ−10TATA$_{-24}$, respectively. Δ−1TATA$_{-24mut}$ contains two point mutations (lowercase letters) in the TATA box of the Δ−1TATA$_{-24}$ mutant. **(B)** The effect of 5′ flanking sequences on the expression of the tRNA$^{Trp1}_{am}$ gene. Carrot protoplasts were cotransfected with 20 μg of *LUC*$_{am}$ reporter plasmid along with 20 μg of different tRNA$^{Trp1}_{am}$ plasmids containing mutations in the 5′ flanking region. All values are represented as the mean percentage of activity relative to the pair LUC$_{am}$/tRNA$^{Trp1}_{am}$. The results shown were obtained with the same preparation of protoplasts to minimize variability due to cell competence or viability. The β-glucuronidase (*GUS*) reporter construct was used as an internal standard to correct for transfection efficiency variability. Error bars represent the standard deviation (n = 3).

mutations in the 5′ flanking region of the tRNA$^{Trp1}_{am}$ gene to eliminate TBP binding (the resulting construct is denoted as Trp1TATA$_{mut}$). The effect of these point mutations upon expression of the tRNA^{Trp} gene was dramatic, because the suppression of LUC was reduced ~15-fold (Figure 6).

The 5′ flank of the tRNA$^{Trp1}_{am}$ gene contains several possible TATA elements in the region of −20 to −32. To define whether a single element was important, a TATA element from the adenovirus 2 major late promoter was introduced into the Δ−1 and Δ−10 constructs at nucleotide −32. This increased *LUC* expression approximately fourfold, suggesting that the TATA element in this position is an important modulatory element. However, because this expression is only 40 to 45% of the wild-type level, it is likely that either additional 5′ modulatory elements are required for maximal expression or the position of the newly introduced TATA element is not optimal. A small change in the position of the TATA element in the 5′ flanking sequences had a surprisingly strong effect upon tRNA gene expression; introduction of the adenovirus TATA box at nucleotide −24 relative to the start site profoundly reduced suppression, and this effect was not reversed by the natural immediate 5′ flanking sequences (compare Δ−1TATA$_{-24}$ and Δ−10TATA$_{-24}$ with Δ−1TATA$_{-32}$ in Figure 6B).

We attempted to analyze the RNA products of these genes transfected into carrot protoplasts by primer extension with a primer ending in the unique amber anticodon but were not successful because the reverse transcriptase encountered a major stop at position +25 and generated truncated products (data not shown). According to the published sequence of tRNA^{Trp} from wheat germ (Ghosh and Ghosh, 1984), position +25 is a 2,2-*N*-dimethylguanine, which is likely to block the reverse transcriptase. This problem, together with a very low level of expression of the deletion mutants in transient assays, prevented us from mapping the transcription starts of these constructs expressed in vivo.

Analysis of the in vitro transcription of these constructs with a heterologous extract from human 293 cells proved informative: transcription of the wild-type tRNA$^{Trp1}_{am}$ gene, the deletion mutants, and the constructs with the TATA element at nucleotide −32 yielded transcripts of similar sizes, indicating that the tRNAs were initiated at approximately the same place (Figure 8, lanes 3 to 7). However, transcription of the constructs with the TATA element at nucleotide −24 yielded shortened transcripts, suggesting that transcription of these DNAs might be initiated within the tRNA coding sequences. Such an origin would explain why these constructs inefficiently suppress LUC (Figure 8, lanes 8 and 9). To determine the 5′ ends of these transcripts, we carried out a primer extension analysis of the in vitro transcripts (Figure 9). Results of this analysis indicated that transcription of the tRNA$^{Trp1}_{am}$ and the Δ−10 constructs begins at position −3 (where +1 is the position of the first nucleotide of the mature tRNA; Figure 9, lanes 2 and 6), whereas transcription of the Δ−1 and the Δ−1TATA$_{-32}$ constructs begins predominantly at position −1 (Figure 9, lanes 3 and 4), and transcription of the Δ−1TATA$_{-24}$ and

