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Atomic Determinants for Aminoacylation of RNA Minihelices and Relationship to Genetic Code

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Introduction

The rules of the genetic code are established by the specific aminoacylations of transfer RNAs, which contain the anticodon triplets of the code. RNA minihelices that recreate one of the two domains of the transfer RNA structure are substrates for specific aminoacylations. These minihelices lack the triplets of the genetic code which are contained in the second domain of the tRNA structure. Thus, the sequence and structure of an RNA minihelix contains information that is interpreted as a distinct, operational RNA code for a specific amino acid. This information consists of subtle atomic determinants that provide the signals for specific aminoacylation. Schemes for the duplication or copying of minihelices offer a potential way to build a transfer RNA (tRNA) structure in which the triplets of the code originate at least in part from the determinants for aminoacylation of minihelices.

Investigations that relate to the origin of the genetic code invariably start with the structure of transfer RNA. The tRNAs arose over two billion years ago, appearing in the earliest life forms as an integral part of the genetic code that was adopted by all living organisms. A tRNA-like molecule probably originated in an RNA world where precursors to the present system of decoding genetic information first developed.[1−4] These precursors are imagined to be RNA oligonucleotides that could be aminoacylated (charged) with specific amino acids. This reaction covalently links an amino acid through an ester linkage to a hydroxyl at the 3′-end of the RNA. Because the amide bond of peptides is thermodynamically more stable than an ester linkage, the early synthesis of dipeptide-like molecules could in principle occur by reactions of amino acid progenitors that were esterified to RNAs. The chemical principles for these reactions are well established.

The contemporary transfer RNA is typically comprised of 76 nucleotides that terminate at the 3′-end in the conserved sequence $N^{73}CCA^{76}$.[5] The tRNAs have a cloverleaf secondary structure shared in common (Figure 1). In the cloverleaf, the molecule is organized into four helical stems that are designated as the acceptor stem (typically seven base pairs), the dihydrouridine (D) stem (four base pairs), the TψC stem (five base pairs), and the anticodon stem (five base pairs). The D, TψC, and anticodon stems are closed by single-stranded loops, while the acceptor stem ends with the single-stranded $NCCA^{76}$ tetranucleotide at the 3′-end. The A^{76} nucleotide is the amino acid attachment site. The anticodon triplet of the genetic code is harbored by the nucleotides at positions 34−36 in the anticodon loop.

The rules of the genetic code are established in aminoacylation reactions, whereby a specific amino acid is linked with the tRNA that contains the anticodon triplet for that amino acid (in the algorithm of the code). These aminoacylations are catalyzed by aminoacyl tRNA synthetases.[6−8] In bacteria and the cytoplasm of eukaryotes, there is one synthetase for each amino acid. Because the genetic code is degenerate (there are 61 triplets that code for 20 amino acids), there is typically more than one tRNA for each amino acid. These tRNA isoacceptors have distinct anticodons and yet must be charged by the same enzyme.

In three dimensions the cloverleaf is folded into two major domains [9−11] (Figure 1). These domains are oriented at a right angle to each other to form an L-shaped molecule. The two domains are formed by the coaxial stacking of the acceptor stem with the TψC stem to form the acceptor−TψC minihelix domain, and by the stacking of the D stem with the anticodon stem. These two domains contain, respectively, the amino acid attachment site and the anticodon trinucleotide. That is, the amino acid attachment function (minihelix domain) and the template-reading head function (anticodon-containing domain) are physically segregated into separate domains. In this arrangement, the anticodon triplet of the code is separated by 75 Å from the amino acid attachment site.

In protein biosynthesis, the two domains of the contemporary tRNA interact with distinct ribosomal RNAs

