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Nucleotides that determine *Escherichia coli* $tRNA^{Arg}$ and $tRNA^{Lys}$ acceptor identities revealed by analyses of mutant opal and amber suppressor tRNAs

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ABSTRACT **We have constructed an opal suppressor system in *Escherichia coli* to complement an existing amber suppressor system to study the structural basis of tRNA acceptor identity, particularly the role of middle anticodon nucleotide at position 35. The opal suppressor tRNA contains a UCA anticodon and the mRNA of the suppressed protein (which is easily purified and sequenced) contains a UGA nonsense triplet. Opal suppressor tRNAs of two $tRNA^{Arg}$ isoacceptor sequences each gave arginine in the suppressed protein, while the corresponding amber suppressors with U35 in their CUA anticodons each gave arginine plus a second amino acid in the suppressed protein. Since C35 but not U35 is present in the anticodon of wild-type $tRNA^{Arg}$ molecules, while the first anticodon position contains either C34 or U34, these results establish that C35 contributes to $tRNA^{Arg}$ acceptor identity. Initial characterizations of opal suppressor $tRNA^{Arg}$ mutants by suppression efficiency measurements suggest that the fourth nucleotide from the 3′ end of $tRNA^{Arg}$ (A73 or G73 in different isoacceptors) also contributes to $tRNA^{Arg}$ acceptor identity. Wild-type and mutant versions of opal and amber $tRNA^{Lys}$ suppressors were examined, revealing that U35 and A73 are important determinants of $tRNA^{Lys}$ acceptor identity. Several possibilities are discussed for the general significance of having tRNA acceptor identity in the same positions in different tRNA acceptor types, as exemplified by positions 35 and 73 in $tRNA^{Arg}$ and $tRNA^{Lys}$.**

The aminoacylation specificity of tRNA ("tRNA acceptor identity") is essential for protein synthesis. The structural features in tRNA that determine tRNA acceptor identity have been studied in several ways: by measuring *in vitro* aminoacylation of tRNAs that differ structurally from corresponding wild-type tRNAs (1–16); by determining the interacting surfaces in complexes of tRNAs and aminoacyl-tRNA synthetases (17–23); by comparing tRNA sequences (24–27); and by determining the *in vivo* amino acid specificities of suppressor tRNAs (28–44). The acceptor identity of tRNA results from the tRNA's productive interaction with the cognate aminoacyl-tRNA synthetase and nonproductive interactions with all other aminoacyl-tRNA synthetase enzymes. The net outcome of both types of interactions is obtained with a suppressor tRNA. For analysis, a suppressor tRNA gene present in a plasmid is inserted into a cell, and, because of its distinctive codon recognition properties, the acceptor identity of the transcribed suppressor tRNA is mirrored by the amino acid recovered in a suppressed protein. Mutants of a suppressor tRNA can pinpoint the specific nucleotides that determine tRNA acceptor identity when the amino acid recovered in the suppressed protein is altered.

We previously reported (38) that the acceptor identity of *Escherichia coli* $tRNA^{Arg}$ is partially determined by the adenosine residue at position 20 (A20) in the variable pocket (38). Our work was based on a computer analysis of tRNA sequences and subsequent sequencing of suppressed protein produced by mutants of amber suppressor tRNAs. We also suggested that the cytidine residue at position 35 (C35) in the wild-type anticodon contributes to $tRNA^{Arg}$ acceptor identity because amber suppressor (U35) $tRNA^{Arg}$ inserted both arginine and lysine into suppressed protein. In amber suppressor $tRNA^{Arg}$, U35 replaces the wild-type C35 to allow the anticodon (CUA) of the tRNA to pair with the amber triplet (UAG) in the mRNA. The insertion of lysine into suppressed protein by amber suppressor $tRNA^{Arg}$ also suggested that U35, which is present in wild-type $tRNA^{Lys}$, contributes to $tRNA^{Lys}$ acceptor identity. We report here the construction of an opal suppressor system in which the suppressor tRNA contains a UCA anticodon and the mRNA of the suppressed protein contains a UGA nonsense triplet. Analysis using this system has allowed us to confirm and extend the description of nucleotide residues that contribute to the acceptor identities of $tRNA^{Arg}$ and $tRNA^{Lys}$.