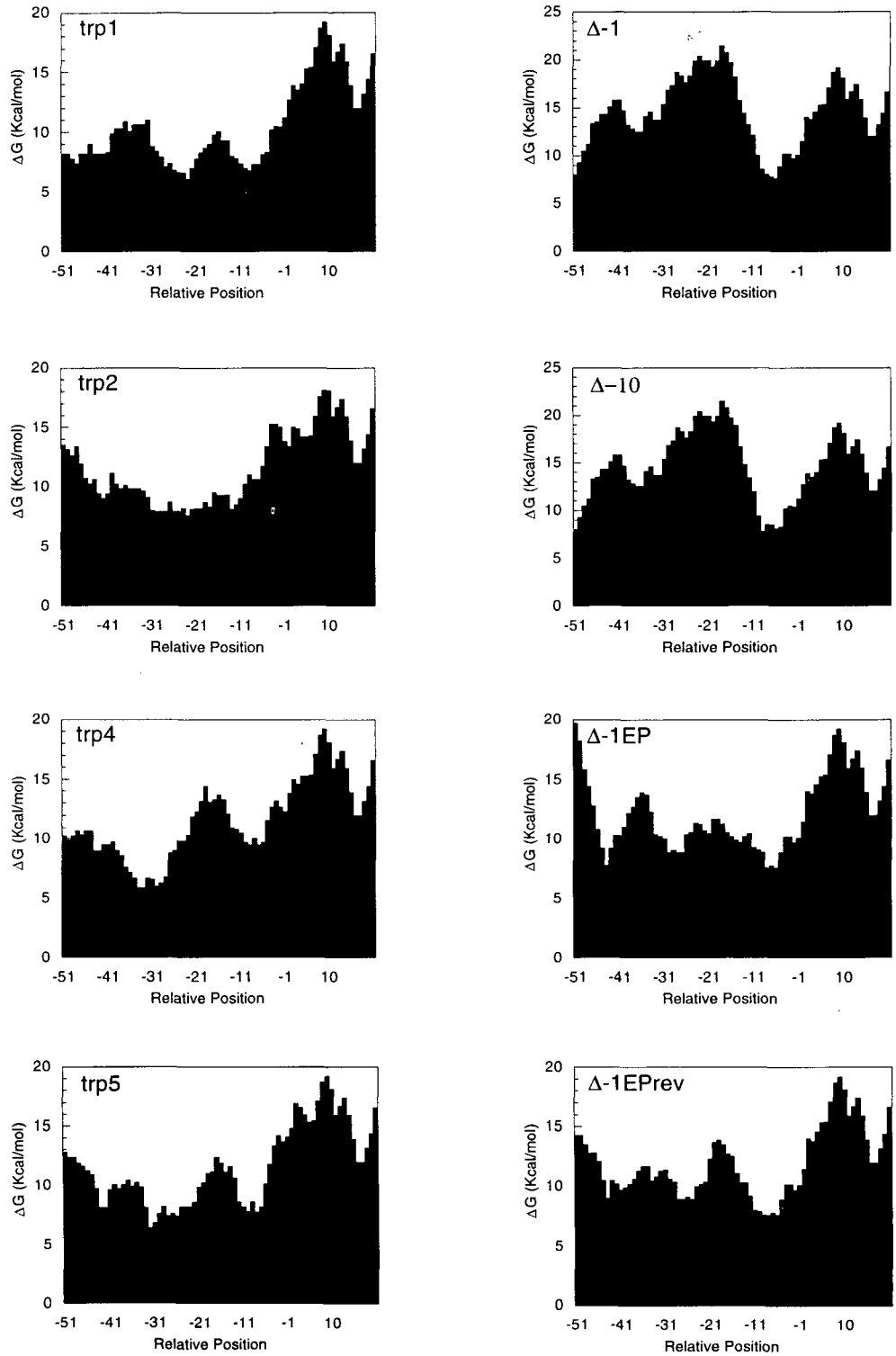

Figure 7. Computer-Calculated DNA Helical Stability.

DNA helical stability for 5′ flanking sequences of natural $tRNA^{Trp}$ genes and for 5′ flanking sequences of mutant $tRNA^{Trp1}_{am}$ genes was calculated using the Thermodyne program described by Natale et al. (1992) with the default set at 25°C and 10 mM Na^+. The window size used in the calculations was 10 nucleotides.

