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Novel non-suppressing mutants of *Escherichia coli* $tRNA^{Tyr}$ su_3^+

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ABSTRACT: Several addition and deletion mutations were constructed in the region of the gene for Escherichia coli $tRNA^{Tyr}$ su_3^+ corresponding to the dihydrouracil loop of the mature tRNA. None of these resulting mutants had detectable suppressor function compared to the parent gene yet some directed the synthesis of mature tRNA. These latter mutants may affect the ability of the tRNA to be aminoacylated or to interact with the translational machinery on the ribosome.

INTRODUCTION:

Many mutant derivatives of Escherichia coli $tRNA^{Tyr}$ su_3^+ have been isolated which are defective in suppressor function (for a compilation see Celis and Piper, 1982). The loss of suppressor function is usually well correlated with the relative amount of mature tRNA made in vivo (1-6). Base substitution mutations near the aminoacyl stem of the tRNA have also been shown to effect suppressor function by altering the specificity of aminoacylation (3,7). In addition, other base substitutions in the D-loop and anticodon stem affect the kinetics of aminoacylation and protein synthesis in vitro (2,3,8) whereas the anticodon suppressor mutation, G35-C35, does not. The base substitution, G15-A15, makes only 5% the usual amount of mature tRNA yet has no detectable suppressor activity indicating that post-transcriptional function of the tRNA may be severely affected. These data, and those concerning su_7^+ function (9) and various studies of the cross-linking of aminoacyl synthetases to tRNA (10-12) suggest that, at a minimum, the D-loop region of tRNAs may be an important contact region in tRNA function during aminoacylation and translation.

We have taken advantage of a unique restriction site in the D-loop of $tRNA^{Tyr}$ su_3^+U43 to create in vitro a series of deletion and addition mutations in this region of tRNA. All the deletion mutants lack at least A14, an invariant nucleotide thought to be important for

maintaining the tertiary structure of the mature tRNA while the addition mutants contain extra nucleotides between A14 and G15. None of the new mutants suppress amber mutations in the T4 encoded lysozyme gene or gene 14, or in the E. coli gene for beta galactosidase. However, the gene transcripts can be processed both in vivo and in vitro by RNase P. As with the mutant G15-A15, mature tRNA is produced in vivo but these new mutant tRNAs must be defective in some aspect of aminoacylation or translation.

METHODS

Bacterial and bacteriophage strains: BF266 (13), A49 (14), $CA27_4$ (15), HB101 (16), and 5K (17) have all been described previously as have the bacteriophage ø80, ø80psu$_3^+$ (15), and ø80 DHA61.3 (6). T4 strains eL1 and B20 were gifts of Dr. W.H. McClain (University of Wisconsin).

Enzymes: Restriction enzymes (New England Biolabs), S1 nuclease (Boehringer-Mannheim), mung bean nuclease (PL Biochemical), DNA polymerase, Klenow fragment (New England Biolabs), calf intestinal phophatase (Boehringer-Mannheim) and T4 DNA ligase (New England Biolabs) were used under the conditions recommended by the manufacturers.

Suppression assays: Assays for suppression of $lacZ_{am}$ mutants were carried out using a blue plaque or blue colony assay as generally described by Russell et al (15) and Feinstein (35). Plaque assays for suppression of T4 amber mutants were carried out by plating on non-suppressor hosts with suitable controls for leakiness. The absence of suppressor function is well correlated with the absence of suppressor tRNA in both assays we used (35).

Subcloning of p61.3: p61.3, a plasmid carrying the $tRNA^{Tyr}$ su_3^+U43 gene, was subcloned to produce a recombinant plasmid containing the su_3^+U43 gene which was devoid of all Ava I sites except the site found in the D-loop of the tRNA gene (Fig. 1). p61.3 was used as the parent plasmid since plasmids containing the intact su_3^+ gene are not amplified significantly (6,36).

1 µg of pBR322 DNA was digested to completion with Ava I, treated with ribonuclease S1, recircularized with DNA ligase and used to transform competent 5 K cells. DNA from each transformant was purified by the mini-screen method (18). Each recombinant was digested with both Ava I and Pst I (there is one Pst 1 site in pBR322 at position 3612, (19)) and electrophoresed on a 1% agarose gel to distinguish between

plasmids which had not been digested with S1 nuclease (cut by both Ava I and Pst I) and those which had been digested with S1 nuclease and had thus lost the Ava I site. One transformant, pBR322delA, was chosen which no longer contained an Ava I site and was used in subsequent experiments. pBR322delA DNA was digested with Bam HI, dephosphorylated, and used as a vector to clone the Sau 3A I fragment containing the su_3^+ gene from p61.3.

The Sau 3A I fragment containing the su_3^+ gene from p61.3 was isolated by digesting p61.3 DNA with Sau 3A I and electrophoresing the digest on a 2% LGT agarose gel. The band containing the su_3^+U43 gene was identified by size after ethidium bromide staining and isolated from gel slices by phenol extraction and ethanol precipitation. The fragment was ligated to the pBR322delA vector and used to transform 5K cells. Individual colonies were isolated which were phenotypically Amp^r Tet^s. A clone containing the Sau 3A I fragment from p61.3 in the same orientation as the Tet^r gene in the pBR322delA vector was identified and named p61.3-S.