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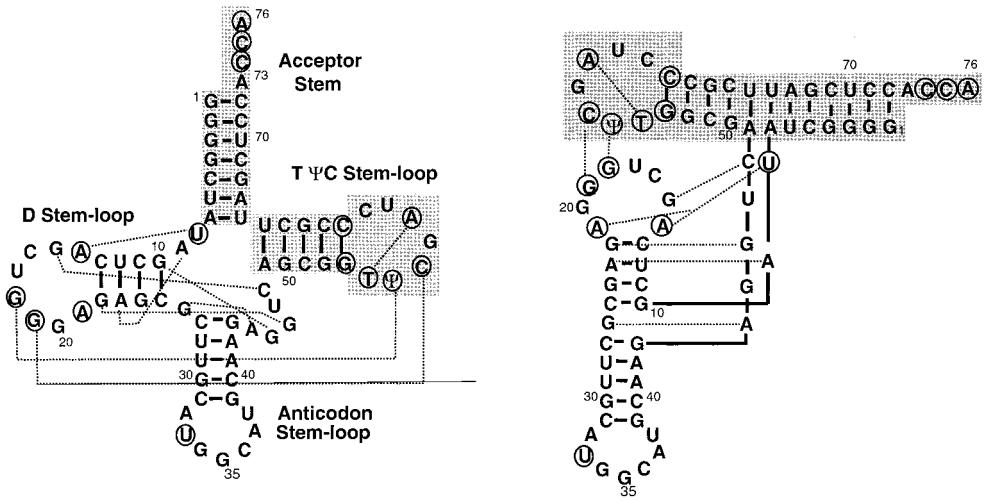

FIGURE 1. Cloverleaf secondary structure (left) and two-dimensional representation of the three-dimensional L-shaped structure (right) of *E. coli* tRNA$^{Ala/GGC}$. Nucleotides that are highly conserved in nonmitochondrial tRNAs are circled.[5] The four major domains are labeled, with the acceptor−TψC minihelix domain being shaded. Dotted lines connect bases involved in tertiary interactions, based on the structure of yeast tRNA^{Phe}.[9,10]

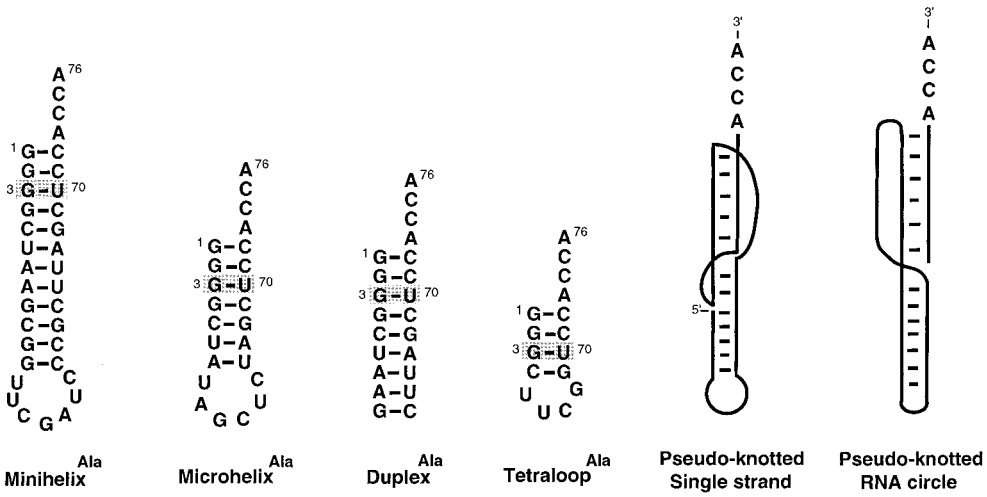

FIGURE 2. Six RNA oligonucleotide substrates for aminoacylation. Minihelix^{Ala} is derived from the acceptor−TψC domain of *E. coli* tRNA$^{Ala/GGC}$, while microhelix^{Ala} is based on the acceptor helix alone (with a loop comprised on nucleotides U8 to C13 (see Figure 1).[16] Duplex^{Ala} is based on the first 9 base pairs of the acceptor−TψC domain,[17] and tetraloop^{Ala} consists of only the first three base pairs of the acceptor stem appended to a UUCG tetraloop that is closed with a C:G pair.[19] The aminoacylation of these four oligonucleotides by alanyl-tRNA synthetase is dependent on the G3:U70 base pair (shaded). The single-stranded pseudoknot minihelix mimics the amino acid acceptor arm that forms at the 3′-end of turnip yellow mosaic virus (TYMV) RNA. This minihelix is a substrate for aminoacylation with yeast histidyl-tRNA synthetase.[20] The circular pseudoknot is hybridized with a single strand to generate a minihelix-like structure that is also charged by yeast histidyl-tRNA synthetase. [21]

imbedded with the ribosome. Thus, the minihelix domain interacts with 23S rRNA in the 50S particle, and the anticodon-containing domain interacts with 16S rRNA in the 30S particle.[3] This segregation of interactions is consistent with the two domains of tRNA having separate origins. The minihelix itself is thought to be the more ancient. This motif, with the CCA trinucleotide at the 3′-end, is found in RNA genomes, as a tag for marking sites of replication, and as a motif that is used by ancient and contemporary reverse transcriptases to initiate or prime reverse transcription.[1,12] It is also a substrate for aminoacylation, where subtle chemical determinants are interpreted by synthetases in terms of specific amino acids.[13−15] Thus, minihelix-like molecules were probably the early progenitors of tRNAs.

