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A nucleotide change in the anticodon of an *Escherichia coli* serine transfer RNA results in *supD*$^-$ amber suppression

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ABSTRACT

The tRNAs specified by the wild type and amber suppressor alleles of the Escherichia coli supD gene have been identified, and their primary structures determined. The sequences differ by a single nucleotide in the middle of the anticodon. A CUA anticodon allows the suppressor tRNA to read the UAG stop codon; the CGA anticodon in the minor serine tRNA species from which the suppressor is derived is specific for the serine codon UCG.

INTRODUCTION

In Escherichia coli strains bearing the supD$^-$ nonsense suppressor, UAG codons are translated as serine (1,2,3). SupD$^-$ suppression is mediated by a minor species of serine tRNA that comprises less than 5% of seryl tRNA (4-8). Low concentrations of the suppressor species in supD$^-$ strains hampered early efforts both to obtain direct evidence that supD$^-$ is the structural gene for a serine tRNA and to determine the nucleotide change that leads to recognition of UAG codons instead of serine codons. Specialized transducing bacteriophages for the supD$^-$ gene were isolated several years ago as a means of increasing suppressor tRNA synthesis. However, in cells infected with the λpsupD$^-$ phage of Yamao et al. (9), stimulation of asparagine and aspartic acid tRNA synthesis, but not of serine tRNA synthesis, was seen. In cells producing the λdsupD$^-$ phage of Steege and Low (10), amplified synthesis of a serine tRNA with suppressor activity was observed, but this tRNA species was not readily detectable in ^{32}P-labeled tRNA preparations (11).

An alternative strategy for preferential labeling of transcripts from genes located near a lambda prophage takes advantage of the phenomenon of escape synthesis. After prophage induction, increased synthesis of the products of the galactose and biotin operons, which flank the normal λ attachment site (att$_\lambda$), has been observed (12,13). This escape synthesis has been interpreted as a consequence of local transcription activity downstream from derepressed phage promoters. Induction of a λ prophage inserted at att$_\lambda$

also leads to a three-fold increase in lysine tRNA acceptor activity (14). Using a rare lysogen in which the λ prophage has been mapped near the supD locus (15,10), we have exploited this phenomenon to enhance ^{32}P-labeling of supD gene transcripts. This report describes the purification of the wild type ($tRNA^{ser}_2$) and suppressing ($tRNA^{ser}_{2am}$) tRNAs specified by the supD locus, and the steps used to establish their nucleotide sequences.

MATERIALS AND METHODS

General

The sources of materials for RNA sequencing and polyacrylamide gel electrophoresis have been described (16,17). Carrier-free (^{32}P)orthophosphoric acid, cytidine 3,5'-[5-^{32}P]bisphosphate (2300 Ci/mmol), and [^{3}H]serine (2.76 Ci/mmol) were obtained from New England Nuclear. [γ-^{32}P]ATP was synthesized by the Glynn-Chapell phosphate-ATP exchange reaction, as given in (18). Phage T4 polynucleotide kinase was from P-L Biochemicals, and T4 RNA ligase, from New England Biolabs. E. coli strains DS65 and DS68 carry the wild type supD^{+} and amber suppressor mutant supD32 alleles, respectively, a λcI857 prophage located near his (15,10), and other markers as follows: Δ(att$_{\lambda}$-bio), arg-47, trp-49(Am), lacZ53(Am), rpsL150, rel-1. The supD32 allele originated in the Garen strain S26rle^{-} (19). The Sup^{+} strain LS289 (pro-48, trpR55, trpA9605(Am), his-85(Am), ilv-632) was used as a source of unlabeled tRNA. Culture media and supplements were as previously described (10). Phage DNA was isolated from a lysate containing the defective transducing phage λdsupD32 and the helper phage λcI857 (20).

tRNA preparation

Unlabeled tRNA was extracted from LS289 by the standard phenol extraction method, followed by a DEAE-cellulose (Whatman DE52) chromatography step (21). ^{32}P-labeled tRNA was prepared from induced cultures of strains DS65 and DS68 as follows. Overnight cultures grown at 30°C in 10 ml of TG medium supplemented with 0.2% glucose, 50 μg/ml thiamine, 0.1 μg/ml D-biotin, 20 μg/ml arginine and tryptophan, 1x10^{-5} M $FeCl_3$, and 0.3 mM KH_2PO_4 were diluted into 15 ml of the same medium that contained 0.15 mM KH_2PO_4. When the culture had reached a density of 2-3x10^{8}/ml, $MgSO_4$ was added to 0.01 M, and the temperature was shifted from 30°C to 43°C (time 0). (^{32}P)Orthophosphoric acid (3-5 mCi) was added at 10 min, the culture returned to 39°C at 15 min, and chloramphenicol (25 μg/ml) added at 25 min to prevent cell lysis. After a 45 min labeling period, the nucleic acids were isolated by direct phenol extraction of the culture in the presence of 100 μg of unlabeled LS289 tRNA,