Mutagenesis of the dihydrouridine loop of $tRNA^{Tyr}$: p61.3-S plasmid DNA, containing a unique Ava I site in the dihydrouridine loop of the su_3^+U43 gene, was digested to completion with the restriction enzyme Ava I. Since Ava I generates 5' protruding single stranded ends after digestion, small deletions and additions were introduced at this site by treating the linearized plasmid with Klenow polymerase in the presence of different combinations of nucleoside triphosphates. Following this reaction, S1 or mung bean nuclease was used to produce a plasmid with two blunt ends, ready for recircularization. Deletions extending four nucleotides and larger were produced by prolonged S1 nuclease treatment alone. Table 1 describes the combination of reactions needed to produce a particular addition or deletion mutation at this site.

The plasmid derivatives were circularized with T4 DNA ligase and were used to transform 5K cells. Twelve individual colonies which were Amp^r and Tet^s were isolated from each transformation and grown in 5ml LB with 50 μg/ml Amp. DNA was isolated from these cultures by the miniscreen procedure, digested with both Ava I and Pst I, and electrophoresed in a 1% agarose gel. Plasmids which had lost the Ava I site in the su_3^+U43 gene were screened further by a series of methods. In cases where new restriction sites were predicted to be generated as a result of the mutagenesis (see Table 1), the clones were screened for

the appearance of a new restriction pattern when digested with this enzyme. In order to identify mutations which did not generate a new restriction site such as the +4 and the deletion mutations larger than -4, small fragments produced by a variety of restriction digests were compared for differences in electrophoretic mobilities on agarose gels. Plasmids which had lost the Ava I site and produced restriction fragments carrying the su_3^+U43 gene which showed a very small difference in size (or no difference at all, depending on the ability of different electrophoretic conditions to distinguish small changes in fragment size) were considered for further analysis.

The final screening method involved sequencing the DNA of all potential mutant derivatives through the site of mutagenesis. Each plasmid DNA was digested with Bst E II and the bank containing the su_3^+U43 gene was purified after electrophoresis on a 2% LGT agarose gel. This Bst E II fragment was digested with Hinf I and labeled at the Hinf I site using alpha-^{32}P- deoxy-ATP and DNA polymerase (Klenow fragment). Preparation in vivo of precursor and mature $tRNA^{Tyr}$: ^{32}P-labeled RNA was prepared from cells carrying plasmids. Preparations from ø80-infected cells were performed as described previously (3).

^{32}P labeled RNA was purified from cells containing plasmid derivatives carrying $tRNA^{Tyr}$ genes (20) as follows. 5K cells containing plasmids carrying mutant derivatives of su_3^+ tRNA genes were grown in 20 ml LP media containing 50 µg/ml Amp (LPA medium) to a concentration of 5 x 10^8 cells/ml. Chloramphenicol (Sigma) was added to a final concentration of 170 µg/ml and the cells were incubated for 12 hours at 37°. The cells were pelleted by centrifugation (7000 rpm for 10 minutes), washed twice with 20 ml fresh LPA medium and resuspended in 20 ml LPA medium. After incubating the cells at 37° for 10 minutes, 10 mCi ^{32}P orthophosphate was added and allowed to incorporate for 20 minutes at 37° (only 1 mCi was used for cells containing pBR322 or p61.3-S alone.) The RNA was extracted with 20 ml liquified phenol and purified in the same manner as labeled RNA after phage infection. Hybrid selection of $tRNA^{Tyr}$ transcripts: $tRNA^{Tyr}$ transcripts were selected from total RNA labeled in vivo (prepared as described above) by a modification of the procedure of Weiner (21). Plasmid DNA containing the $tRNA^{Tyr}$ gene was immobilized on nitrocellulose filter paper and used to select $tRNA^{Tyr}$ transcripts from a mixture of RNA labeled in vivo. The filters were prepared in triplicate as follows: 5 µl 0.2 N NaOH was

added to 5 µg of the appropriate plasmid DNA in 5 µl TE buffer and incubated at 65° for 5 minutes. 2 µl 4 M NaCl and 3 µl 2 M Tris pH 5 were added and the mixture was spotted on 1 cm circles of nitrocellulose filters which had been previously rinsed in water, then in 20X SSC (1X = 0.15 N NaCl, 15 mM sodium acetate, pH 7.0), and allowed to dry. After spotting, the filters were dried, rinsed in 2X SSC, and baked overnight at 60°. The filters were placed in 500 µl hybrid selection buffer (5X SSC, 50% deionized formamide). RNA labeled in vivo with ^{32}P was added to the hybridization reaction and incubated at 42° for 16 hours. The filters were removed and washed three times in 500 µl hybrid selection buffer at 23° for 15 minutes. The tRNA transcripts which remained on the filters were eluted by placing the filters in 400 µl water for 3 minutes at 80° and the RNA was precipitation with ethanol. Further purification was performed by electrophoretic separation of the $tRNA^{Tyr}$ transcripts on polyacrylamide sequencing gels.

Preparation in vitro of precursor $tRNA^{Tyr}$: Precursor $tRNA^{Tyr}$ transcripts were made according to the procedures of Daniel et al., (22) and Farnham and Platt (23). Transcription reactions were performed in 30 µl containing 0.2 pmol DNA restriction fragment as a template, 100 mM KCl, 0.1 mM DTT, 0.1 mM EDTA, 4 mM magnesium acetate, 20 mM Tris-acetate pH 7.9, 10% glycerol, 200 µm each of ATP, CTP, UTP, 10 µCi alpha-^{32}P-GTP (Amersham, 310 Ci/mmole), and 3 units of E. coli RNA polymerase. The reactions were incubated at 37° for 30 minutes and stopped by adding 2 µl 20 mg/ml carrier tRNA, 1 µl 10% SDS, 225 µl 0.3 M sodium acetate followed by an extraction with 250 µl phenol saturated with 100 mM Tris pH 7.5. The reactions were precipated in 95% ethanol and the transcripts were separated on a 5% polyacrylamide sequencing gel.

