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Specific function of a G·U wobble pair from an adjacent helical site in tRNA^{Ala} during recognition by alanyl-tRNA synthetase

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ABSTRACT

G·U wobble pairs are crucial to many examples of RNA–protein recognition. We previously concluded that the G·U wobble pair in the acceptor helix of *Escherichia coli* alanine tRNA (tRNA^{Ala}) is recognized indirectly by alanyl-tRNA synthetase (AlaRS), although direct recognition may play some role. Our conclusion was based on the finding that amber suppressor tRNA^{Ala} with G·U shifted to an adjacent helical site retained substantial but incomplete Ala acceptor function in vivo. Other researchers concluded that only direct recognition is operative. We report here a repeat of our original experiment using tRNA^{Lys} instead of tRNA^{Ala}. We find, as in the original experiment, that a shifted G·U confers Ala acceptor activity. Moreover, the modified tRNA^{Lys} was specific for Ala, corroborating our original conclusion and making it more compelling.

Keywords: helical irregularity; in vivo analysis; nonsense suppressor; tRNA identity

INTRODUCTION

Biological RNA–protein interactions require the stable merging of two complementary tertiary structures. The properties of unique small structural features, like G·U wobble pairs within helical tracts, may be essential for specificity in many such interactions. The hydrogen bonding between G and U displaces these bases into the helical grooves relative to the bases in G·C and A·U Watson-Crick base pairs (Ladner et al., 1975; Quigley & Rich, 1976). This not only creates a distinctive array of functional groups, but also weakens the helix and causes local distortions (Rhodes, 1977; He et al., 1991; Allain & Varani, 1995b). Thus, a ligand might recognize a G·U pair in a regular A-type helix (direct recognition) or a G·U pair in an irregular helix (indirect recognition). A dynamic mechanism involves recognition of G·U in a helix that alternates between regular and irregular forms, with ligand binding and intrinsic instability of G·U mediating transition between forms.

The G·U wobble pair at the third site in the acceptor helix of *Escherichia coli* tRNA^{Ala} (G3·U70) (Fig. 1) is a major determinant of tRNA^{Ala} acceptor identity (Hou & Schimmel, 1988; McClain & Foss, 1988a). Tertiary structural data are not available for tRNA^{Ala} or AlaRS. Nevertheless, we concluded previously that recognition of tRNA^{Ala} by AlaRS proceeds by both indirect and direct recognition mechanisms (McClain et al., 1988). This conclusion was based, in part, on our observation that G3·U70 could be shifted to G4·U69, an adjacent helical site, without substantial loss of aminoacylation specificity in amber suppressor tRNA^{Ala}. The redesigned G4·U69 tRNA^{Ala} was less active but, more importantly, it was substantially aminoacylated with Ala (79% Ala and 7% Lys, with no other amino acid in significant yield). Other workers concluded that aminoacylation of tRNA^{Ala} proceeds by direct recognition only (Shi et al., 1990; Musier-Forsyth et al., 1991; Musier-Forsyth & Schimmel, 1992). However, an exclusive direct recognition mechanism would not tolerate the shift of G·U. We report here a repeat of our original experiment using suppressor tRNA^{Lys} instead of suppressor tRNA^{Ala}. We find, as in the original experiment, that the G4·U69 pair confers tRNA^{Ala} acceptor identity. Moreover, the modified tRNA^{Lys} was specific for Ala acceptance, confirming our original conclusion that an indirect recognition mechanism is required to explain the data.