followed by precipitation with two volumes of ethanol. A 1 hr incubation at 37°C in 0.1 M Tris-HCl, pH 9.0 was used to deacylate the tRNA. Two further steps produced tRNA samples suitable for analysis on two-dimensional polyacrylamide gels: 1) 10 min digestion with DNase I (Worthington DPFF) at 4°C in 0.01 M Tris-HCl, pH 8, 0.01 M $MgCl_2$, 0.01 M $CaCl_2$, and 2) chromatography on 2.5 ml columns of DEAE-cellulose (21). The two-dimensional polyacrylamide system of Garel et al. (22) was employed for tRNA separations, but an 0.089 M Tris-borate, pH 8.3, 0.0025 M EDTA buffer was used, and pH adjustments with HCl were omitted. A third gel, consisting of 20% polyacrylamide and 7 M urea, was the final purification step. tRNA was recovered from macerated polyacrylamide particles by extraction with 0.5 M NaCl, 0.01 M Mg acetate, 0.1% SDS (23) in the presence of 50 µg unlabeled tRNA. Unlabeled $tRNA_2^{Ser}$ was purified via the same steps, visualized by staining with ethidium bromide, and eluted electrophoretically. Previously reported procedures for hybridizations on nitrocellulose filters were used (24), except that RNase treatment of the hybrids was omitted.

Assay for amino acid acceptor activity

A dialyzed DEAE-cellulose fraction prepared as given by Muench and Berg (25) from strain LS289 served as the aminoacyl synthetase preparation. ^{32}P-labeled tRNA used in standard assays for amino acid acceptor activity (26) contained phage R17 RNA rather than unlabeled LS289 tRNA as carrier.

Sequencing methods

Standard procedures (27,28) were used for analysis of tRNAs labeled with $^{32}PO_4$ in vivo. To prepare 5' end-labeled tRNA fragments, nested sets of hydrolysis products were generated by heat treatment for 3-6 min at 80°C in H_2O or for 5-10 min at 100°C in formamide. Following lyophilization to dryness, they were labeled with [γ-^{32}P]ATP (29), fractionated on 15% sequencing gels, and transferred to thin layers of PEI-cellulose (30). Each fragment was eluted with 30% triethylamine carbonate, pH 10, containing 50 µg/ml yeast RNA, and subsequently digested with nuclease P1 (31). The resulting nucleoside 5' monophosphates were separated on Avicel thin layer plates, using the solvents isobutyric acid/NH_4OH/H_2O (250:5.5:144) (28) and t-butyl alcohol/HCl/H_2O (70:15:15). Partial enzymatic digestion products of [5'-^{32}P]pCp-labeled tRNA (32), generated as described by Donis-Keller et al. (33), were fractionated in parallel with the corresponding formamide hydrolysis products on sequencing gels of 20% acrylamide, 8.3 M urea.

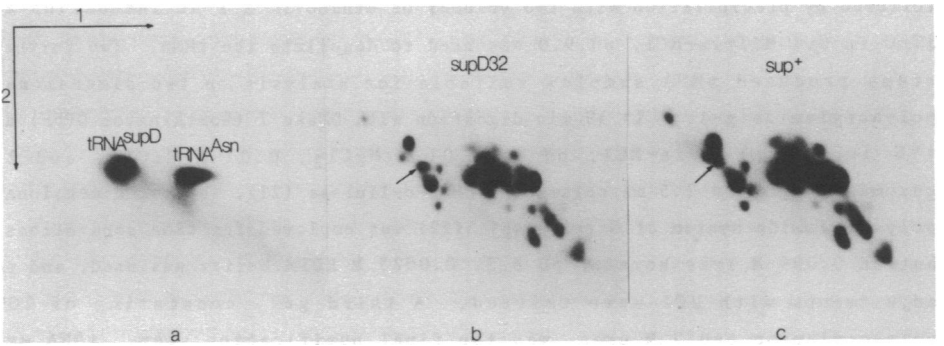

Figure 1: Autoradiogram of ^{32}P-labeled tRNAs separated by two-dimensional polyacrylamide gel electrophoresis. The first dimension was 9.6% polyacrylamide, 7 M urea, and the second, 20% acrylamide, 4 M urea. The tRNAs purified by annealing to λdsupD32 DNA are shown in (a). The total tRNAs from supD32 (b) and supD$^+$ (c) strains labeled after heat induction of a λcI857 prophage near the supD locus are displayed. Arrows mark the positions of the corresponding suppressor and wild type tRNA species.