Preparation of S100 extract for processing reactions in vitro: 5g MRE600 cells were added to 10g alumina and ground in a mortar and pestle to a paste. 5 ml JAS buffer (50 mM Tris pH 7.5, 10 mM magnesium acetate, 60 mM NH_4Cl, 6 mM mercaptoethanol), 10 µl DNase (5 mg/ml in JAS buffer) and 10 µl PMSF (made fresh at 10 mM in isopropanol) was added to the cells and incubated for 30 minutes on ice. The mixture was centrifuged for 16 minutes at 7000 rpm to remove the cell debris. The supernatant was spun for 30 minutes at 15500 rpm in an SS34 rotor (Sorvall) to produce an S30 extract. This supernatant was centrifuged at 42000 rpm for 2 hours at 4° in an SW65 rotor (Beckman) and the resulting S100 supernatant was divided into 100 µl aliquots that were

immediately frozen in liquid nitrogen and stored at -70°.

RNase P assays: Assays for RNase P activity were carried out as described by Kole and Altman (24) and Guerrier-Takada et al (25).

RESULTS

Mutagenesis of the D-loop of tRNA^{Tyr}: su_3^+U43 p61.3-S plasmid DNA containing a unique Ava I site in the D-loop region of the su_3^+U43 gene (Fig. 1) was mutagenized in vitro and the resulting plasmid DNAs

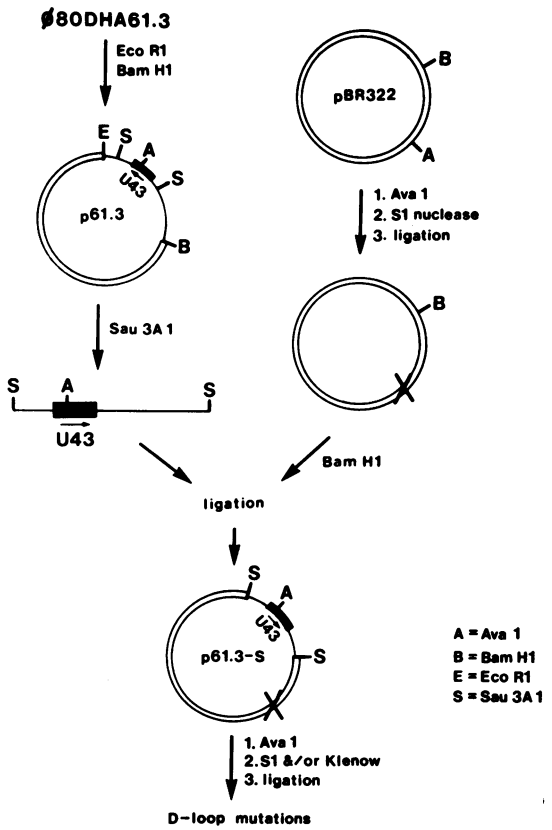

Figure 1: Flow diagram for the production of the D-loop mutations. pBR322 was digested at the unique Ava I site, treated with S1 nuclease, and recircularized to produce a cloning vector which contained no Ava I sites. A Sau 3A1 DNA restriction fragment from p61.3 which contained the U43 tRNA^{Tyr} gene (6) was inserted into the Bam HI site of the pBR322 'Avaless' vector resulting in the subclone p61.3-S. This clone contains a unique Ava I site in the U43 gene which was used to introduce the D-loop mutations into this gene.

screened as described in the Methods section. Several potential deletion and addition mutants were selected on the basis of the restriction pattern or electrophoretic mobility of their DNA. Nucleotide sequence analysis was then performed on selected restriction fragments to determine the exact nature of the changes in the DNA (26). The mutations which were well characterized are diagrammed in Figure 2. The mutations identified are as follows: a) -1, the deletion of A14 of $tRNA^{Tyr}$ b) -2, the deletion of A14 and G15 c) -4, the deletion of nucleotides CCGA from positions 11 to 14; d) -6, the delection of CCGAGC from positions 11 to 16; e) -16, the deletion of GGGTTCCCGAGCGGCC from positions 5 to 20; f) +4, the addition of nucleotides CCGA between A14 and G15; g) +23, the addition of nucleotides ATACCCTTGAGCGCCATTACCGC between A14 and G15. Although the sequencing reactions were performed on one strand only, the results obtained are consistent with other corroborative evidence. The sequence surrounding each of the deletions is consistent with published sequence for the $tRNA^{Tyr}su_3^+$ gene from $\phi 80psu_3^+$ (27). New restrictions sites which were created by the D-loop mutations (Table 1) were also confirmed by the DNA sequence analysis.

Origin of the +23 addition mutation: The tyrT locus from the plasmid carrying the +23 mutation was mapped with restriction enzymes to determine whether any rearrangements of this locus had accompanied the formation of this unusual mutation in the D-loop. A Bst E II/Alu 1 DNA restriction fragment was mapped with a variety of restriction enzymes according to the procedure of Smith and Birnstiel (28). The resulting restriction map (data not shown) showed that no rearrangements had occured in this region of the plasmid DNA. In order to determine the origin of the additional 23 nucleotides in this mutant, a computer program (29) was used to search for this sequence in the tyrT locus and in pBR322. In both cases, the +23 sequence was not found suggesting that it may have originated elsewhere in the E. coli chromosome.