RNA Minihelix Substrates for Aminoacylation

In general, six types of RNA oligonucleotide constructions as substrates for aminoacylation have been examined (Figure 2). These consist of the minihelix domain itself, the seven-base-pair microhelix (first seven base pairs of the minihelix,[16] which corresponds to the acceptor stem of the tRNA), RNA duplexes that lack the single-stranded loop of the minihelix and may lack some of the base pairs of the minihelix,[17,18] RNA tetraloop substrates that consist of the first four base pairs of the minihelix which are thermodynamically stabilized by a rationally chosen loop of four nucleotides,[19] an RNA pseudoknot that recreates a minihelix-like structure,[20] and a circular RNA that rearranges into a pseudoknot.[21] (The RNA pseudoknot can

Table 1. RNA Oligonucleotide Substrates for Aminoacylation by tRNA Synthetases

amino acid specificity	RNA oligonucleotide[a]	species	ref
Ala	minihelix, microhelix	*E. coli*	16
	duplex	*E. coli*	17
	tetraloop	*E. coli*	19
	duplex	human	49
	duplex	*T. thermolphilus*	50
Asp	minihelix	yeast	13
	microhelix, tetraloop	yeast	
Cys	minihelix, microhelix	*E. coli*	51
Gln	microhelix	*E. coli*	52
Gly	microhelix	*E. coli*	42
	tetraloop	*E. coli*	19
	minihelix	human	53
His	minihelix, microhelix	*E. coli*	54
	tetraloop	*E. coli*	19
	pseudoknot−minihelix	yeast	20
	circular pseudoknot		21
Ile	minihelix, microhelix	*E. coli*	55, 56
Met	minihelix		
	microhelix, duplex	*E. coli*	18, 56
	tetraloop	*E. coli*	19
Ser	minihelix, microhelix	*E. coli*	15, 57
		human	58
Tyr	microhelix	*E. coli*	59
	microhelix	*P. carinii*	59
	microhelix	human	60
Val	minihelix	yeast	61

[a] RNA oligonucleotides are as defined in Figure 2.

recreate a minihelix by an unusual pairing of nucleotides in two loops. It is found at the 3′-end of plant viral RNAs. These viral RNAs are charged with specific amino acids that are attached to the 3′-end of the minihelix pseudoknot.[8,22−24]) These substrates have been used interchangeably in many of the studies reported so far.

Substrates for charging RNA oligonucleotides with 11 different amino acids have been reported (Table 1). Thus, for many amino acids the anticodon per se is not essential to relate a given amino acid to its cognate tRNA. The RNA oligonucleotide substrates vary considerably in their activity, depending on the system, but in all cases, aminoacylation is sequence-specific. Moreover, the sensitivity to sequence is typically the same as that seen with the full tRNA; that is, the same mutations in the tRNA acceptor stem that affect activity also affect the charging efficiency of the cognate RNA oligonucleotide substrate.

Atomic Determinants of Minihelix Aminoacylation

The alanine system was the first to be investigated for the possibility that a minihelix based on a tRNA acceptor−TψC stem might be charged.[16] Since the early studies of this system, fine structure functional interactions have been determined in some detail. Because this system has been the most thoroughly investigated from a chemical standpoint, it is considered here as an example.

The alanine minihelix was tested as a potential substrate because of strong evidence for the role of the acceptor stem in determining aminoacylation efficiency and specificity. In particular, a single G3:U70 base pair was shown to mark a tRNA for charging with alanine.[25,26] Transfer of this base pair into other tRNAs conferred alanine acceptance on them. Footprint analysis established that the enzyme makes no contact with the anticodon.[27]

Minihelices, microhelices, tetraloop structures with only four base pairs, and RNA duplexes were all charged with alanine, provided they contained the critical G3:U70 base pair.[16,17,19] (For the microhelix, the k_{cat} parameter was close to that seen with the full tRNA; K_m was elevated by at least 10-fold.[16]) Transfer of this base pair into other oligonucleotide sequence frameworks conferred alanine acceptance on them. Thus, the oligonucleotide substrates recapitulated the behavior seen with the full tRNA.