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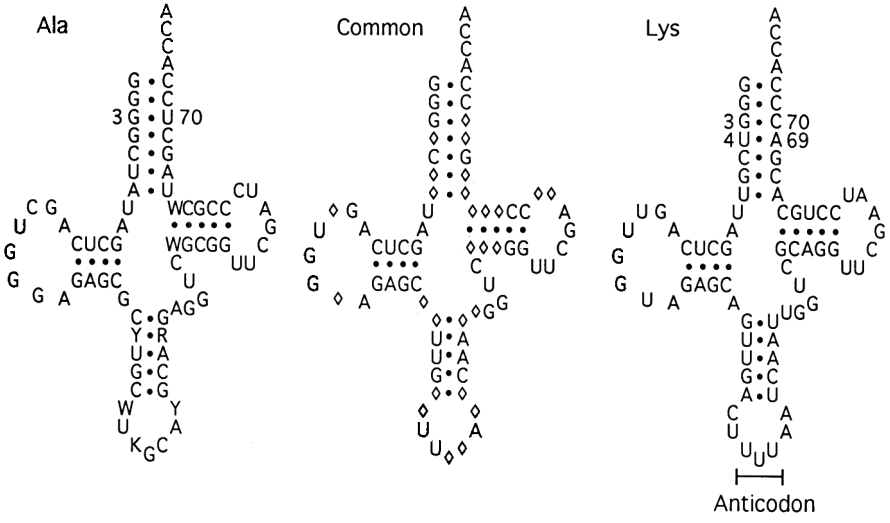

FIGURE 1. Comparison of two isoacceptors of $tRNA^{Ala}$ with $tRNA^{Lys}$ of *E. coli*. The middle tRNA shows common bases and indicates positions containing different bases (Nicholas et al., 1987) with diamonds. The sequences are from a tabulation (Steinberg et al., 1993). Nucleotide positions in the anticodon and in positions 3·70 and 4·69 are indicated. The anticodon of $tRNA^{Lys}$ was changed to UCA in opal suppressors and CUA in amber suppressors; these respective anticodon changes were combined with other changes indicated in the text. The modified nucleotides are not indicated. Abbreviations: R = A, G; W = A, U; Y = C, U; K = G, U.

RESULTS AND DISCUSSION

Rationale

The best way to prove that G4·U69 can preserve the function of G3·U70, if indeed it can, is to show that the acceptor identity of another tRNA switches to that of $tRNA^{Ala}$ when the tRNA is mutated to G4·U69 with a Watson–Crick pair at 3·70. The essential role of G4·U69 in this identity switch is unmistakable. In contrast, maintaining acceptor identity when $tRNA^{Ala}$ is similarly mutated, as in our previous work, is less decisive because a qualitative functional change is not involved.

A tRNA identity switch requires that the mutant tRNA is a good substrate for the new aminoacyl-tRNA synthetase and also a poor substrate for the other 19 enzymes. We used *E. coli* amber suppressor $tRNA^{Lys}$ in the present experiments because previous work (McClain et al., 1988) established that mutating its G3·C70 to G3·U70 switched the molecule's identity from $tRNA^{Lys}$ to $tRNA^{Ala}$. This experiment demonstrated the important point that, even though the nucleotide sequences of $tRNA^{Lys}$ and $tRNA^{Ala}$ differ at 27 positions (Fig. 1), an appropriately modified $tRNA^{Lys}$ becomes a good substrate for AlaRS. The dynamics of this identity switch reflect the importance of G3·U70 to $tRNA^{Ala}$ and G3·C70 to $tRNA^{Lys}$ identities, and also the smaller importance of anticodon nucleotides. The anticodon plays a role in the acceptor identity of $tRNA^{Lys}$ (McClain et al., 1990), but not of $tRNA^{Ala}$ (Hou & Schimmel, 1988; McClain & Foss, 1988a). The CUA amber suppressor tRNA anticodon (which pairs with amber codon UAG) contains the U35 determinant of $tRNA^{Lys}$ identity, but U35's importance to $tRNA^{Lys}$ identity is outweighed by that of G3·U70 to $tRNA^{Ala}$ identity.

We expect that amber suppressor G4·U69 $tRNA^{Lys}$ will exhibit some Lys specificity, but also that this specificity will diminish in the opal suppressor derivative. Lys specificity is expected because it was observed in the corresponding mutant G4·U69 $tRNA^{Ala}$ (McClain et al., 1988). That work also revealed that G4·U69 is active in $tRNA^{Ala}$ when combined with A3·U70, but is inactive with G3·C70. The diminished Lys specificity of opal suppressor $tRNA^{Lys}$ is anticipated because C35 replaces U35 in the UCA opal suppressor anticodon (which pairs with opal codon UGA). In summary, these considerations led us to test opal suppressor $tRNA^{Lys}$ containing A3·U70 and G4·U69.