System Design

The opal suppressor system was constructed from an existing amber suppressor system (34, 44). An oligodeoxynucleotide was synthesized as an exact copy of the wild-type tRNA gene sequence with the anticodon sequence changed to UCA to make an opal suppressor tRNA that will pair with UGA in the mRNA. Mutants of the opal suppressor tRNA gene combined the UCA anticodon with other nucleotide changes. Each synthetic gene was separately inserted into a pBR322-type plasmid and introduced into *E. coli* cells. The sequence of the cloned gene was confirmed, but the transcribed tRNA was not examined for sequence or base modification. Two functional properties of the synthesized tRNA were determined, the suppression efficiency and the amino acid specificity. The suppression efficiency, which is the percent of growing protein chains translated beyond a nonsense triplet, was measured in the *lacI–Z* fusion system, where the type of amino acid inserted in the *I* segment has little effect on β-galactosidase enzyme activity. The amino acid specificity of the suppressor tRNA was determined by sequencing suppressed dihydrofolate reductase protein translated from a mRNA with a UGA nonsense triplet at codon position 10. The dihydrofolate reductase protein was expressed from a second, pACYC184-type plasmid compatible with the plasmid expressing the suppressor tRNA. The dihydrofolate reductase gene containing the UGA triplet was derived by mutagenesis from the corresponding gene containing the UAG triplet. Initial results with this system have shown that the yield of suppressed dihydrofolate reductase protein is lower in the opal system than in the amber system. Opal mutants in the *lacI–Z* fusion system were available (45).

A characteristic property of the opal suppression system is that, even in the absence of an opal suppressor tRNA, the

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UGA triplet is translated as amino acid at a low but significant level (45). For the *U4* allele in *lacI–Z* used here, a 1% suppression efficiency was the basal level (see Table 1 legend). Because of this property, an opal suppressor tRNA had to exhibit a suppression efficiency >1% to be useful for our analyses.

Anticodon and Variable Pocket

The anticodon of *E. coli* $tRNA^{Arg}$ is ICG (I is inosine, a modified adenosine) in the major isoacceptor and CCU in a minor isoacceptor (ref. 46; Fig. 1 *A* and *B*). Opal suppressors of each were constructed by changing the anticodon sequences to UCA. The suppressors were termed ARG for the major $tRNA^{Arg}$ isoacceptor and ARGII for the minor $tRNA^{Arg}$ isoacceptor. The suppression efficiencies were 19% and 10%, respectively (Table 1), and each suppressor $tRNA^{Arg}$ inserted only arginine into suppressed protein (Table 2). In contrast, the corresponding amber suppressor $tRNA^{Arg}$ molecules with a CUA anticodon sequence inserted a second amino acid in addition to arginine (ref. 38 and Table 2). Opal and amber suppressors of the minor $tRNA^{Arg}$ isoacceptor had lower suppression efficiencies than the corresponding suppressors of the major $tRNA^{Arg}$ isoacceptor. This could reflect poorer aminoacylation or ribosomal function of the minor $tRNA^{Arg}$ isoacceptor.

The specificity of the $tRNA^{Arg}$ opal suppressors corroborates the importance of C35 to $tRNA^{Arg}$ acceptor identity. Two additional points are noteworthy. (*i*) The anticodon sequences of the opal and amber suppressor tRNAs differ from one another in two bases, and the data do not independently demonstrate that the middle base is responsible for the different acceptor identities of the two tRNA molecules. However, all known *E. coli* $tRNA^{Arg}$ isoacceptors contain C35, whereas the base at position 34 varies. (*ii*) Even though the opal suppressors of both $tRNA^{Arg}$ isoacceptors contain mutant bases at positions 34 and 36 while retaining a $tRNA^{Arg}$ acceptor function, the wild-type bases might still play a supporting role in $tRNA^{Arg}$ acceptor identity. A contribution of <5% would escape us, since this is the lower limit of detecting a secondary amino acid in the suppressed protein (37).