Role of G3:U70. The G:U base pair was established by NMR to be in the wobble configuration.[28,29] It presents specific atomic determinants on both the major and the minor groove sides of the RNA helix. In RNA helices, the minor groove is wider and more accessible to interactions with protein motifs than is the major groove. To understand the basis for recognition of G3:U70, multiple substitutions were made into chemically synthesized RNA duplex substrates (Figure 3). These substitutions were chosen to vary both major and minor groove atomic determinants. Inactive natural base pair substitutions included G:C, A:U, and U:G, while the nonnatural pairs 2-aminopurine (2-AP):U, 2-aminoadenosine (2-AA):U, and inosine (I):U were also inactive.[28,30] Of these the most significant is the comparison between G:U and I:U. Both present the same major groove atomic determinants. Only the free exocyclic 2-amino group in the minor groove distinguishes G:U from I:U.

A 2-amino group in the minor groove is also presented by the G:C, 2-AP:U, and 2-AA:U pairs. In these instances, the amino group is paired in the Watson−Crick configuration. The distinguishing feature for G:U is the presence of an unpaired 2-amino group, in the wobble configuration. The question was whether a free 2-amino group could be placed in the minor groove with a pair other than G:U. Pairing 2-AA with isocytidine (isoC) places a free 2-amino group in the same location as that seen with G:U (Figure 3). The duplex substrate with a 2-AA:isoC pair at 3:70 was highly active for charging.[30] These results strongly supported the idea that a G:U pair marked a helix for charging with alanine by presenting an unpaired exocyclic 2-amino group in the RNA minor groove.

Potential Role of a Helix Irregularity. McClain and coworkers report that alanine-inserting amber suppressors can be charged with alanine, with base pairs at 3:70 other than G:U.[31] These suppressor tRNAs were studied using an in vivo assay. Examples of substitutions reported to be functional in vivo included G:A, A:C, and C:A. As a consequence of these observations, a helix irregularity was suggested as the major basis for the recognition of $tRNA^{Ala}$.

Substrates with these and other substitutions were each investigated in vitro, as RNA duplexes and as full tRNA substrates.[32] The A:C and C:A substitutions at 3:70 reduced charging efficiency (k_{cat}/K_m) by over 3000-fold. This change corresponds to a loss of about 5 kcal mol^{-1} of apparent free energy of transition state stabilization. The G3:A70 substrate was reduced less, about 100-fold in charging