Suppression propeties of the D-loop mutants: Plasmids containing each of the mutants described above were tested by two separate methods for their ability to produce functional suppressor $tRNA^{Tyr}$. First, plasmid DNA was used to transform CA274 (lac Z_{am}) and the transformants were examined for the appearence of blue colonies on plates containing IPTG and XG. Under conditions where p61.3-S gave rise to dark blue colonies, no blue color was observed at 37° in CA274 containing any of the deletion or addition mutants. This assay was also conducted at 30°

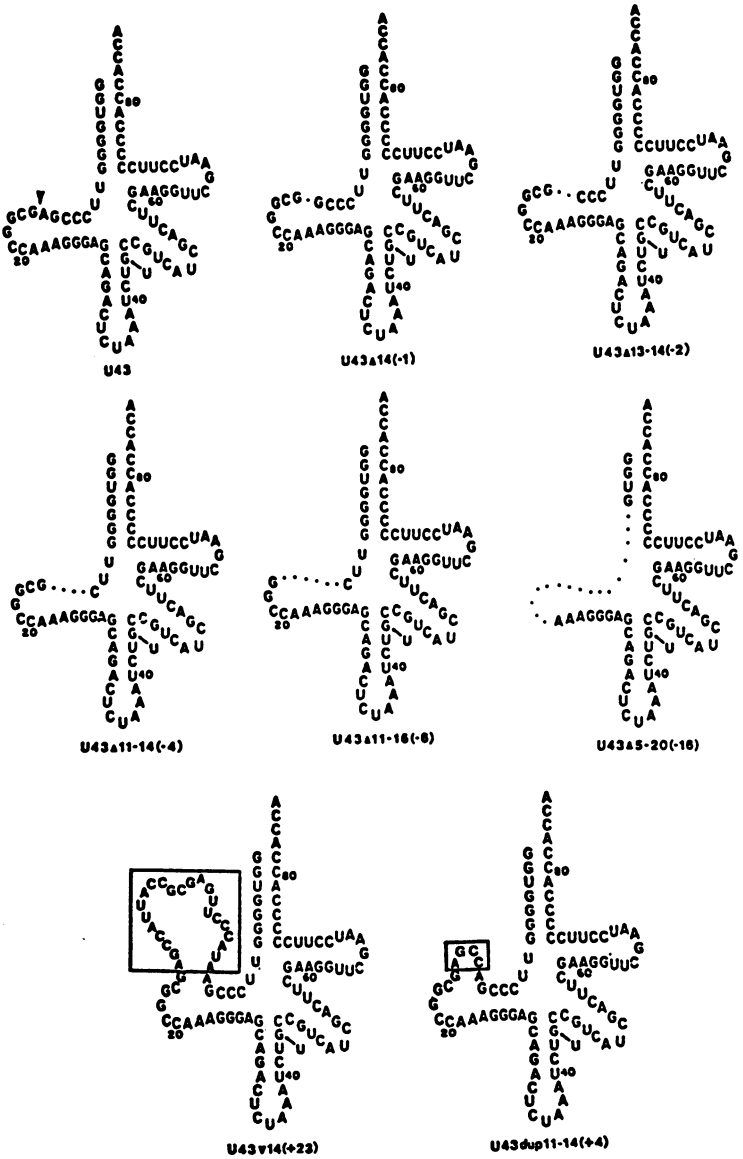

Figure 2: Diagramatic representation of the D-loop mutations. Each of the tRNA^{Tyr} molecules with a deletion or addition mutation in the D-loop region are displayed in the standard clover leaf structure. In the upper left, the parent mutant U43 is shown with the cut produced by Ava I identified with an arrow head. Nucleotides which are absent in individual mutants are represented with a dot (.) and added nucleotides are enclosed in a box. In all cases, the U43 mutation is also indicated.

Table 1: Construction of the D-loop mutations

Mutation	How constructed	New Site
U43	---	Ava 1
-1	Klenow + CTP + GTP	Msp 1
-2	Klenow + CTP	Sac II
-4	S1	Fnu DII
-6	S1	None
+4	Klenow + NTP	None

and at 42° to determine if any of these mutants were temperature sensitive suppressors. In both cases, the results were identical to those observed at 37°.

Suppression was also tested by infecting CA274 carrying these plasmids with phage T4 derivatives containing a lethal amber mutation in one of the late genes. The assay for suppression was the appearance of plaques which resulted from suppressing the effect of the lethal T4 amber mutation. Two different T4 mutants were used: eL1, containing an amber mutation in the lysozyme gene and B20, containing an amber mutation in gene 14. Both these T4 B20 amber mutants have a burst size of about 100 when plated on CA274 containing p61.3-S. Neither, however, produce any plaques when plated on CA274 containing plasmids carrying the -1, -2, -4, or +4 mutations. When the T4 B20 strain was used for infection, an aliquot of infected cells plated on CA274 containing p61.3-S which would produced 10^4 plaques per plate produced no plaques on the indicator strain containing the deletion and addition mutants. Thus, the mutations isolated in the D-loop prevent the production of detectable suppresor tRNA. No revertants to suppressor function, as assayed by the appearance of blue colonies, were detectable at a level of 1 in 10^4 for any of the mutants.