The role of U35 in $tRNA^{Lys}$ acceptor identity was suggested by the finding that several amber suppressor (U35) tRNAs including $tRNA^{Arg}$ inserted some lysine into suppressed protein (35, 37, 38, 41, 44). In the present study, the conversion of the anticodon of the only known acceptor of *E. coli* $tRNA^{Lys}$ from mam^5s^2UUU (Fig. 1*C*) to UCA resulted in opal suppressor $tRNA^{Lys}$ that had a suppression efficiency of 1% (Table 1). This indicates that opal suppressor $tRNA^{Lys}$ is inactive, since 1% is the basal enzyme activity present in cells without an opal suppressor tRNA (Table 1). This result contrasts with amber suppressor $tRNA^{Lys}$, which had a suppression efficiency of 38% (ref. 41; Table 1). The following results further indicate that opal suppressor $tRNA^{Lys}$ is nonfunctional. Northern analyses (38) indicated that similar amounts of suppressor $tRNA^{Lys}$ were present in cells containing opal or amber suppressors (data not shown). Also, different mRNA codons in *lacI–Z* surround UGA and UAG

Table 1. Suppressor tRNA efficiency

tRNA	Suppression efficiency, %
Opal suppressor	
ARG	19
ARGG20	1*
ARGU20	1*
ARGG73	16
ARGU73	5
ARGC73	3
ARGII	10
ARGIIG73	11
ARGIIU73	5
LYS	1*
LYSA20	2
Amber suppressor	
ARG	29
ARGII	18
LYS	38
LYSA20	36
LYSG73	13
LYSU73	7

Fig. 1 shows the wild-type tRNA sequences. Suppression efficiency measurements used opal UGA allele *U4* (codon 189) or amber UAG allele *A16* (codon 153) in a *lacI–Z* fusion. Suppression efficiency is the percentage of enzyme activity relative to the wild-type *lacI–Z* fusion (which averaged 191 units) and has not been corrected for the value found for cells without a suppressor tRNA (1.0% for *U4* and <0.00% for *A16*). Methods were described (37, 38).
*Value similar to that for cells without a suppressor tRNA.

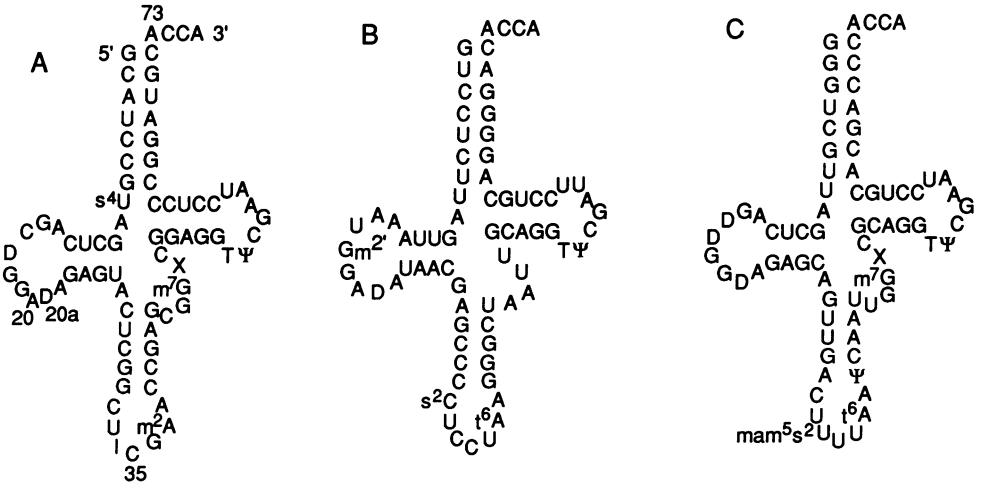

FIG. 1. Cloverleaf arrangement of nucleotide sequences of *E. coli* major $tRNA^{Arg}$ isoacceptor (*A*), minor $tRNA^{Arg}$ isoacceptor (*B*), and $tRNA^{Lys}$ (*C*). The sequences and nucleotide modifications are described elsewhere (46). The I at position 34 of the major $tRNA^{Arg}$ isoacceptor is inosine. Opal or amber suppressor derivatives of the tRNA sequences change the anticodon sequences (positions 34, 35, and 36) to UCA or CUA, respectively. The suppressor genes and transcribed tRNAs are designated ARG for the major $tRNA^{Arg}$ isoacceptor, ARGII for the minor $tRNA^{Arg}$ isoacceptor, and LYS for $tRNA^{Lys}$.