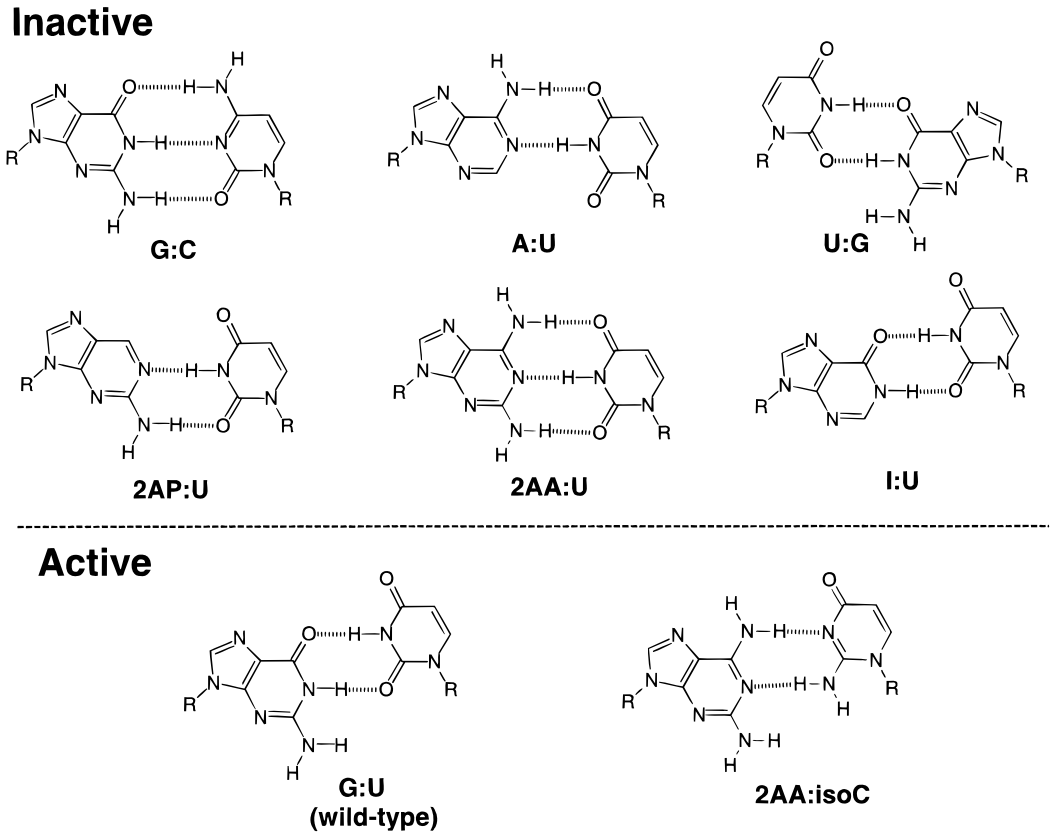

FIGURE 3. Substitutions made to probe the 3:70 position in RNA oligonucleotide substrates for alanyl-tRNA synthetase.[28,30] Inactive variants are shown above the dotted line, whereas active variants are listed below. Abbreviations are 2AP = 2-aminopurine, 2AA = 2-aminoadenosine, I = inosine, and isoC = isocytidine.

efficiency. Interestingly, this pair presents an unpaired 2-amino group (from G) in the minor groove. These results show that a helical distortion is not as important as specific atomic groups in determining aminoacylation efficiency. They also show that the level of charging of mutant tRNAs in vivo is dependent on factors other than aminoacylation kinetic efficiency.

Other Minor Groove Interactions. The 2′-hydroxyl groups line the minor groove and are prominent sites for hydrogen-bonding interactions. By making single 2′-deoxy and 2′-OCH_3 substitutions, functional contacts with specific 2′-OH groups were mapped. [33] The result was the delineation of three functional contacts with 2′-OH groups that affected the efficiency of aminoacylation by at least 1 kcal mol^{-1} . These are at positions 4, 70, and 71 in the helix. In contrast, 2′-deoxy substitutions at positions 1, 2, 3, 5, 68, 69, and 72 had little effect. Thus, there is a highly specific constellation of functional contacts with 2′-OH groups. Further work delineated an important contribution from the exocyclic 2-amino group of the G2:C71 base pair that is adjacent to the critical G3:U70 base pair. This interaction, and the functional 2′-OH contacts, all lie within 5 Å of the 2-amino group of the G3:U70 base pair. Of all of these minor groove elements, the 2-amino group of G3 makes the greatest (more than 3 kcal mol^{-1}) contribution to recognition.

Major Groove Atomic Interaction at the End of the Helix. Although the crystal structure of alanyl-tRNA synthetase is not yet determined, structurally related synthetases approach the top of the acceptor stem from the major groove side (as exemplified by the cocrystal structures of aspartyl-tRNA synthetase complexed with $tRNA^{Asp34}$ and seryl-tRNA synthetase complexed with $tRNA^{Ser35}$). In accordance with these observations, the results of biochemical studies in the alanine system support major groove discrimination at the terminal (1:72) base pair.

The first indication that the G1:C72 base pair was important in the alanine system came from in vivo studies by McClain and co-workers.[36] More recently, in vitro work elucidated the atomic group interactions at this site. Attempts to aminoacylate (with alanine) a G3:U70-containing duplex based on the acceptor stem of *E. coli* $tRNA^{Pro}$ failed.[37] In addition to the presence of a G3:U70 pair, a C1:G72 to G1:C72 base pair transversion was necessary and sufficient to confer alanine acceptance on $duplex^{Pro}$ and full $tRNA^{Pro}$.[37,38] In particular, the G72 nucleotide in the $duplex^{Pro}$ acceptor stem appeared to block charging by AlaRS. Incorporation of nucleotide base analogues further narrowed the blocking effect to the 6-keto oxygen and/or N1 proton of G72.[38] Atomic group "mutagenesis" was done by incorporating over 30 standard and modified base pair combinations at the 1:72 position of $duplex^{Ala}$ variants.[39] The results confirmed the blocking effect of a major groove carbonyl oxygen presented by either a G or a U at position 72. Major groove functional groups of the wild-type G1:C72 base pair were also shown to make modest contributions to positive