Mutant construction and testing

By directed mutagenesis (McClain & Foss, 1988b), we modified a gene for $tRNA^{Lys}$ so that the transcribed tRNA would contain A3·U70, G4·U69, and the UCA opal anticodon. When this gene was expressed from plasmid pGFIB (Masson & Miller, 1986) in *E. coli*, the resulting tRNA was specifically aminoacylated with Ala, as determined by the N-terminal sequence of a reporter protein. The reporter protein, dihydrofolate reductase, was synthesized from mRNA containing a UGA opal codon corresponding to the third amino acid from the N-terminus (McClain et al., 1991). Figure 2 shows chromatograms of cycles 1–5 obtained by automated gas-phase sequencing. The yield of Ala in cycle 3 was 92%, and no other amino acid yield exceeded 5%, which is our detection limit (McClain & Foss, 1988b). An independent determination of tRNA specificity using a reporter protein gene with the UGA codon at residue 10 (McClain & Foss, 1988b; Normanly et al., 1990) instead of residue 3 confirmed that this mutant tRNALys inserted only Ala (93% Ala).

Figure 3, section b shows that opal suppressor A3·U70, G4·U69 $tRNA^{Lys}$ suppressed a β-galactosidase opal mutation and formed blue colonies on an X-gal indicator plate. Two control tRNAs described previously are

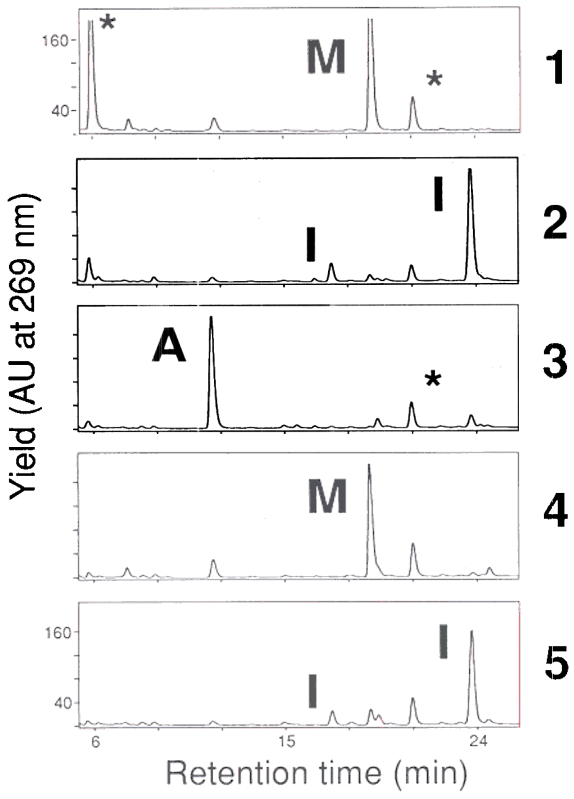

FIGURE 2. Chromatograms of the first five cycles of dihydrofolate reductase protein sequence. Cells grown at 37 °C expressed opal suppressor A3·U70, G4·U69 $tRNA^{Ala}$ from plasmid pGFIB (Masson & Miller, 1986) and dihydrofolate reductase protein with a UGA at codon 3 from plasmid pD3op (McClain et al., 1991). The protein was purified by affinity chromatography and analyzed on an Applied Biosystems gas-phase protein sequencer as described (McClain & Foss, 1988b). Cycles 1–5 (number to the right of each chromatogram) yielded one amino acid each as follows: 1, 86% Met; 2, 93% Ile; 3, 92% Ala; 4, 92% Met; 5, 96% Ile. Yields ≤5% are not indicative of the protein sequence, but were retained in amino acid calculations. Cys was not analyzed. Two peaks characteristic of Ile are noted. Asterisks indicate dithiothreitol at 6 min and diphenyl thiourea at 21 min.