Table 2. Suppressor tRNA specificity

tRNA	Amino acid(s) (%) in suppressed protein
Opal suppressor	
ARG	Arg (92)
ARGII	Arg (97)
LYSA20	Arg (84*)
Amber suppressor	
ARG†	Arg (37), Lys (55)
ARGII	Arg (66), Gln (17)
LYS†	Lys (94)
LYSA20	Lys (99)
LYSG73	Lys (60), Gln (29‡)

The amino acid corresponding to the UGA or UAG nonsense triplet at position 10 in dihydrofolate reductase is reported. Amino acid yield <5% is not reported; thus, the combined reported yield is <100%. Protein purification and data analyses were described (37).
*Corrected by subtracting 7% Met present in a contaminating protein sequence reading (residues 1–12) Ala-Lys-Val-Leu-Val-Leu-Tyr-Tyr-Ser-Met-Tyr-Gly.
†Published previously (37, 38, 41).
‡Includes 3% Glu.

triplets (45) and could contribute to the different suppression efficiencies of the two $tRNA^{Lys}$ suppressors. However, the UGA and UAG triplets are present at the same position in the mRNA of dihydrofolate reductase, and two attempts to purify suppressed protein produced by opal suppressor $tRNA^{Lys}$ revealed little or no protein. All these results indicate that the change from U35 to C35 severely damages the interaction of $tRNA^{Lys}$ with the lysyl-tRNA synthetase. This demonstrates that U35 is essential to $tRNA^{Lys}$ acceptor identity.

In addition to the work with the anticodons, two types of suppressor tRNA mutants confirmed the contribution of A20 in the variable pocket to $tRNA^{Arg}$ acceptor identity. Wild-type $tRNA^{Arg}$ contains the base sequence G19-A20-D20a-A21 (D is a modified U), whereas $tRNA^{Lys}$ contains G19-D20a-A21 (Fig. 1). While residues G19 and A21 are common to all *E. coli* tRNA sequences, the dinucleotide A20-D20a is distinctive of $tRNA^{Arg}$ species (38). The first type of mutant changed the opal suppressor of the major isoacceptor of $tRNA^{Arg}$ from A20-D20a to either G20-D20a (mutant ARGG20) or U20-D20a (mutant ARGU20). The resulting suppression efficiency of each mutant was 1% (Table 1), indicating that $tRNA^{Arg}$ was inactive. The second type of mutant (LYSA20) changed D20a to A20-D20a in $tRNA^{Lys}$. The amber suppressor $tRNA^{Lys}$ mutant was essentially normal, indicating that the nucleotide spacing between G19 and A21 does not contribute directly to $tRNA^{Lys}$ acceptor identity. However, both functional properties of the opal suppressor $tRNA^{Lys}$ mutant were altered: the suppression efficiency was 2% (Table 1) and arginine was present in suppressed protein (Table 2). The conversion of $tRNA^{Lys}$ to $tRNA^{Arg}$ acceptor identity can be explained since the known nucleotide residues needed for $tRNA^{Arg}$ acceptor identity, A20 and C35 (and possibly A73; see below), were present, while U35, needed for $tRNA^{Lys}$ acceptor identity, was absent. The 2% suppression efficiency for this mutant indicates that additional structural features of $tRNA^{Arg}$ acceptor identity remain to be identified.

Several observations in these and earlier experiments (38) indicate that A20 plays a larger role than C35 in determining $tRNA^{Arg}$ acceptor identity. (*i*) Changing A20 to either G20 or U20 in opal suppressor $tRNA^{Arg}$ inactivated the molecules, while changing C35 to U35 (i.e., changing opal suppressor $tRNA^{Arg}$ to amber suppressor $tRNA^{Arg}$) left the molecules active and with a substantial $tRNA^{Arg}$ acceptor identity. (*ii*) Every opal and amber suppressor tRNA with a substantial $tRNA^{Arg}$ acceptor identity examined to date contains A20, while, in contrast, tRNA molecules with this acceptor identity may or may not contain C35.