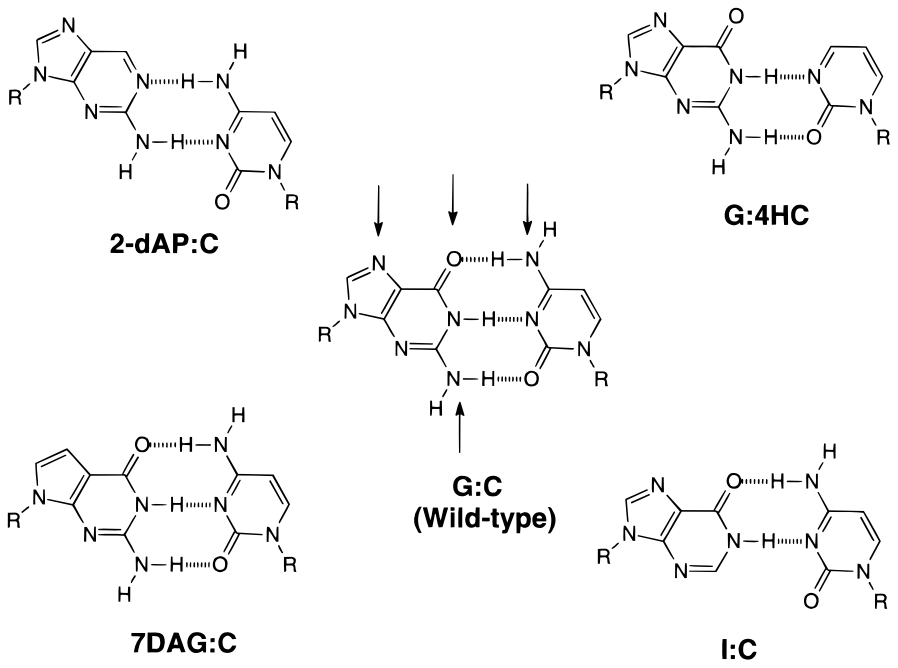

FIGURE 4. Substitutions at position 1:72 in duplex substrates for alanyl-tRNA synthetase.[39] The naturally occurring G1:C72 pair is shown in the center, with base pair replacements that examined functional groups (arrows) in the major and minor grooves. Abbreviations are 2′-dAP = 2′-deoxy-2-aminopurine, 7DAG = 2′-deoxy-7-deazaguanosine, and 4HC = 2-pyrimidinone.

recognition by AlaRS. Deletion of the carbonyl oxygen and N1 proton of G1 resulted in a 6.4-fold decrease in k_{cat}/K_m, corresponding to a 1.1 kcal mol^{-1} contribution to the apparent free energy of transition state stabilization, while the 4-amino group of C72 contributes 0.65 kcal mol^{-1} (Figure 4). However, none of the major groove atoms in the first base pair contribute as much toward transition state stabilization as the minor groove elements in and around the G3:U70 base pair.

The Discriminator Base. The unpaired nucleotide that is stacked on the terminal 1:72 base pair is sometimes referred to as the "discriminator" base.[40] In the alanine system, this base has been shown to modulate G3:U70-dependent aminoacylation with alanine and is needed for transition state stabilization.[41] Although the 2′-hydroxyl group has been shown to be dispensable at this site,[33] further work is necessary to establish the atomic determinants involved in the function of this key single-stranded nucleotide.

Relationship of Minihelix Aminoacylation to That of Full tRNA

The sequence-specific aminoacylation of RNA oligonucleotides based on tRNA acceptor stems can be compared with the aminoacylation of the full tRNA, on one hand, and, on the other hand, with the CCA trinucleotide that occurs at the end of all tRNAs. The latter trinucleotide can be used at high concentrations in an attempt to force it into the active site. By comparing the rate of aminoacylation of a minihelix or minihelix-like structure with that of the CCA triplet, the contribution of acceptor stem nucleotides to aminoacylation efficiency can be calculated. Similarly, by comparing the full tRNA to that of the minihelix, the contribution of the second domain of the tRNA is obtained. This domain contains the anticodon triplet.

Even for a system such as glycine, where the anticodon triplet has an important role in aminoacylation efficiency, the thermodynamic contribution of the acceptor stem to aminoacylation efficiency is greater than that of the second domain of the tRNA.[42] For example, in the glycine system, the rate of charging of the full tRNA is about 5 orders of magnitude greater than that of a microhelix. But the enhancement of charging obtained when the acceptor stem is joined to the CCA triplet is greater than the enhancement of the full tRNA over that of the acceptor-stem-based substrate. (In fact, with an assay that could detect as little as 5 ppm of charging, no aminoacylation of CCA could be detected.) While an analysis of this sort has not been done with every tRNA, the results establish that, even when the rate of charging of a minihelix is several orders of magnitude below that of the full tRNA, the minihelix may make a greater contribution to charging efficiency than that of the second domain of the tRNA.