Table 1. Average ΔG for the Native and Modified 5′ Flanking Sequences of tRNA^{Trp} Genes

Constructs	ΔG(kcal/mol)[a]
Trp1	8.7
Trp2	8.7
Trp4	9.7
Trp5	9.7
Δ − 1	16.8
Δ − 10	16.8
Δ − 1EP	10.8
Δ − 1EP$_{rev}$	10.7
Trp1TATA$_{mut}$	9.7
Δ − 1 + TATA$_{-32}$	15.2
Δ − 10 + TATA$_{-32}$	15.3
Δ − 1 + TATA$_{-24}$	14.5
Δ − 10 + TATA$_{-24}$	14.5
Δ − 1 + TATA$_{-24mut}$	14.6

[a] ΔG was calculated within the region from nucleotides − 10 to − 40 (+1 is the first nucleotide of the mature tRNA^{Trp} sequence). kcal, kilocalorie.

Δ−10TATA$_{-24}$ constructs begins at position +9, inside the sequences of the mature tRNA (Figure 9, lanes 5 and 8). These results help explain the analysis of in vivo expression of these tRNA gene constructs in carrot protoplasts: transcription of the wild-type gene, genes lacking TATA elements, or genes having a single TATA element at +32 is initiated outside the coding sequence of the mature tRNA; transcription of the constructs with the single TATA element at −24 is initiated within the coding sequence. Therefore, the poor expression of the Δ−1TATA$_{-24}$ mutant in the suppression assay is probably due to those transcripts' inability to function as tRNAs. The good correlation between the expression in vivo, as measured by the suppression assay, and the products transcribed in the human extract suggests that this is a reasonable explanation for the importance of the location of the TATA element upon expression of the tRNA^{Trp} genes.

Analysis of 3′ Sequence Elements Required for tRNA Expression

Deletion of the natural 3′ flanking sequences of tRNA$^{Trp1}_{am}$ (yielding construct Trp1$_{am}$ (−3′); Figure 10A) diminished LUC expression, indicating that expression of the tRNA genes was impaired. Analysis of the in vitro transcription of this construct revealed that termination occurs at a site in the plasmid sequence farther downstream (Figure 8, lane 10). Introduction of a heterologous SV40 sequence capable of serving as a terminator for wheat germ RNA polymerase III transcription (Wang and Folk, 1994) at the approximate position of the natural terminator (yielding construct Trp1$_{am}$(−3′) term; Figure 10A) completely restored LUC activity, indicating that a properly positioned terminator element is the only 3′ flanking sequence required for efficient expression of these tRNA genes (Figure 8, lane 11, and Figure 10B).

DISCUSSION

This study demonstrates the DNA sequence requirements for plant tRNA gene transcription. We defined a modulatory role for sequences in the 5′ and 3′ flanking regions. In addition, our experiments provide insights into the role of 5′ flanking sequences in tRNA gene expression in all eukaryotes. We believe that most of the effects of changes in the 5′ and 3′ flanking experiments we have observed through these in vivo assays are caused by alterations in transcription, although in vitro assays using a homologous system must be performed to prove this point formally.

Despite the fact that the tRNA internal control regions contain elements essential for expression of most eukaryotic tRNA genes, many studies have demonstrated that 5′ flanking sequences also modulate their transcription (for recent reviews, see White, 1994; Sprague, 1995). In several instances, the importance of extragenic 5′ and 3′ sequences has been demonstrated in vivo (Shaw and Olson, 1984; Allison and Hall, 1985; Campbell et al., 1985; Marschalek and Dingermann, 1988; Tapping et al., 1993); however, it is unclear how these sequences affect transcription initiation, because neither the proteins binding these sequences nor the structural features the sequences might confer on the DNA have been well defined.

Sequences that might influence transcription initiation are those bound by TBP, which is required for transcription of all class III templates, including tRNA genes (Cormack and Struhl, 1992; Schultz et al., 1992; White et al., 1992). However, how TBP interacts with the template and with the rest of the transcription machinery has not been fully resolved. The paradigm at present is the one developed for transcription of yeast tRNA genes, which suggests that TBP does not directly interact with