RESULTS

Identification of the supD tRNAs

To facilitate detection of the suppressor tRNA specified by the supD32 gene, an E. coli strain which carries λcI857 inserted near supD was labeled with $^{32}PO_4$ following heat induction of the prophage. Phage RNAs were removed from the ^{32}P-labeled total cellular RNA by hybridization to λcI857 DNA. tRNAs complementary to DNA from the transducing phage λdsupD32 were then purified by annealing to a λdsupD32, λcI857 DNA mixture. The material released from the hybrids, less than one percent of the total ^{32}P-labeled RNA, was separated in two dimensions on polyacrylamide gels. An autoradiogram of the gel (Fig. 1a) revealed two tRNA species. Fingerprints obtained for the species on the right were identical to published fingerprints of E. coli tRNA^{Asn} (34). A T1 RNase fingerprint suggested that the tRNA on the left was also a unique species, but one that had not been detected in previous experiments. Fingerprint analysis was then used to locate this species in the two-dimensional display of total ^{32}P-labeled tRNA derived from the supD32 strain (Fig. 1b). The corresponding tRNA from the isogenic supD$^+$ strain, labeled as described above, had a slightly reduced mobility in the two-dimensional gel system (Fig. 1c). These tRNAs were identified as serine tRNAs by aminoacylation using crude synthetase preparations. As shown by the T1 RNase fingerprints of Fig. 2, they are nearly identical. Oligonucleotide analysis showed that 2-methylthio-N^6-isopentenyl adenosine, a modified nucleoside characteristically found in

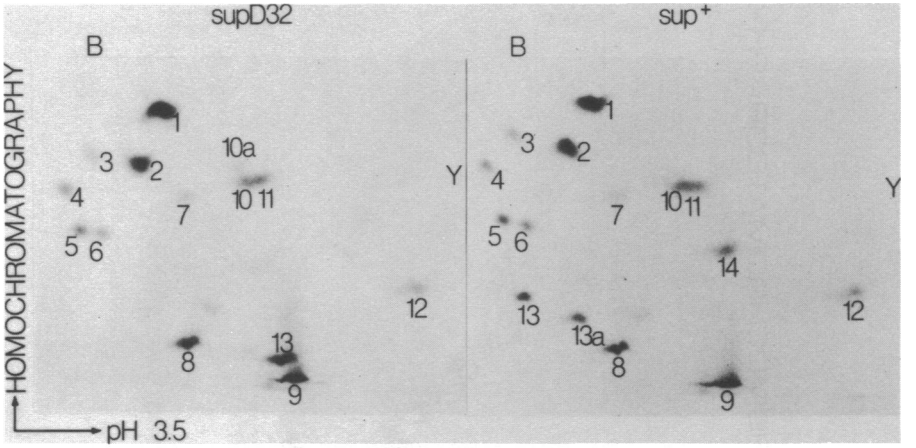

Figure 2: T1 RNase fingerprints of uniformly labeled $tRNA^{ser}_{2am}$ (supD32) and $tRNA^{ser}_{2}$ (sup^{+}). The first dimension was electrophoresis on cellulose acetate (Cellogel) strips in pyridinium acetate, pH 3.5, 7 M urea; the second was homochromatography on PEI-cellulose thin layer plates in homomixture C (27). B and Y, positions of the blue (xylene cyanole FF) and yellow (orange G) marker dyes, respectively.

the anticodons of some tRNAs, was present in a large oligonucleotide (numbered 13) in the tRNA from the suppressing strain, and in a smaller oligonucleotide with variable mobility (spots 13 and 13a), in the tRNA from the nonsuppressing strain. The latter fingerprint also included an extra oligonucleotide (labeled 14). This differential oligonucleotide pattern was the expected result of a nonsense suppressor mutation, namely, a single nucleotide change in a 5'CGA 3' anticodon for serine (UCG) to give the 5'CUA 3' anticodon which reads UAG stop codons.