Products of transcription in vivo: Transcripts from the tyrT locus of the different plasmids containing D-loop mutants were selected by hybridization from cells labeled with $^{32}PO_4^{2-}$. The electrophoretic pattern of the RNA from each of the D-loop mutants is shown in Figure 3. When compared to pBR32 alone or p61.3-S, it is apparent that the -1, -2, -4, +4, and +23 derivatives code for unique transcripts larger than 5S

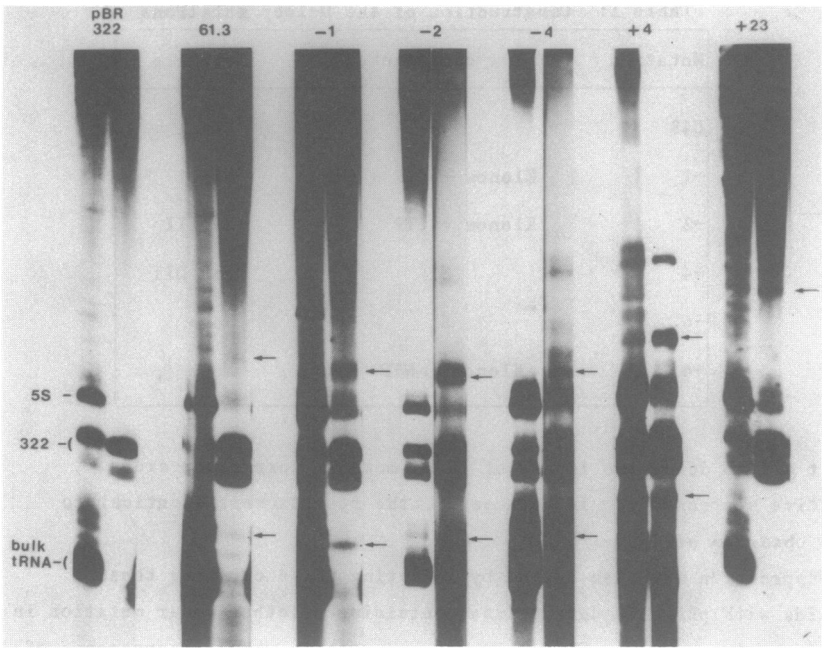

Figure 3: Hybrid selection of $tRNA^{Tyr}$ transcripts containing D-loop mutations. Plasmid DNA immobilized on nitrocellulase filters were used to hybrid select $tRNA^{Tyr}$ transcripts for each of the D-loop mutant derivatives (see Methods) except that cells carrying the parent plasmid p61.3-S or pBR322 alone were labeled with only 1 mCi rather that 10 mCi. In each set, the left lane shows the total pattern of in vivo labeled RNA after electrophoresis in a 10% polyacrylamide gel (see Methods). The right lane of each set shows RNA eluted from the nitrocellulose filters after hybrid selection. The upper arrows (above 5S RNA) indicate the presence of a unique transcript the size of precursor $tRNA^{Tyr}$ while the lower arrows (above bulk tRNA) indicate the production of a transcript the correct size for the corresponding mature $tRNA^{Tyr}$. Appropriate bands were eluted from the gel of the total extract prepared in vivo (left lane of each pair) and the amount of the precursor tRNA and mature tRNA produced from these mutants was compared to that produced by p61.3. by counting Cerenkov radiation. 5S RNA, bulk tRNA, and two pBR322 encoded transcripts are also indicated in the figure. It should be noted that these autoradiographs do not reflect quantitative yields of transcripts. Mutant extracts had ten times as much radioactivity present as the parent extract, the relevant autoradiographs are over expired to show minor species and species not related to $tRNA^{Tyr}$ are apparent in non-quantitative yields in the hybrid selection experiments.

RNA. In addition to these tRNA precursor molecules, RNA of the size of mature tRNA can be seen in the lanes corresponding to the -1, -2, -4 and +4 mutants. Fingerprint analysis showed that only molecules in the

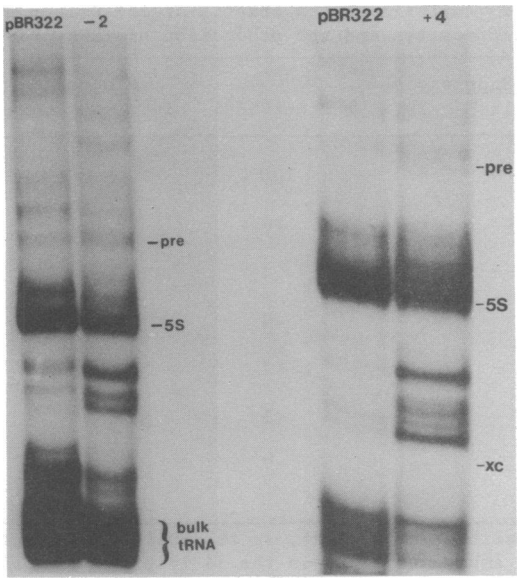

Figure 4: Large scale preparation of +4 and -2 precursor tRNAs. Left panel: Autoradiograph of polyacrylamide gel separation of RNA extracted from cells containing the plasmid pBR32 (left lane) or the plasmid containing the +4 mutant (right lane). The positions of precursor tRNA, 5S RNA and bulk tRNA are shown.
Right panel: As in left panel: left lane, pBR322; right lane, plasmid containing -2 mutant. The positions of precursor tRNA, 5S RNA, and the dye marker xylene cyanol (XC) are shown.

indicated positions for precursor and mature tRNA had $tRNA^{Tyr}$ sequences. No partial processing products were observed.