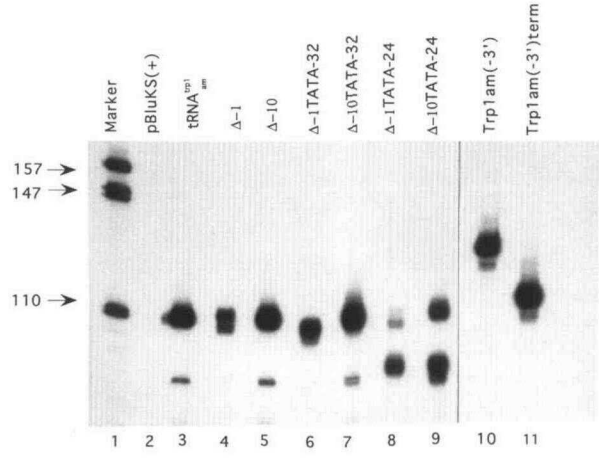

Figure 8. In Vitro Transcription Analysis of tRNA^{Trp} Constructs.

The transcription of tRNA$^{Trp1}_{am}$ genes with different 5′ and 3′ regions (see Figures 6 and 10) was assayed using a human 293 cell S100 extract and analyzed on a 6% polyacrylamide gel. The pBluescript SK−/HpaII digests were used as molecular length markers, which are given at left in base pairs.

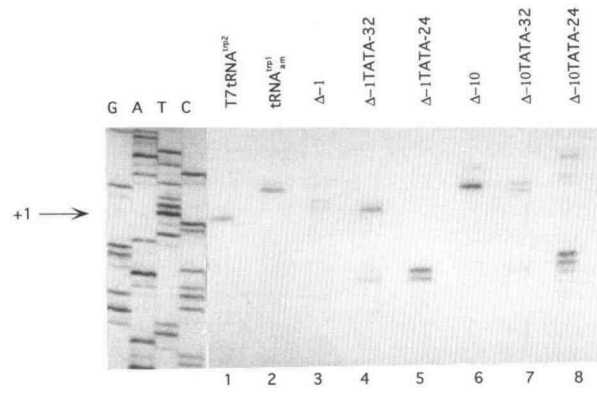

Figure 9. Primer Extension Analysis of RNA Species Produced by Human Extracts.

An oligonucleotide complementary to nucleotides +72 to +44 of tRNA^{Trp1} was used to prime the reverse transcription of in vitro transcription products. The tRNA^{Trp} sequence ladder was generated using the wild-type tRNA^{Trp} gene and the same primer. The lane adjacent to the sequence ladder contains the primer extension product of a synthetic tRNA^{Trp2} transcript, produced by T7 RNA polymerase with a size corresponding to that of the mature tRNA. The +1 indicates the first nucleotide of the coding region of the tRNA$^{Trp1}_{am}$ gene.

upstream DNA. Yeast tRNA genes rarely contain TATA boxes in their 5′ flanking sequences, and efforts to cross-link the TFIIIB complex to DNA resulted in the cross-linking of 70- and 90-kD polypeptides to the 5′ flanking sequences but not to TBP (Kassavetis et al., 1992; Bartholomew et al., 1993). In the same vein, a mutation of TBP that blocks its binding to TATA boxes in vitro has no effect on the ability of TFIIIB to bind to DNA and to promote transcription of tRNA genes (Schultz et al., 1992).

On the other hand, circumstantial evidence suggests that upstream sequences provide a site of TBP interaction with tRNA genes, either directly or indirectly (reviewed in White, 1994; Sprague, 1995). Upstream sequences frequently contribute to transcription efficiency, and these upstream regions often contain AT-rich elements that resemble class II TATA boxes. For example, in *Bombyx mori*, two AT-rich sequences in the 5′ flank of the tRNA^{Ala} gene modulate transcription (Palida et al., 1993) and may act by promoting binding of TBP-associated TFIIIB. The 5′ flanking regions of plant tRNA genes have AT-rich sequences, and plants have the highest AT content (between nucleotide positions −29 and −33 upstream of the tRNA genes) among five different groups (insects, fungi, protozoa, plants, and vertebrates; Palida et al., 1993). A role for TATA elements in positioning the 5′ ends of transcripts of yeast tRNA genes has also recently been reported by Chalker and Sandmeyer (1993). There, introduction of a TATA element proximal to a tRNA gene caused transcription to initiate at several new positions; however, the effect was relatively minor compared with that we have observed.