Nucleotide sequence analysis

Sanger RNA sequencing methods were employed for initial characterization of the ^{32}P-labeled suppressor and wild type serine tRNAs, hereafter referred to as $tRNA^{ser}_{2am}$ and $tRNA^{ser}_{2}$. The products of complete secondary digestions established the sequences of the smaller RNase T1 and RNase A oligonucleotides, and when combined with partial spleen phosphodiesterase digestion data, gave the sequences of several longer oligonucleotides. Oligonucleotides were ordered within the tRNA sequence by using information derived from limited digestion with RNase T1. However, the tRNAs contain stretches of pyrimidine residues that were not easily ordered by these sequencing methods. One of these occurs in the suppressor tRNA at the 5' end

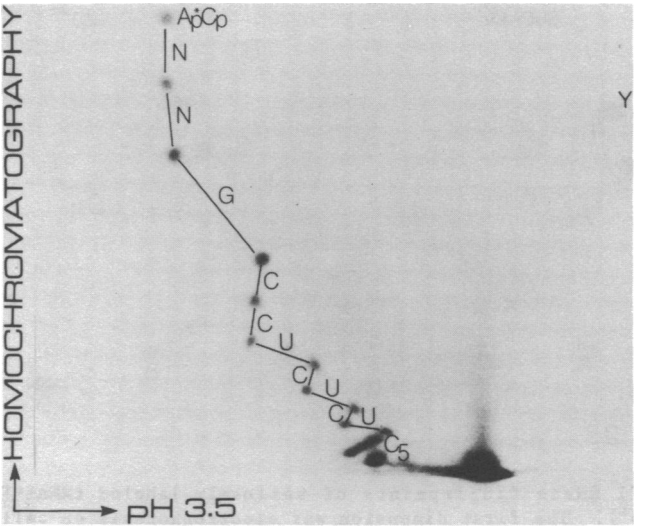

Figure 3: Sequence from the 3' terminus of [5'-^{32}P]pCp-labeled tRNA$^{ser}_2$ through the pyrimidine-rich TΨC-stem. Hydrolysis products were generated at 100°C for 10 min in 100% formamide. Two-dimensional separation was as in Figure 2. Since removal of the C residues closest to the labeled end does not give diagnostic mobility shifts, these nucleotides are designated N. The smallest labeled product derived from formamide hydrolysis, as determined from analysis by TLC chromatography, is the dinucleotide A*pCp, where the asterisk indicates the position of the labeled phosphate.

of oligonucleotide 13 from the anticodon loop, and another, in both tRNAs within a very long oligonucleotide (Fig. 2, spot 9) that contains the modified nucleosides T and Ψ. In addition, an AUG triplet later placed at the base of the aminoacyl acceptor stem was not found in molar yield in fingerprints of uniformly ^{32}P-labeled material. Information deduced from RNase A fingerprints likewise left gaps in the sequence, particularly at the 5' and 3' ends of the molecules.

Several methods designed for analysis of RNAs labeled *in vitro* at a 5' or 3' end were thus applied to obtain the data needed for unambiguous sequence assignments. Unlabeled tRNA$^{ser}_2$ was used for these purposes, since the suppressor species could not be obtained as a homogeneous chemical species. In a first approach, nested sets of tRNA$^{ser}_2$ hydrolysis products generated by limited hydrolysis in H_2O or formamide were labeled with [γ-^{32}P]ATP, and separated on sequencing gels. Following direct transfer of the fragments to PEI-cellulose thin layer plates, elution, and digestion with nuclease P1, the resulting labeled 5' terminal nucleoside monophosphates, including modified

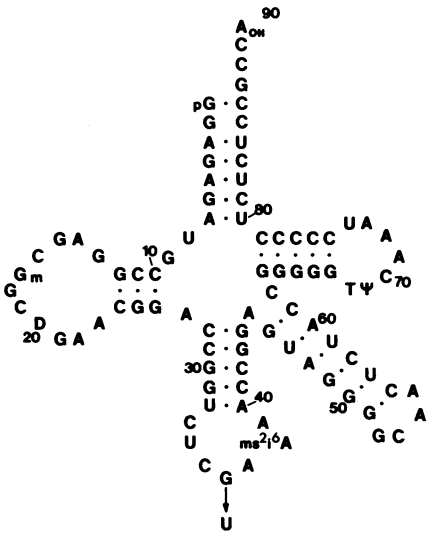

Figure 4: Cloverleaf model of $tRNA_2^{ser}$. In the amber suppressor tRNA, the central anticodon nucleotide is U.

nucleotides, were identified by thin layer chromatography. Taken together, the 5' labeled fragments provided sequence data for positions 2-62, and 68-73 of the tRNA. The gap was due to a series of G residues in the TΨC-stem which were not clearly resolved, although earlier oligonucleotide analysis indicated that the sequence here was G_5T. In a second approach designed to obtain information from the 3' terminal region of the molecule, $tRNA_2^{ser}$ was labeled at this end with [5'-^{32}P]pCp, using the reaction catalyzed by T4 RNA ligase. The products of limited digestion with RNase A, T1, U2, and PhyI provided data that spanned much of the 3' region. However, the lack of a C-specific cleavage reaction and the presence of residual secondary structure at the temperature used for digestions (55°C) again resulted in incomplete data for the region of the TΨC-stem. Thus to complete the 3' terminal sequence, (^{32}P)pCp-labeled tRNA was subjected to limited hydrolysis in formamide, and the products were separated in a two-dimensional fingerprint. As shown in the autoradiogram of Fig. 3, this displayed an unambiguous sequence for the pyrimidine-rich 3' end.