Operational RNA Code and Relationship to Genetic Code

The relationship between the sequences/structures within the tRNA acceptor stems and specific amino acids constitutes an operational RNA code for amino acids.[4,43] This "code" is distinct from the nucleotide triplets of the genetic code. The information contained within the acceptor stem is expressed in terms of a constellation of atomic groups which is interpreted as a specific amino

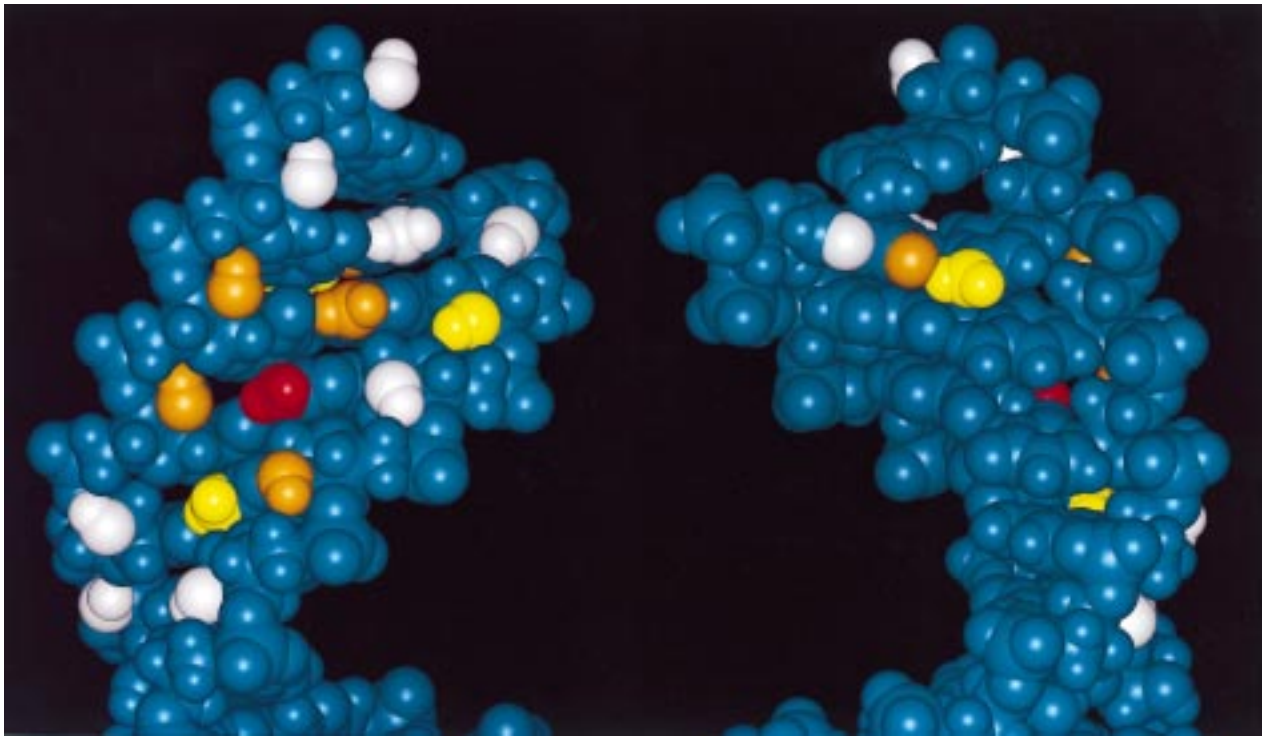

FIGURE 5. Illustration of functional atomic determinants for aminoacylation of a duplex^{Ala} substrate, from the minor (left) and major (right) groove sides of the helix. The model was generated by using Insight II (Biosym Technologies) on an IRIS Indigo XS24 workstation (Silicon Graphics, Inc) to build an A-form helix based on the duplex^{Ala} sequence. The duplex was energy minimized using Discover and the AMBER force field.[62] The CCA 3′-end is deleted for clarity. The view shows the "discriminator base" A73 (top) to base pair 6:67 (bottom). All atoms probed are indicated in a color other than blue. White indicates functional groups whose removal has little effect ($<$0.5 kcal mol^{-1}) on aminoacylation efficiency, yellow, 0.5−1.0 kcal mol^{-1} ; orange, 1−3 kcal mol^{-1} ; and red, $>$3 kcal mol^{-1}.