Our data with the plant tRNA^{Trp} genes support the notion that plant TBP may interact directly with upstream DNA. Our experiments reveal that point mutations that inactivate the upstream TATA elements of the tRNA$^{Trp1}_{am}$ gene, or that the substitution of the natural 5′ region with artificial ones lacking TATA elements, profoundly reduce tRNA gene expression in vivo. Furthermore, shifting the position of a single TATA element in the upstream sequences profoundly affects the site of transcription initiation in vitro, and the pattern of expression is consistent with the results of suppression observed in vivo. Introduction of a single TATA element at a position equivalent to that of a highly conserved TATA element in plant tRNA genes

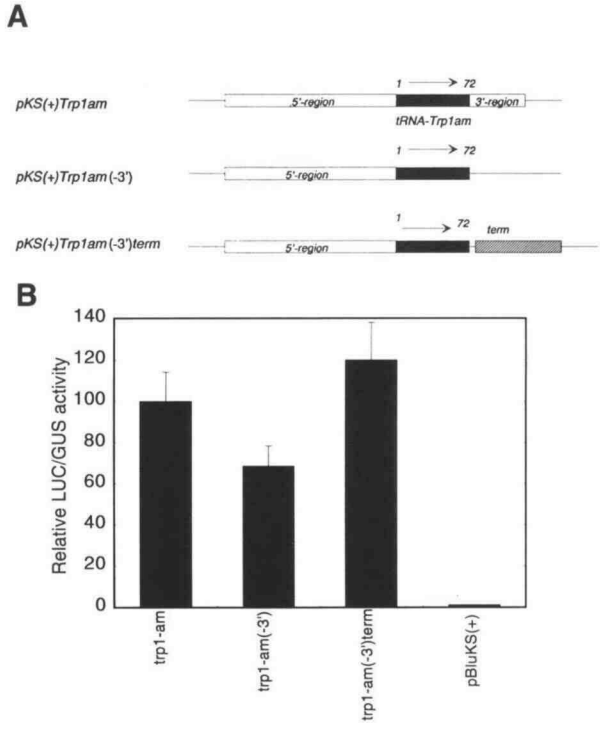

Figure 10. Effect of 3′ Flanking Sequences on Expression of the tRNA$^{Trp1}_{am}$ Gene.

(A) Constructs with sequence rearrangements in the 3′ flanking sequence of the tRNA$^{Trp1}_{am}$ gene. The top line represents the 350 bp of the Arabidopsis tRNA$^{Trp1}_{am}$ gene. The filled boxes represent the 72-bp–long structural gene, and the arrows indicate the direction of transcription. The striped box represents a 179-bp fragment from the SV40 origin of replication that contains a stretch of eight thymidine residues (Wang and Folk, 1994) located ~30 bp from the end of the coding region of the tRNA$^{Trp1}_{am}$ gene.

(B) The effect of 3′ flanking sequences on expression of the tRNA$^{Trp1}_{am}$ gene. Carrot protoplasts were transfected with 20 μg of the *LUC*$_{am}$ reporter plasmid, along with 20 μg of plasmid tRNA$^{Trp1}_{am}$ containing mutations in the 3′ flanking region. All values are represented as the mean percentage of activity relative to the pair LUC$_{am}$/tRNA$^{Trp1}_{am}$. Error bars represent the standard deviation (n = 5). pBluKS+, pBluescript KS+.

enhances transcription of the genes with substituted 5′ flanking sequences but does not completely restore transcription. This failure to restore transcription completely may be due either to suboptimal localization of the mammalian TATA box or to a requirement for additional sequences.

Gerlach et al. (1995) have recently suggested that sequences immediately downstream of the yeast *U6* TATA box influence TFIIIB binding to DNA or subsequent steps in RNA polymerase III transcription initiation. There may be a similar requirement for expression of these plant tRNA genes.

The in vitro transcription system we used to study the effect of the TATA box in these plant tRNA genes is derived from mammalian cells; this suggests that the mammalian transcription apparatus might also respond to TATA elements in the 5′ flanking sequence of tRNA genes, despite these elements not occurring as frequently in mammalian tRNA genes as in genes of plants and invertebrates.