Preparative amounts of tRNA precursor coded for by the -1, -2, and the +4 D-loop mutants (Fig. 4) were made and the mutant transcripts were isolated by excising the appropriate region of a polyacrylamide gel. The transcripts were repurified by electrophoresis through another 10% polyacrylamide gel. The total amount of precursor transcript produced by each mutant derivative was compared to the amount produced by the parent p61.3-S (Table 2).

Processing in vitro of mutant tRNA precursors: tRNA precursors coded for by the +4 and -2 mutants were processed in vitro by M1 RNA, the RNA subunit of E. coli RNase P (25). The resulting products were compared to those produced by processing the precursors to su_3^+. Figure 5, lanes A4 and B4 show a band corresponding to the 5' product for each of the mutants which has a mobility identical to the 5' product from su_3^+U43 and

Table 2: Analysis of the effects of the D-loop mutations on suppression and the production of tRNA precursor

Mutation	Suppression[a] lacZ	T4	% Pre-tRNA[b]	Processing in vivo[c]	in vitro[d]
su_3^+	+	+	100	100*	100
U43	+	+	20	20	20
-1	-	-	4	1	2
-2	-	-	4	2	2
-4	-	-	<1	N.D.	1
-6	-	-	<1	N.D.	2
+4	-	-	7	2	3

a) Measured as described in the text, as the ability to suppress a lacZ amber mutations or amber mutations in the T4 lysozyme or head protein genes.
b) % Pre-tRNA reflects the amount of $tRNA^{Tyr}$ precursor containing D-loop mutations compared to su_3^+ precursor that accumulates in vivo after ^{32}p-labeling. The percentages were derived by first comparing the Cerenkov radiation of precursor $tRNA^{Tyr}$ containing the D-loop mutations to su_3^+U43 precursor (control) as explained in the legend to Fig. 3. These ratios were then normalized with the value of 20% for su_3^+U43 precursor since U43 produces 1/5 the amount of precursor compared to su_3^+ (6).
c) RNase P processing in vitro represents the amount of mature $tRNA^{Tyr}$ containing D-loop mutations compared to mature su_3^+ $tRNA^{Tyr}$ that accumulates after ^{32}p-labeling. The percentages were derived by first comparing the Cerenkov radiation of mature $tRNA^{Tyr}$ containing the D-loop mutations to mature su_3^+U43 tRNA (control). These ratios were then normalized with the value of 20% for su_3^+U43 tRNA as in (a).
d) RNase P processing in vitro represents the relative level of processing of mutant precursor $tRNA^{Tyr}$ compared to su_3^+ precursor by RNase P in the assay in vitro.
*We compared the production and processing in vivo of the su_3^+ gene transcript with that of the su_3^+U43 gene transcripts in cells infected with Ø80 carrying either of these genes. We have not yet been able to clone the su_3^+ locus with intact flanking sequences into pBR322 or any of its derivatives.

the expected band for the tRNA-containing product (Fig. 5, lanes A2 and B2). The RNA in these bands as well as the RNA from the su_3^+U43, +4, and -2 precursors were digested with RNase A and fingerprinted. A comparison of the resulting fingerprints from su_3^+U43 and the mutants confirmed that the precursors isolated with +4 and -2 mutations were those for $tRNA^{Tyr}$ containing an unprocessed 5' end. Similarly, the products of processing the mutant and parent gene trascripts have the