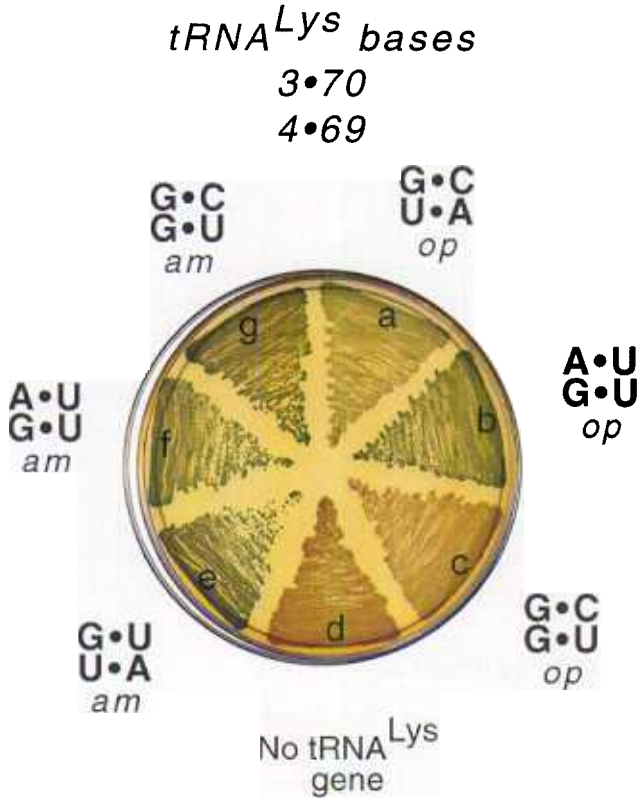

FIGURE 3. Streak plate containing 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside. The bases at positions 3·70 and 4·69 of opal suppressor ("op") and amber suppressor ("am") of $tRNA^{Lys}$ are shown. The tRNA genes were expressed from plasmid pGFIB in *E. coli* strain XAC/U4 (opal suppressors) or strain XAC/A16 (amber suppressors). Cells in section d contained plasmid pGFIB without a tRNA gene. Freshly transformed cell cultures were grown in LB broth plus ampicillin at 37 °C to 3 × 10^8 cells/mL then streaked on LB indicator plates with ampicillin and incubated 17 h at 37 °C.

included for comparison. The unmutated opal suppressor $tRNA^{Lys}$ is inactive and forms light blue colonies (Fig. 3, section a) (McClain et al., 1990), whereas amber suppressor G3·U70 $tRNA^{Lys}$ is active with a β-galactosidase amber mutation and forms blue colonies (Fig. 3, section e) (McClain et al., 1988). In the X-gal assay (Miller & Albertini, 1983; McClain et al., 1988), a blue pigment is produced in cells when β-glactosidase cleaves a colorless substrate, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside. Only the full-length enzyme, arising from nonsense suppression, is active. Occasional readthrough of the opal mutation in cells without an opal suppressor tRNA produces low levels of enzyme (Fig. 3, section d). Thus, cells producing an active suppressor tRNA form blue colonies, whereas those producing an inactive or metabolically unstable suppressor tRNA form light blue colonies.

When we constructed and tested opal suppressor $tRNA^{Lys}$ containing G3·C70 and G4·U69, it produced light blue colonies on an X-gal indicator plate (Fig. 3, section c). We argue that this mutant tRNA is inactive rather than metabolically unstable because the corresponding mutant of amber suppressor $tRNA^{Lys}$ is active (see below). Nevertheless, the inactivity of tRNA containing G3·C70 and G4·U69 is striking and suggests interdependence of the third and fourth pairs in $tRNA^{Ala}$ for aminoacylation.