Schulman and Pelka (13) reported on *in vitro* aminoacylation of unmodified tRNA transcripts by the arginyl-tRNA synthetase. It was concluded that residues corresponding to A20 and C35 of $tRNA^{Arg}$ contribute to the binding and catalytic parameters of the aminoacylation reaction. Moreover, in the wild-type and mutant $tRNA^{Met}$ molecules used for the measurements, C35 was 40-fold more important than A20. We note that this is opposite the bases' relative importance in $tRNA^{Arg}$ suppressors. The *in vivo* and *in vitro* systems agree on the qualitative importance of A20 and C35 to $tRNA^{Arg}$ acceptor identity; however, they need not yield the same relative relationship between the two nucleotides. For example, the *in vivo* system examines the competition among all 20 aminoacyl-tRNA synthetase enzymes for the suppressor tRNA. Further, the *in vitro* measurements were conducted with mutants of $tRNA^{Met}$, which differ from the $tRNA^{Arg}$ isoacceptors by 24–33 nucleotide residues. These differences could bias the relative contribution of A20 and C35.

Position 73

Some years ago, mutants of amber suppressor $tRNA^{Tyr}$ that allowed aminoacylation with tyrosine and glutamine were isolated. One mutation changed A73 to G73 in the fourth base from the 3′ end of the $tRNA^{Tyr}$ chain (28, 29). Residue G73 is present in *E. coli* $tRNA^{Gln}$ and apparently gives the 3′ end of the $tRNA^{Gln}$ chain a distinctive loop conformation that is important for interaction with the glutaminyl-tRNA synthetase (22). Although the structural basis is unknown, the nucleotide at position 73 is known to contribute to the acceptor identities of *E. coli* $tRNA^{Ser}$ (34) and $tRNA^{Phe}$ (37) and to the *in vitro* aminoacylation of *E. coli* $tRNA^{Met}$ (6), $tRNA^{Asp}$ (11), and $tRNA^{His}$ (12).

We have examined position 73 for a contribution to $tRNA^{Lys}$ acceptor identity by changing A73 to either G73 (LYSG73) or U73 (LYSU73) in amber suppressor $tRNA^{Lys}$. Both mutants exhibited a reduced suppression efficiency (Table 1). Further analysis of the G73 mutant by sequencing suppressed protein revealed that lysine and glutamine were present (Table 2). Since only lysine is present in the protein from wild-type amber suppressor $tRNA^{Lys}$ (refs. 37 and 41; Table 2), these results demonstrate that A73 contributes to $tRNA^{Lys}$ acceptor identity. Residue U35 was shown above to play an essential role in $tRNA^{Lys}$ acceptor identity and thus appears to be more important than A73 in $tRNA^{Lys}$ acceptor identity.

We surmise that the presence of G73 in $tRNA^{Lys}$ allows glutaminyl-tRNA synthetase to effectively compete with the lysine enzyme for aminoacylation of the mutant $tRNA^{Lys}$ molecule. While a successful competition does not require that the interaction with the cognate lysyl-tRNA synthetase be damaged, this apparently has occurred, since mutant G73 $tRNA^{Lys}$ has a reduced suppression efficiency. The interaction needs to be examined by *in vitro* aminoacylation or other experiments, since suppression efficiency also can be reduced by deficiencies in the synthesis (47) or ribosomal function (31) of a suppressor tRNA. To the extent that deficiencies in synthesis or translation successfully discriminate against incorrectly aminoacylated tRNAs, tRNA acceptor identity should be viewed as a property of several cellular systems, of which the aminoacyl-tRNA synthetases are but one component. At present, we suggest that residue A73 in $tRNA^{Lys}$ contributes to $tRNA^{Lys}$ acceptor identity in two different ways: directly, by promoting the productive interaction with the cognate lysyl-tRNA synthetase, and

indirectly, by preventing interactions with noncognate aminoacyl-tRNA synthetases, as exemplified by the glutaminyl-tRNA synthetase enzyme.

