Single Atomic Group in RNA Helix Needed for Positive and Negative tRNA Synthetase Discrimination

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To maintain the fidelity of protein synthesis, 20 aminoacyl-tRNA synthetases must discriminate among the approximately 60 cellular tRNAs.[1,2] Both positive recognition elements in the cognate systems and negative elements used for noncognate discrimination contribute to the so-called "identity set" of each tRNA.[2−4] While positive recognition elements have now been identified for all 20 *Escherichia coli* synthetases, only a few negative or blocking elements have been elucidated to date.[5,6] In this work, we identify a specific atomic group in the acceptor helix of a tRNA that contributes significantly to both positive recognition by the cognate synthetase and negative discrimination by a noncognate enzyme.

The G3:U70 wobble base pair in the acceptor stem of tRNA^{Ala} is a major recognition element for alanine-tRNA synthetase (AlaRS),[7,8] and synthetic minihelices or duplexes that contain this element are substrates for AlaRS.[9,10] Despite the fact that these small RNA substrates are aminoacylated at reduced rates relative to full-length tRNAs, they provide useful mechanistic information regarding tRNA activation.[11] We have recently shown that the incorporation of the critical G3:U70 base pair into an RNA duplex derived from the acceptor stem of *E. coli* tRNA^{Pro} does not confer alanine acceptance on this duplex.[12] However, duplex^{Pro} can be converted into an efficient substrate for *E. coli* AlaRS by changing an additional base pair in the terminal (1:72) acceptor stem position.[12] We now show that these nucleotide changes proximal to the site of amino acid attachment also allow efficient aminoacylation with alanine of a full-length tRNA^{Pro} transcript.[13] The alanine acceptance activity of a U70-tRNA^{Pro} variant is still reduced 8700-fold relative to a wild-type tRNA^{Ala} transcript (Figure 1). However, the activity of a triple mutant that now contains a C1:G72 → G1:C72 base pair change in addition to G3:U70 is substantially improved. Remarkably, the activity of this mutant is only reduced 11-fold relative to the tRNA^{Ala} transcript (Figure 1). Nucleotides outside of the acceptor stem domain of tRNA^{Pro} apparently contribute very little to negative discrimination by AlaRS *in vitro* or *in vivo*.[14]

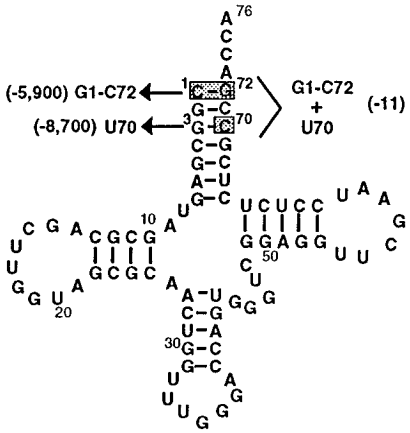

Figure 1. Structure of the UGG isoacceptor of *E. coli* tRNA^{Pro} used in this work. The numbers in parentheses indicate fold decrease in k_{cat}/K_M for aminoacylation with alanine and are reported relative to a wild-type tRNA^{Ala} transcript. The arrows that point to the left indicate the results of single base or base pair changes made. The result shown on the right is for the triple mutant.

A G1:C72 base pair is found at the terminal position of the majority of *E. coli* tRNAs,[15] and is therefore an unlikely position for synthetase discrimination. Nevertheless, substitutions at this position have dramatic effects on aminoacylation by AlaRS and most other class II synthetases examined to date.[12] Rather than participating in direct interactions with AlaRS, the G1:C72 pair may be required for correct presentation of other nearby recognition elements, such as the "discriminator base" at position 73[16,17] or the critical 2-amino group of the G3:U70 wobble base pair.[18] Additionally or alternatively, mutations at 1:72 could introduce negative or blocking elements. The next set of experiments was designed to probe the latter possibility.

As reported previously, both G1:G72 and C1:G72 substitutions eliminate charging of duplex^{Ala} by AlaRS (Figure 2A).[12] We estimate, on the basis of the sensitivity of the duplex assays, that the activity of the G72-containing duplexes is reduced at least 1000-fold relative to the wild-type duplex. The base analog 2′-deoxy-2-aminopurine (2AP) was substituted at position 72 of the two duplex^{Ala} variants shown in Figure 2A.[19] A deoxynucleotide substitution at this position was previously shown to have little effect on aminoacylation.[20] Surprisingly, the G72 → 2AP substitution in both of the duplex^{Ala} variants tested restored aminoacylation with alanine (Figure 2). While the activity of the 2AP variants is still reduced relative to the