The sequence deduced for $tRNA_2^{ser}$ is shown in Fig. 4, with the single base change resulting from the suppressor mutation indicated by the arrow. Evidence that the central anticodon nucleotide in $tRNA_{2am}^{ser}$ is a U residue was provided by the analysis of uniformly ^{32}P-labeled material. Products of RNase T1 and

RNase A digestion ruled out the presence of G or A in this position, and PhyI digestion of the T1 RNase oligonucleotide 13 (Fig. 2) permitted distinction between the pyrimidines. PhyI, which cleaves after G, U, and A residues, gave the complete digestion products Up, CUp, and CCGp, as well as an incomplete product that contained Ap and ms^2i^6Ap. The absence of CCAp among the products suggests that a U precedes A in the anticodon oligonucleotide UCUCUAms^2i^6AAACCG. A thymine in the corresponding position of the *supD32* gene DNA (20) confirms the RNA sequence deduced. Both tRNAs, as determined from TLC chromatographic analysis of uniformly and 5' end labeled material, contain the modified nucleotides Gmp, Dp, ms^2i^6Ap, Tp, and Ψp. s^4Up was not found in any of the preparations, nor was there evidence for additional modified nucleotides in the anticodon. The low molar yields observed for the AUG triplet which would contain s^4U, however, could be accounted for by degradation during the course of analysis due to the presence of this modified nucleotide.

DISCUSSION

The nucleotide sequences of $tRNA_2^{ser}$ and $tRNA_{2am}^{ser}$ provide direct evidence that *supD*$^-$ amber suppression in *Escherichia coli* is the result of a mutational alteration in a serine tRNA anticodon. Among the tRNA suppressors of nonsense, missense, and frameshift mutations analyzed to date at the molecular level, a nucleotide substitution or addition in the anticodon is nearly always the outcome of the suppressor mutation (35). The UGA suppressing activity of a $tRNA^{trp}$ derivative that has acquired a G→A base change in the D stem (36), however, shows that other tRNA structural changes can lead to aberrant codon recognition.

The novel *E. coli* tRNA species identified by our analysis is the third among the family of serine isoacceptors to be purified to homogeneity and subjected to nucleotide sequence analysis. $tRNA_1^{ser}$, a major species with a 5'VGA 3' anticodon (37) that responds to serine codons UCA and UCG, is specified by the *serT* gene, which has been located (38) in the region of minute 16 on the Taylor and Trotter map. $tRNA_3^{ser}$, which carries a 5'GCU 3' anticodon specific for serine codons AGU and AGC (39,40), is the product of a gene *serW*, located near minute 61 (38). The *supD* gene has previously been mapped near *his* at 43 minutes (19,41). Since it has now been identified as the structural gene for a serine tRNA, the gene will be designated *serU*. The product of the wild type *serU*$^+$ gene, $tRNA_2^{ser}$, has a 5'CGA 3' anticodon that should respond uniquely to the serine codon UCG; the corresponding product of

the amber suppressor allele used here (serU132), $tRNA^{ser}_{2am}$, reads the UAG stop codon. The primary structures and genetic map locations for additional serine tRNAs, including those specific for UCU and UCC codons, remain to be established.

As determined from inspection of the sequences, $tRNA^{ser}_2$ has numerous features in common with E. coli $tRNA^{ser}_1$ and $tRNA^{ser}_3$ as well as with the serine tRNA specified by bacteriophage T4 (42). These tRNAs characteristically have 3 base pairs in the D stem, and large variable stem-loop structures containing 16-21 nucleotides. There is little homology among the extra arm sequences, however. The anticodon stem and loop sequence of $tRNA^{ser}_2$ is nearly identical to those of $tRNA^{ser}_1$ and T4 $tRNA^{ser}$. In the region of the TΨC-loop, $tRNA^{ser}_2$ has a sequence TΨCAAAU identical to that found in T4 $tRNA^{ser}$, but the very stable stem of 5 GC base pairs that closes this loop is strikingly homologous to the T-stem of $tRNA^{ser}_3$.

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