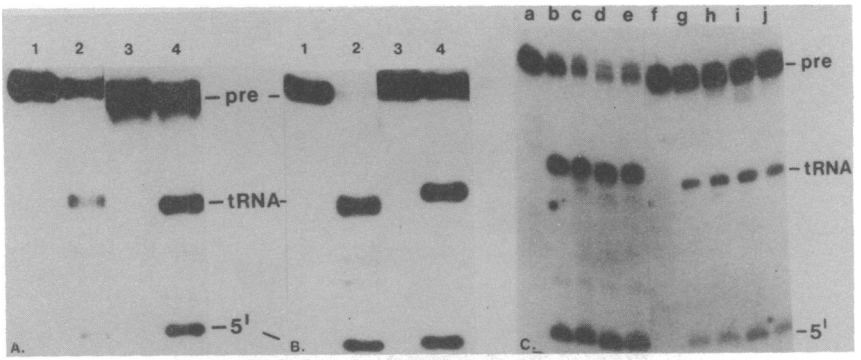

Figure 5: Electrophoretic separation of mutant RNAs before and after cleavage by purified M1 RNA. A. Precursor $tRNA^{Tyr}$ from 61.3 (lane 2) and from +4 (lane 4) were treated respectively with 30 ng and 360 ng of M1 RNA and electrophoresed on a 10% assay gel. Lanes 1 and 3 represent control reactions with no M1 RNA added. The precursor, mature tRNA, and 5' fragments are indicated. A difference in mobility for the +4 precursor and the 61.3 precursor as well as the +4 tRNA product and the 61.3 tRNA product can be seen. The amount of Cerenkov radiation produced by the RNA in these bands and bands from similar experiment showed that the +4 precursor was processed approximately 10% as efficiently as the 61.3 precursor. B. Precursor $tRNA^{Tyr}$ from 61.3 (lane 2) and from -2 (lane 4) were treated respectively with 30 ng and 360 ng of M1 RNA and electrophoresed on a 10% assay gel. Lanes 1 and 3 represent control reactions with no M1 RNA added. The precursor, mature tRNA, and 5' fragments are indicated. A difference in mobility for the -2 precursor and the 61.3 precursor as well as the -2 tRNA product and the 61.3 tRNA product can be seen. The amount of Cerenkov radiation produced by the RNA in these bands and bands from similar experiment showed that the +4 precursor was processed approximately 10% as efficiently as the 61.3 precursor. C. Precursor $tRNA^{Tyr}$ from 61.3 (lanes a-e) and -1 (lanes f-j) were treated with RNase P purified to different extents (Kole and Altman, 1981) and electrophoresed on a 5% sequencing gel. Untreated precursor RNA (lanes a and f), precursor RNA treated with 30 ng M1 RNA (lanes b and g), with reconstituted M1 RNA and C5 protein (lanes c and h), with Sepharose 4B fraction (lanes d and i) and DEAE fraction (lane e and j) are shown. A slight increase in the electrophoretic mobility of the tRNA product for the -1 mutants can be seen in lanes g through j, most likely the result of a change in tertiary structure of the tRNA molecule. Precursor, mature tRNA, and the 5' fragment are indicated.

expected oligonucleotide composition and, most importantly, pGGU at their 5' termini (data not shown).

The -1 precursor transcript was also obtained and processed in vitro with RNase P purified to varying degrees (see legend Figure 5C). Insufficient amount of this substrate were available to allow fingerprinting of the RNase P cleavage products. These products,

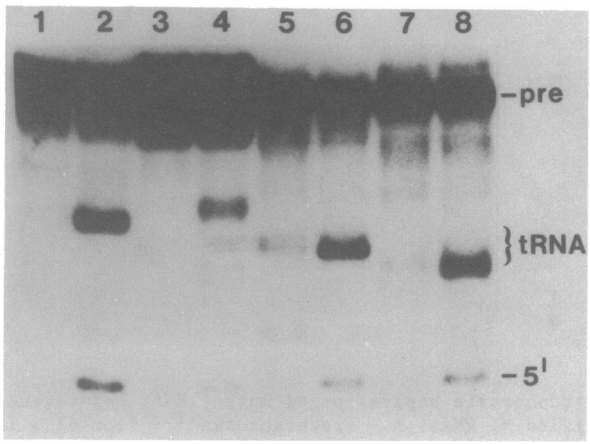

Figure 6: In vitro transcription and processing of the -4 and -6 deletion mutants. Cleavage products of -4 and -6 precursors processed with 360 ng of M1 RNA were separated on a 10% polyacrylamide assay gel. su_3^+ precursor assayed without (lane 1) and with (lane 2) 30 ng M1 RNA, su_3^+U43 precursor assayed without (lane 3) and with (lane 4) 30 ng M1 RNA, -4 precursor assayed with (lane 5) and with (lane 6) 360 ng M1 RNA, and -6 precursor assayed without (lane 7) and with (lane 8) 360 ng M1 RNA are shown. In all cases, the mobility of the 5' fragment is identical. The different mobilities of the tRNA products from the deletion mutant transcripts are apparent in comparison to the mobility of the tRNA products from the controls.

nevertheless, had the anticipated electrophoretic mobilities of the correct RNase P cleavage products. In all cases, the extent of processing relative to su_3^+U43 precursor is approximately 10% as determined by counting Cerenkov radiation of appropriate gel slices. The efficiency of processing the -2 and +4 precursors were estimated in a similar fashion.

Transcription and processing in vitro of precursors to mutant tRNAs: Precursor transcripts containing -4 and -6 mutations were obtained in vitro as generally described in the Methods section. A Bst EII restriction fragment from the plasmid derivatives containing each of these D-loop mutations was purified from a 2% LGT agarose gel. These restriction fragments were digested with Hph 1 which produces a DNA restriction fragment that extends 38 nucleotides beyond the 3' end of the $tRNA^{Tyr}$ sequence. The entire digest was used as template for transcription reactions in vitro under conditions where polymerization was allowed to terminate randomly along the DNA template. Using this

method, transcripts close to the size of the 131 su_3^+ precursor were isolated after electrophoresis on a 5% polyacrylamide sequencing gel. Transcription of the mutant genes appeared as efficient as transcription of the parent genes su_3^+U43 or su_3^+. After purification, the transcripts were treated with M1 RNA in the standard assay (see Methods) and electrophoresed on a 10% assay gel. Figure 6 shows the appearance of the expected 5' fragment and tRNA species as products of processing of each of the D-loop mutant transcripts. These products were identical to those from su_3^+U43 as judged by their electrophoretic mobility. The transcript from the -4 mutant was further digested with RNase T_1 and the resulting fingerprint compared to that of su_3^+U43. They were identical except that the 3' proximal oligonucleotide is missing in the -4 fingerprint since this oligonucleotide does not end in G (which was used for labeling) if transcription is allowed to terminate randomly along the DNA template. The deletion of four nucleotides in the D-loop does not change the remaining pattern of spots in the fingerprint.

DISCUSSION

Identification and processing of gene products: The dihydrouridine loop region of the su_3^+U43 $tRNA^{Tyr}$ gene has been mutagenized at a unique Ava I site by altering the DNA at this restriction site using a combination of Klenow polymerase and S1 or mung bean nucleases. The collection of plasmid derivatives containing the mutated gene include -1, -2, -4, -6, -16, +4, and +23 mutations in the D-loop region. The identities of these mutations have been confirmed by restriction mapping and by sequencing across the site of each mutation.