Our current thinking about the relative contributions of the TATA element and the internal promoter elements has been strongly influenced by the elegant studies of the yeast *U6* gene in which both a TATA element and the classical A and B box elements appear to contribute to transcription efficiency and to initiation site selection by RNA polymerase III (Burnol et al., 1993; Chalker and Sandmeyer, 1993; Eschenlauer et al., 1993; Gerlach et al., 1995). There, the roles of both the TATA element and the A block in helping position TFIIIB suggest that TBP (as part of the TFIIIB complex) might be capable of contacting DNA specifically. There may be alternative forms of TFIIIB, one of which is capable of interacting with the TATA element and the other with TFIIIC bound to the A block, or TFIIIB might be capable of interacting with both the TATA element and TFIIIC simultaneously. The plant TFIIIB complex might require such coordinate interactions for transcription of tRNA genes; the comparable TFIIIB complexes from yeast might not. Clearly, additional work is required to test these thoughts, and such studies are in progress.

Our goal is to develop tRNA suppressors that function efficiently in plants to modify amino acid composition of storage proteins and to use tRNA genes to result in the synthesis of antisense RNAs (Bourque and Folk, 1992) and ribozymes (X. Bu, A. Kenzior, M. Zwick, and W. Folk, unpublished data). These insights into the DNA sequence elements affecting tRNA gene expression should help us to control the expression of tRNA toward those ends.

METHODS

Isolation and Sequence Analysis of tRNA^{Trp} Genes

An *Arabidopsis thaliana* ecotype Columbia EMBL3 genomic library (generously supplied by H. Klee, Monsanto Corp., St. Louis, MO) was screened by using an amplified polymerase chain reaction (PCR) fragment as a probe containing the sequence of tRNA^{Trp2} from Arabidopsis (Lin et al., 1992). DNAs were transferred to nylon membranes and prehybridized in 5 × SSPE (1 × SSPE is 180 mM NaCl; 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4) buffer containing 1% SDS and 100 μg/mL heparin at 58°C for 20 hr. Hybridization was performed at 58°C for 20 hr, using the same buffer containing 50 ng of a 350-bp PCR product containing the tRNA^{Trp2} ^{32}P-labeled DNA by random priming (Feinberg and Vogelstein, 1983). After hybridization, the filters were washed once with 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) buffer containing 0.1% SDS at room temperature, then washed twice at 65°C for 20 min, then twice in 0.3 × SSC buffer containing 0.1% SDS at 65°C for 20 min. Five phage isolates were purified, and their DNAs were sequenced directly in phage with the *fmol* DNA sequencing system (Promega). Specific primers to the tRNA^{Trp} coding region in opposite orientations were used to sequence 5′ and 3′ regions of new tRNA genes, using phage DNA as a template, and then primers specific to the 5′ and 3′ regions were used to sequence coding regions of tRNA^{Trp} genes. tRNA^{Trp} genes were subcloned into the EcoRV site of pBluescriptII KS+ (Stratagene), yielding constructs designated as Trp1, Trp2, Trp3, and Trp5.

Construction of Suppressor tRNA^{Trp} Genes and Mutant Luciferase Genes

The anticodon sequences of four tRNA$^{Trp}_{cca}$ genes (1, 2, 4, and 5) were altered to CTA (complementary with the amber UAG stop codon). Mutagenesis was performed using PCR and trimolecular ligation. This approach utilized two oppositely directed specific primers (one of them with a 5′ end at the amber codon and the other with one at the adjacent nucleotide), and the reverse and forward M13 primers, to prepare two pieces of tRNA^{Trp} sequence in pBluescriptII KS+, thus introducing specific mutation(s). Each PCR product was digested with a unique restriction enzyme from the polylinker of pBluescriptII KS+. The vector pBluescriptII KS+ DNA was digested with the same enzymes and ligated with the two PCR products in one ligation reaction. Templates used for mutagenesis were derivatives of the plasmid pBluescriptII KS+, with the tRNA^{Trp} gene cloned in the EcoRV site of the polylinker. The plasmid with 24 copies of tRNA$^{Trp2}_{am}$ was constructed by ligation of the 350-bp StyI PCR product of tRNA$^{Trp2}_{am}$ with the pBluescriptII KS+ vector digested by SmaI in the presence of polyethylene glycol.