The role of the base at position 73 in $tRNA^{Arg}$ acceptor identity was also investigated. The two isoacceptors of $tRNA^{Arg}$ studied here contain A73, although other isoacceptors of *E. coli* $tRNA^{Arg}$ contain G73 (46). Mutants of the two opal suppressors containing either G73, U73, or C73 in the major $tRNA^{Arg}$ isoacceptor and either G73 or U73 in the minor $tRNA^{Arg}$ isoacceptor were constructed. The G73 mutants of both $tRNA^{Arg}$ isoacceptors were little affected in suppression efficiency; in contrast, the efficiencies of the U73 and C73 mutants were reduced (Table 1). While the characterization of these mutants is incomplete, the results strongly indicate that position 73 contributes to $tRNA^{Arg}$ acceptor identity. Since only one arginyl-tRNA synthetase has been found in *E. coli* (48), these results raise the possibility that two different nucleotide residues, A73 and G73, specify one function.

Conclusions and Implications

Analyses of wild-type and mutant opal and amber suppressor tRNAs have demonstrated that residues A20 and C35 (and possibly A73 and G73) contribute to $tRNA^{Arg}$ acceptor identity and that residues U35 and A73 contribute to $tRNA^{Lys}$ acceptor identity. Functional analyses of mutant suppressor tRNAs defective in these individual residues allowed an assessment of their relative importance to tRNA acceptor identity.

Many, but not all, of the nucleotides that determine tRNA acceptor identity are localized in common regions and positions of the different tRNA molecules. This feature is illustrated by the importance of residues 35 and possibly 73 in $tRNA^{Arg}$ and $tRNA^{Lys}$ acceptor identities. The individual importance of residue 20 to $tRNA^{Arg}$ and not to $tRNA^{Lys}$ acceptor identity demonstrates that the localization is incomplete. The involvement of residue 73 in the acceptor identity of several additional *E. coli* tRNAs was noted above. Other examples where residue 35 contributes to *E. coli* tRNA acceptor identity include $tRNA^{Gln}$ (22, 30), $tRNA^{Met}$ (8), and $tRNA^{Trp}$ (30).

Having tRNA acceptor identity in the same position in different tRNA molecules was predicted from the results of computer comparisons of 67 *E. coli* tRNA sequences: two regions of the tRNA molecules were repeatedly found to correlate with different tRNA acceptor types. These two regions were in the anticodon and in the acceptor end, the latter region including position 73 and the four adjoining base pairs of the helix (27). The involvement of residue 73 in tRNA acceptor identity had been suggested earlier (24).

We suggest several possibilities that are interrelated but not mutually exclusive for the general significance of having tRNA acceptor identity in the same position in different tRNA acceptor types.

(*i*) Some of the positions for tRNA acceptor identity could have been present in the tRNA molecules earlier in evolution and been retained. This possibility was suggested for residue 73 (24). The aminoacyl-tRNA synthetase enzymes would have coevolved with the tRNAs.

(*ii*) The presence of similar catalytic mechanisms in many contemporary aminoacyl-tRNA synthetases (2, 48) may necessitate a certain degree of similarity in the locations of the nucleotides that determine tRNA acceptor identity.

(*iii*) Just as the anticodon of the tRNA molecule recognizes the cognate codon in the mRNA, the anticodon can also recognize the aminoacyl-tRNA synthetase for the cognate amino acid (7–9, 30, 33, 49).

(*iv*) Having tRNA acceptor identity in the same position in different tRNAs uses less of the sequences for this function, leaving more of the sequences for the shared functions and common three-dimensional structure of the tRNA molecules.

(*v*) Having tRNA acceptor identity in the same position in different tRNA molecules helps tRNAs discriminate against the noncognate aminoacyl-tRNA synthetase enzymes because the position will more often hold other bases. For example, a tRNA with a larger base or a charged base in the tRNA acceptor identity position can ignore enzymes with cavities designed to accept only smaller or differently charged bases at that position.

(*vi*) The bases in the tRNA acceptor-identity positions must be exposed on the surface of the tRNA molecules for the interactions with the cognate and noncognate aminoacyl-tRNA synthetases. Residues 20, 35, and 73 meet this requirement, as they are in single-stranded regions of the tRNA structure (50, 51). However, a conformational change in tRNA structure can expose a base that is internal to the static tRNA structure (22, 41, 42). It was noted previously that most of the bases in the tRNA structure, except those specifically concerned with recognition by other molecules, are internal, similar to hydrophobic groups in proteins (52).

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