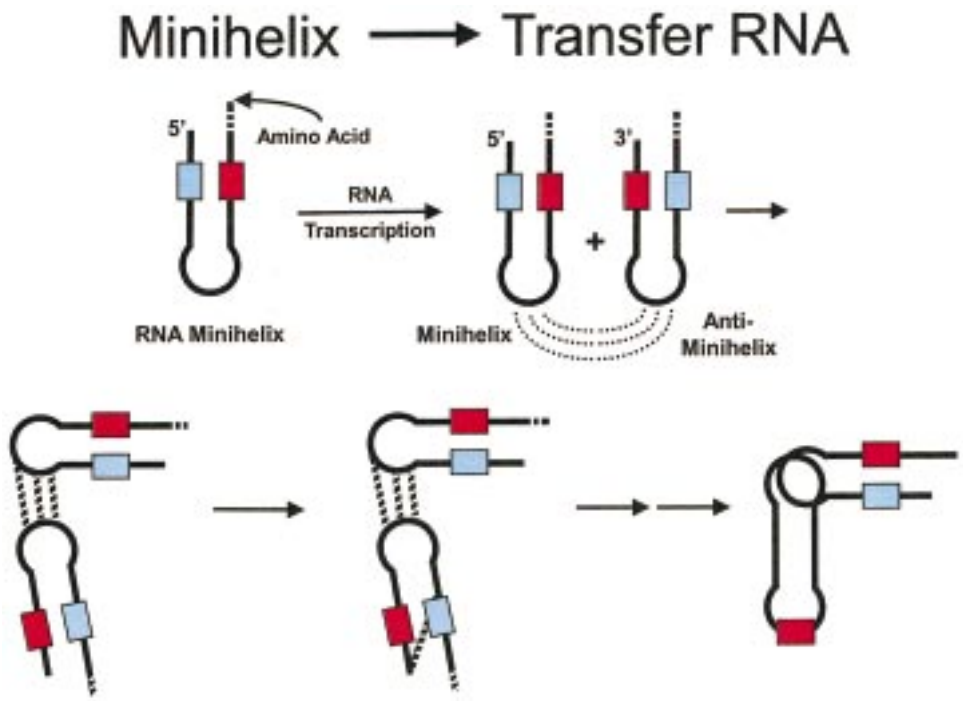

FIGURE 6. Interaction between a minihelix and a complementary anti-minihelix to create a tRNA, with nucleotides of the operational RNA code (shown in color) incorporated into the anticodon loop of the tRNA.

acid. Figure 5 is an example of such a constellation for alanine. Whether this constellation originated in an RNA world from a motif that physically interacted with alanine itself is not known.

At least two general schemes can be considered for connecting the operational RNA code of the minihelix domain to the genetic code. In one scheme, RNA oligonucleotide substrates for aminoacylation became joined

to other RNA oligonucleotides that acted as template-reading heads.[4,43] These reading heads interacted with primordial messenger RNAs and established template-dependent peptide synthesis by virtue of bringing together the charged acceptor-stem-like oligonucleotides. In this scenario, the template-reading head had an origin distinct from that of the minihelix domain.

In a second scenario, the ancient minihelix domain was itself the precursor of the template-reading head that constitutes the second, anticodon-containing domain of the contemporary tRNA. Thus, the primordial minihelix was the precursor of both the acceptor−TψC structure and the anticodon-containing second domain. For example, the minihelix is self-complementary, so that it can be unfolded and recombined by dimerization with itself into a hydrogen-bonded secondary structure. This minihelix dimer can then rearrange to give a tRNA cloverleaf.[44,45] A different scheme involves the creation of a complementary copy of the minihelix to give an anti-minihelix (Figure 6). By associating as "kissing" hairpins through complementary loop−loop interactions, the tRNA-like L-shaped structure is generated. Complementary loop−loop interactions between RNA hairpins have been described and characterized by Marino et al.[46]

In scenarios where the primordial minihelix is the precursor to both domains of the tRNA, the anticodon triplets can be seen as orginating from acceptor stem nucleotides.[47,48] This circumstance would mean that the genetic code came at least in part from the operational RNA code.

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