For the construction of luciferase (*LUC*) genes containing nonsense codons, the plasmid pGEM-luc (Promega) was used as a source of *LUC* DNA and plasmid p33TSN (kindly provided by A. Franco, University of Missouri–Columbia) was used as a source of the cauliflower mosaic virus (CaMV) 35S promoter, the tobacco mosaic virus (TMV) Ω leader, and the nopaline synthase (*NOS*) terminator. An EcoRI fragment of p33TSN containing the CaMV 35S promoter, TMV Ω leader (followed by the NcoI site), and a *NOS* terminator was subcloned into the EcoRI site of the polylinker of pBluescriptII KS−. The resulting plasmid (pKS[−]35SE) was mutagenized using PCR and trimolecular ligation. Nonsense mutants of the *LUC* gene fused to the CaMV 35S promoter and TMV translational enhancer were constructed by trimolecular ligation of the NcoI-SalI fragment of the vector pKS(−)35SE and a NcoI-SpiI–digested PCR product of the *LUC* gene carrying the specified mutations and the SpiI-SalI *LUC* gene fragment from pGEM-luc. All mutations were confirmed by sequencing using Sequenase (U.S. Biochemical Corp.).

Transformation of Carrot Protoplasts

Carrot (*Daucus carota*) protoplasts were prepared as described previously (Franklin et al., 1992). Introduction of DNA into carrot protoplasts

was performed as described for tobacco (*Nicotiana tabacum*) protoplasts (Goodall et al., 1990), with modifications; the yield of carrot protoplasts was up to 1.5 × 10^8 from a 4-day-old 40-mL suspension culture, which is approximately sixfold greater than that reported for tobacco protoplasts. Protoplasts were pelleted at 60*g* for 5 min and then washed twice with 30 mL of W5 solution (154 mM NaCl, 5 mM KCl, 125 mM $CaCl_2$, and 5 mM glucose, pH 6.0) and resuspended in W5 at 2 to 4 × 10^6 protoplasts per mL. The protoplasts were stored in this solution for 0.5 to 4 hr at 5 to 8°, and immediately before transformation, the protoplasts were sedimented by centrifugation and resuspended at the same concentration in solution MC (5 mM Mes, 20 mM $CaCl_2$, 0.5 M mannitol, pH 5.7). Plasmid DNA (40 μg, prepared by alkaline lysis and purified through CsCl gradients) was mixed at room temperature with 10^6 protoplasts in 0.3 mL of MC solution. Three-tenths of a milliliter of 40% polyethylene glycol (PEG) 8000 solution (40 g of PEG 8000 was dissolved in 70 mL of solution containing 0.1 M $Ca(NO_3)_2$ and 0.4 M mannitol; the solution was adjusted to pH 10 with KOH, filter sterilized, and stored frozen in aliquots) was added, and the samples were gently mixed and incubated at room temperature for 5 min. Samples were diluted with 4 mL of hormone-free carrot suspension culture medium, using 4.3 g/L Murashige and Skoog salts (Sigma) with 1 mg/L each of thiamine, pyridoxine, and nicotinic acid, 4 mg/L glycine, 4% sucrose, pH 5.8, and incubated at room temperature for 24 hr.

LUC Assays

Carrot protoplasts were pelleted at 200*g* for 15 min, the supernatant was removed, and protoplasts were resuspended in 100 μL of cell culture lysis reagent (Promega). From 2 to 20 μL of cell extract was mixed with 100 μL of room temperature luciferase assay reagent (Promega). The reaction was placed in a luminometer (model 3010; Analytical Scientific Instruments, Alameda, CA) and counted for 10 sec. Assays from cells transformed with the wild-type *LUC* gene were performed after 1:200 dilutions of extracts so that the assays remained within the linear range. β-Glucuronidase assays were performed as described by Jefferson (1987).