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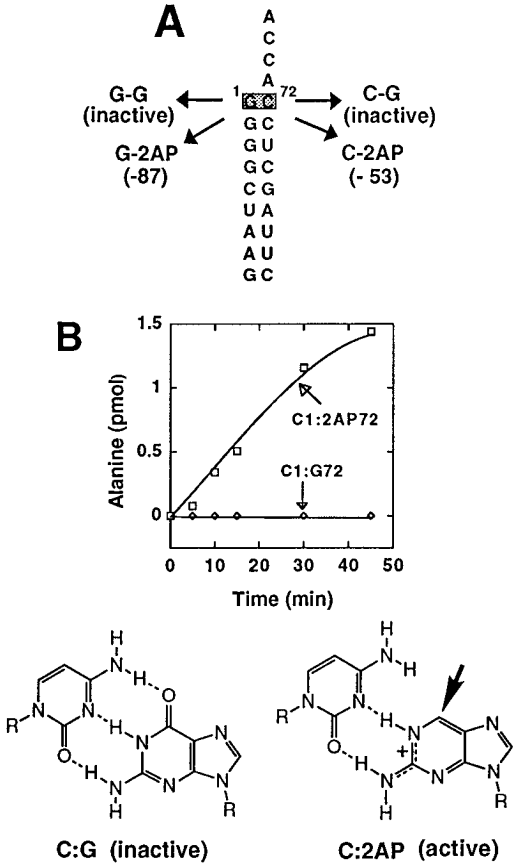

Figure 2. Aminoacylation with alanine of RNA duplex substrates corresponding to the acceptor stem of *E. coli* $tRNA^{Ala}$. (A) Structure of $duplex^{Ala}$. Arrows point to changes made at the 1:72 position (shaded). 2AP is 2′-deoxy-2-aminopurine. Numbers in parentheses indicate fold decrease in k_{cat}/K_M for aminoacylation with alanine and are reported relative to the wild-type $duplex^{Ala}$. (B) Typical aminoacylation assay of two of the RNA duplexes shown in part A with alanine. The structures of the base pair substitutions at position 1:72 are also shown. The C:2AP base pair is shown in the protonated form predicted by NMR.[26] The arrow points to the location of the functional group probed by this study.

wild-type $duplex^{Ala}$ substrate (by 53- and 87-fold), a substantial portion of the activity is restored. Figure 2B shows the proposed structure of the C:2AP base pair. We conclude, on the basis of this study, that the 6-keto group in the major groove of G72 contributes significantly to negative discrimination by AlaRS.

We had shown previously that G72, which is unique to *E. coli* $tRNA^{Pro}$, is an important positive recognition element for proline-tRNA synthetase (ProRS).[21] In contrast, the first nucleotide, C1, can be deleted without reducing aminoacylation efficiency.[21,22] To determine whether a specific functional group of G72 is critical for ProRS recognition, two purine base analogs were substituted at this position. This was accomplished by preparing semisynthetic $tRNA^{Pro}$ molecules as described previously.[23−25] Deoxynucleotide analogs were also shown previously to be tolerated at this position.[23] A deoxyinosine (dI) substitution had no effect on aminoacylation. This base analog lacks the minor groove 2-amino group but is otherwise identical to G. Next, 2AP was substituted at position 72. This base analog lacks the 6-keto group and the N1 hydrogen of G. In a Watson−Crick base pair, the latter is normally unavailable for hydrogen bonding to an enzyme functional group. The 50-fold reduction in aminoacylation observed upon making this substitution in the context of ΔC1-$tRNA^{Pro}$ suggests that in addition to its role in negative discrimination by AlaRS the 6-keto group of G72 is an important positive recognition element for ProRS.

Major tRNA identity elements generally lie in the same positions in different tRNAs.[5] It has been suggested that the overlapping recognition sites of tRNAs allows nucleotides to make positive contacts with cognate synthetases and negative contacts with noncognate enzymes.[5] The results of our study offer direct support for this hypothesis. Base analog substitutions suggest that both ProRS and AlaRS discriminate the terminal base pair via the major groove. In particular, the 6-keto group of G72 appears to perform a dual function. It is responsible for positive recognition of the first base pair of $tRNA^{Pro}$ by *E. coli* ProRS, and its presence also contributes significantly to negative discrimination by AlaRS. We cannot rule out the possibility that an indirect mechanism is responsible for the effects observed in this study. For example, the addition or deletion of a single atomic group may confer positive or negative effects by altering the RNA conformation. The conformational effects of 1:72 substitutions at the end of the $tRNA^{Ala}$ acceptor stem helix are currently under investigation.

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