To gain more perspective on the role of the G·U pair in $tRNA^{Ala}$ identity, and on the dynamics of aminoacyl-tRNA synthetase competition, we modified the two opal suppressor $tRNA^{Lys}$ genes just described so that the transcribed tRNAs would contain CUA anticodons of amber suppressors. The appropriate reporter protein gene with a UAG amber codon at residue 3 was already available (McClain et al., 1991). When the modified tRNAs were tested, we observed that amber suppressor G3·C70, G4·U69 $tRNA^{Lys}$ was specifically aminoacylated with Lys (93% Lys) and formed blue colonies on an X-gal plate (Fig. 3, section g). The other mutant, amber suppressor A3·U70, G4·U69 $tRNA^{Lys}$,

was aminoacylated with 46% Ala and 43% Lys, and also produced blue colonies on an X-gal plate (Fig. 3, section f). As reported previously (McClain et al., 1988), amber suppressor A3·U70 $tRNA^{Lys}$ was specifically aminoacylated with Lys (95% Lys). It seems that the Lys acceptor activity of two derivative amber suppressor $tRNA^{Lys}$ molecules can be attributed to U35 present in the CUA amber anticodon. It is also reasonable that amber suppressor A3·U70, G4·U69 $tRNA^{Lys}$ accepts more Lys (43% Lys) than the corresponding mutant $tRNA^{Ala}$ (7% Lys) because $tRNA^{Lys}$ is optimal for its cognate LysRS enzyme.

However, the most impressive observation made with the amber suppressor $tRNA^{Lys}$ derivatives is that the mere introduction of G4·U69 into A3·U70 $tRNA^{Lys}$ allows AlaRS to successfully compete with LysRS in the dynamic competition for aminoacylation (Yarus, 1972; Swanson et al., 1988). This provides yet another demonstration of indirect recognition of G3·U70.

Concluding remarks

The results support our original conclusion that the G3·U70 wobble pair in $tRNA^{Ala}$ contributes more than sequence to AlaRS recognition. A direct recognition mechanism relying on only the array of functional groups projecting from G3·U70 in an A-type helix is an oversimplification. We have argued that the AlaRS directly recognizes G3·U70 because this pair is optimal for aminoacylation (McClain et al., 1988). However, direct recognition is only a sub-part of the overall recognition process because this alone cannot explain our finding that the G4·U69 pair marks tRNA for specific aminoacylation by AlaRS in vivo. Direct recognition also cannot explain our previous observation that alternative 3·70 pairs in $tRNA^{Ala}$, such as G3·A70, U3·U70, and C3·A70, allow specific aminoacylation by AlaRS in vivo (McClain et al., 1988). The internal consistency of our results reinforces our conviction that both indirect and direct mechanisms contribute to AlaRS recognition of $tRNA^{Ala}$ in vivo.

An unresolved puzzle is why tRNA with G4·U69 only functions in combination with A3·U70 but not with G3·C70. This is true in $tRNA^{Ala}$ and in $tRNA^{Lys}$ (McClain et al., 1988; this study). The weaker A3·U70 pair might better allow distortion of the helix by G4·U69; alternatively, or in addition, the G3·C70 pair may inhibit local metal binding (Limmer et al., 1993; Allain & Varani, 1995a) or clash with AlaRS. Moreover, the base pairs flanking G3·U70 play an important role in aminoacylation (McClain et al., 1988), which may limit perturbations the sequence will tolerate. Regardless of the mechanism, the dependency of G4·U69 on A3·U70 can explain the apparent discrepancy of our work with that subsequently reported by others (Musier-Forsyth et al., 1991). These workers were unable to aminoacylate a minihelix G4·U69 $tRNA^{Ala}$ with AlaRS in vitro; however, because the minihelix contained G4·U69 and G3·C70, its inactivity is not surprising. (Minihelix with G4·U69 and A3·U70 was not tested.) Nevertheless, it is clear that the dependency of G4·U69 on A3·U70 in mutant tRNAs is yet another indication that AlaRS relies on a distinctive structure in this region of the molecule.

A tertiary structure has not been obtained for the $tRNA^{Ala}$ system, so the particular irregularity associated with G3·U70 is matter of speculation. Either free $tRNA^{Ala}$, enzyme-bound $tRNA^{Ala}$, or the $tRNA^{Ala}$ in both states could manifest structural irregularities. Examples of sequence-dependent distortions have now been established in several other RNA systems (Rould et al., 1989; Ruff et al., 1991; Allain & Varani, 1995b). Ultimately, three-dimensional structures and thermodynamic parameters will be required to understand the role of G3·U70 in $tRNA^{Ala}$ during aminoacylation.

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