Construction of 5′ and 3′ Deletion Mutants of $tRNA^{Trp1}_{am}$

A 5′ deletion mutant of the $tRNA^{Trp1}_{am}$ gene was constructed by BamHI-digestion of $Trp1_{am}$, followed by religation, yielding the deletion mutant Δ−1, lacking the natural 5′ flanking sequence up to and including nucleotide −1. The Δ−1TATA$_{-32}$ and Δ−1TATA$_{-24}$ 5′ mutants were constructed by cloning an annealed double-stranded oligonucleotide containing the adenovirus 2 major late promoter (a gift of M. Martin, University of Missouri–Columbia) into the NotI and XbaI sites of Δ−1, respectively. The Δ−10, Δ−10TATA$_{-32}$, and Δ−10+TATA$_{-24}$ mutants were constructed by reintroducing the first 10 nucleotides (−1 to −10) of the 5′ flanking region of the $tRNA^{Trp1}$ gene into Δ−1, Δ−1TATA$_{-32}$, and Δ−1+TATA$_{-24}$ constructs, respectively. Mutants containing the early palindrome in either orientation (Δ−1EP and Δ−1EP$_{rev}$, the forward and reverse orientations, respectively) were constructed by introduction of a double- stranded oligonucleotide containing the sequence of the early palindrome of simian virus 40 (SV40) replication origin (Lin and Kowalski, 1994) into the XbaI site of the Δ−1 mutant. The point mutations in the TATA elements of the 5′ regions of the $tRNA^{Trp1}_{am}$ gene were generated by PCR (resulting in a Trp1TATA$_{mut}$ construct). A 3′ deletion mutant of the $tRNA^{Trp1}_{am}$ gene was constructed using a PCR product containing the coding region of the $tRNA^{Trp1}_{am}$ gene and the 5′ flanking region cloned into the EcoRV site of pBluescript KS+. The deletion mutant $Trp1_{am}$(−3′) lacks the natural 3′ flanking sequences (including the putative transcription termination site) up to the first nucleotide downstream from the coding region. A 3′ deletion mutant $Trp1_{am}$(−3′) term with an artificial terminator of transcription was constructed by cloning a PCR product from the origin of replication of SV40, shown to terminate wheat germ polymerase III transcription efficiently (Wang and Folk, 1994) into the SalI-HindIII–digested plasmid $Trp1_{am}$(−3′).

The sequences of all mutants were confirmed by DNA sequencing using Sequenase (U.S. Biochemical Corp.).

DNase I Footprinting

DNA templates used for footprinting on the coding and noncoding strands of $tRNA^{Trp1}$ flanking sequences were prepared by isolating the EcoRI-KpnI and HindIII-SacI fragments from $Trp1_{am}$ and end-labeled with phosphorus-32 by the Klenow fragment of DNA polymerase I. The A Arabidopsis TATA-binding protein (TBP) (At2; Gasch et al., 1990; a gift of N.-H. Chua, Rockefeller University, New York, NY) was purified from *Escherichia coli* as a glutathione *S*-transferase fusion (construct pGEX-2T-At2; kindly prepared and provided by M. Zwick, University of Missouri–Columbia) and cleaved with thrombin to isolate TBP from the glutathione *S*-transferase tag. TBP was incubated with 6 ng of the ^{32}P-labeled DNA fragment in 20 μL of assay buffer (400 ng poly[dG-dC], 10 μg of BSA, 15 mM Hepes, pH 7.9, 50 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 0.2 mM EDTA, 10% glycerol) for 40 min at room temperature. Then, assay buffer was added to each sample up to 200 μL. DNase I (25 ng) was added to each sample, and digestion was continued at room temperature for 2 min. The reaction was terminated by adding 100 μL of stop solution (0.2 M NaCl, 30 mM EDTA, 100 mg/mL yeast RNA). After phenol–chloroform extraction, ethanol precipitation, and washing, the digested DNA was resuspended in 5 μL of gel loading buffer and loaded onto a 6% sequencing gel. DNA bands were detected by autoradiography after overnight exposure at −70°C with intensifying screens.

In Vitro Transcription

Transcription assays were performed using 200 ng of linear DNA in 25-μL reactions containing 35 μL of protein of human 293 cell S100 extracts (Yoshinaga et al., 1987; as described by Scanlon and Folk, 1991). The products were analyzed on 6% polyacrylamide, 8 M urea denaturing gels.

Primer Extension Analysis

An oligonucleotide complementary to nucleotides +72 to +44 of the $tRNA^{Trp1}$ gene was radiolabeled with γ-^{32}P-ATP and used to prime extension reactions of RNAs produced by the human 293 cell extract. To minimize the problems from strong secondary structure of tRNA, extension was performed at 70°C with Tth DNA Polymerase (Promega).

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