The -1, -2, and +4 mutations allow the accumulation of precursor tRNA *in vivo* as seen by the appearance of specific RNA species larger than 5S RNA in hybrid selection experiments. (Species corresponding to precursors coded by other mutations were also observed in hybrid selection experiments but not identified by fingerprinting). The amount of mutant RNA which accumulates is approximately 10% that produced by su_3^+U43 (Table 2) suggesting that these altered precursors are probably more susceptible to degradation than the su_3^+U43 precursor.

The precursor transcripts from the -2 and +4 plasmid derivatives were treated *in vitro* with M1 RNA in the standard assay (25) and found to be processed (although inefficiently) by M1 RNA. Subsequent to processing, these precursors yield two bands after electrophoresis: one

which comigrates with the 5' band from processed su_3^+U43 precursor tRNA and another which migrates similarly to the tRNA product generated from this precursor. The two product bands were fingerprinted with RNase A and were each found to contain the correct number and pattern of oligonucleotides. The fingerprints also showed that cleavage had occurred at position 1 of the mature tRNA sequence as seen by the disappearance of GGUp and the appearance of pGGUp.

The data regarding *in vivo* accumulation of these precursors are consistent with *in vitro* RNase P processing result which show a 10% efficiency of processing of the precursors containing D-loop mutations compared to processing of su_3^+U43 precursor. (Transcripts made *in vitro* from DNA containing the -4 and -6 mutants were also processed at the same rate as the -1 and -2 mutant transcripts.)

Suppressor function of mutant tRNAs: All of the mutations we studied appear to prevent the formation of functional suppressor tRNA. Using both the blue colony assay and the T4 amber mutant rescue assay, which is a simplified version of the burst size test (30), none of the new mutants exhibited suppressor function under optimum conditions for suppression by su_3^+U43 carried by p61.3-S. The T4 amber mutant B20 normally produces 0.01 viable phage per cell as opposed to wild type levels of 150 to 200 (31). In the presence of the su_3^+ gene, the efficiency of chain elongation through this T4 amber mutation is 51%, as determined by Kaplan et al., (32). Since the amount of mature tRNA produced from plasmids containing the D-loop mutations is about 2% that of wild type su_3^+ $tRNA^{Tyr}$ (see Table 2), the efficiency of chain elongation through the T4 amber mutations in the presence of these D-loop mutations would theoretically be in the range of 1%. For the B20 mutation, this would result in the production of greater than 1 to 2 viable phage per cell. This level of phage production would be sufficient to produce plaques, considering that T4 amber mutants are capable of forming plaques when the average burst size is in the range of 0.1 phage per cell (30). Therefore, while some molecules the size of $tRNA^{Tyr}$ are observed in the hybrid selection experiments it is unlikely that these D-loop mutations allow any of it to function as suppressor tRNA.

Since the test for suppression function shows no activity at a level 100-fold lower than (0.01 % compared to 1%) the amount of tRNA which accumulates *in vivo*, the lack of suppressor activity of the mutants may

not be solely the result of inefficient processing of precursor transcripts. The defective step in the function of these mutant tRNAs may be in the charging of the molecules or their ability to interact efficiently with the ribosome, and/or interact efficiently with other components of protein synthesis. Although these mutant genes are carried on a plasmid, there is virtually no amplification of their products and thus insufficient amounts of these mutant mature tRNAs could be isolated to test the various kinetic parameters of the aminoacylation and translation reactions.

Structural considerations: We presume that the effects of the D-loop mutations on function of the $tRNA^{Tyr}$ are independent of those resulting from the U43 mutation. In the case of the U43 mutation production of tRNA, processing of precursor tRNA and amount of suppressor activity are strictly correlated. There seems to be no independent effect of this mutation on aminoacylation or translation function. While there is no detailed structural information regarding the added effect of the D loop mutations on structure-function relationships, we infer that they alone are responsible for the additional ten-fold decrease in processing efficiency and the further hundred-fold decrease in suppressor function. Though there is no rigorous proof for the validity of this inference, all our data are compatible with the notion that the mutations have independent effects on tRNA function.

The D-loop mutations alter the secondary and tertiary structure of $tRNA^{Tyr}$ precursor in a number of ways. Besides the size changes, some of which disrupt the D-loop stem, these mutations also alter the base composition of the loop. All of the deletion mutations are missing the A14 nucleotide which is conserved among prokaryotic tRNAs. This nucleotide is involved in the formation of the A14-U8 base-pair which contributes to the tertiary structure of tRNA (33). We note that the complete phenotype of the mutants is already observable in the -1 deletion. This observation highlights the importance of A14 and the necessity for its local environment to stay intact for tRNA function. Many of the deletion derivatives are missing the two G residents at positions 17 and 18 which form the tertiary interactions between the D-loop and the TψCG loop. The ability to process transcripts of these mutant genes suggests that an intact D-loop is not essential for the 5' processing of tRNA precursors. Finally, we note that the $tRNA^{Tyr}$ precursor containing the -6 mutation has a theoretical clover leaf

structure similiar to the normal human mitochondria $tRNA^{Ser}$ species (34) which is processed efficiently in vivo by a mitochondrial RNase P (C. Doersen, unpublished results).

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