

Enzymatic aminoacylation of single-stranded RNA with an RNA cofactor

(aminoacyl-tRNA synthetase/evolution of coding/RNA hybrids/RNA-DNA hybrids/tRNA recognition)

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ABSTRACT A chemically synthesized single-stranded ribonucleotide tridecamer derived from the 3' end of *Escherichia coli* alanine tRNA can be charged with alanine in the presence of short complementary RNA oligonucleotides that form duplexes with the 3' fragment. Complementary 5' oligomers of 9, 8, 6, and 4 nucleotides all confer charging of the 3' fragment. Furthermore, in the presence of limiting 5' oligomer, greater than stoichiometric amounts of the single-stranded 3' acceptor fragment can be aminoacylated. This is due to a reiterative process of transient duplex formation followed by charging, dissociation of the 5' oligomer, and then rebinding to an uncharged single-stranded ribotrindecamer so as to create another transient duplex substrate. Thus, a short RNA oligomer serves as a cofactor for a charging enzyme, and it thereby makes possible the aminoacylation of single-stranded RNA. These results expand possibilities for flexible routes to the development of early charging and coding systems.

Major determinants for the molecular recognition of transfer RNAs divide broadly into two classes (for recent reviews, see refs. 1 and 2). In one group, major sites for recognition by aminoacyl-tRNA synthetases are outside of the anticodon, the trinucleotide that specifies the amino acid in the framework of the genetic code. Examples include serine (3), alanine (4), and histidine (5, 6) tRNAs. In another group, the anticodon itself is recognized by the cognate synthetase and appears to be the primary element that determines aminoacylation specificity and kinetic efficiency. Examples include methionine- (7), valine- (7), isoleucine- (8), and arginine- (9) tRNA synthetases. In addition, glutamine-tRNA synthetase relies at least to some extent on recognition of the anticodon (10). We suggested recently (6) that this subdivision of recognition according to whether or not the anticodon plays an essential role may correlate in part with at least two different classes of structures of aminoacyl-tRNA synthetases (11–13).

In the case of alanine- and histidine-tRNA synthetases, the enzymes can aminoacylate short hairpin mini- and microhelices that recreate the acceptor-T Ψ C stem and the acceptor stem alone, respectively (6, 14). Thus, more than half of the tRNA structure, including the anticodon stem and loop, is dispensable for aminoacylation. As with the intact tRNA^{Ala} (4), aminoacylation of the alanine mini- or microhelices is dependent on the presence of a G3-U70 base pair that is proximal to the amino acid attachment site, and transfer of this base pair into other hairpin helices confers alanine acceptance on them (14, 15). Although the histidine system is less well characterized, it is now clear that the extra base pair that is located at the end of the acceptor stem, and which is unique to histidine tRNAs, is critical for aminoacylation (5, 6). In both systems, therefore, major elements for recognition

are proximal to the amino acid attachment site. In addition, each enzyme retains specificity for its cognate mini- or microhelix.

The apparent simplicity of the alanine or histidine tRNA motifs that facilitate charging can be explored further through the investigation of single-stranded RNA molecules. However, previous work suggested that single strands *per se* would not be charged by *Escherichia coli* alanine-tRNA synthetase. For example, minihelices that have complete base pairing except for a G3-G70 or U3-U70 mismatch cannot be aminoacylated (15). Thus, unlike the single-stranded anticodon that is recognized by some synthetases, this observation is suggestive of a recognition site (G3-U70) for alanine-tRNA synthetase which must be in a duplex format for efficient aminoacylation. If so, aminoacylation of a single strand might be achieved by the hybridization of a short oligonucleotide to an acceptor strand, so as to form the G3-U70 base pair, at least transiently. Aminoacylation of disconnected complementary strands derived from the acceptor stem was also suggested by early work of Imura *et al.* (16), who reported low levels (3–5%) of charging of complementary fragments of a yeast alanine tRNA.

This possibility was explored in the present investigation. Part of the motivation for these studies was to define the simplest system for amino acid specific aminoacylation. This not only provides insight into molecular recognition by alanine-tRNA synthetase but also expands the conceptual framework for understanding the basis for coding systems and how they could develop. In particular, aminoacylated single-stranded RNA molecules may have been progenitors of contemporary systems for decoding genetic information.

MATERIALS AND METHODS

Materials. Nucleoside triphosphates were purchased from Pharmacia P-L Biochemicals, [γ -³²P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) and [³H]alanine (70 Ci/mmol) were from New England Nuclear, and [¹⁴C]alanine (154 Ci/mol) was from Amersham. T2 ribonuclease was from Sigma. T7 RNA polymerase was purified from *E. coli* strain BL-21/pAR 1219 according to Grodberg and Dunn (17). *E. coli* tRNA^{Ala} (UGC isoacceptor) was purchased from Subriden RNA (Rolling Bay, WA). Oligodeoxynucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer in the Massachusetts Institute of Technology Center for Protein Structure and Engineering.

Preparation and Characterization of RNA. Transcription reactions for RNA synthesis were carried out as described (15, 18), using T7 RNA polymerase at 0.1 mg/ml. The resulting minihelix was purified on a 16% polyacrylamide gel.

Chemical synthesis of RNA oligoribonucleotides was performed on a Gene Assembler Plus (Pharmacia) synthesizer using fully protected diisopropylamino- β -cyanoethyl ribonucleoside phosphoramidites. Phosphoramidites were prepared by using a modification of previously published procedures

(19). Details of these modifications have been published elsewhere (20).

A ribotridecamer corresponding to the 3' CCA stem of tRNA^{Ala} constitutes one strand of all active duplexes that were studied. The initial choice of the length of the 3' oligomer was partially based on the knowledge that the efficiency of trichloroacetic acid precipitation onto the Whatman filter pads employed in the aminoacylation assays is length dependent. It was hoped that a 13-mer could be precipitated with high efficiency. The efficiency of trichloroacetic acid precipitation of RNA substrates was determined by 5'-³²P-end-labeling minihelix^{Ala} and the 13-mer (21). End-labeled RNA was purified by polyacrylamide gel electrophoresis, combined with all aminoacylation assay components, and spotted onto trichloroacetic acid-soaked filter pads. The amount of ³²P which bound to pads that were not washed was compared to that on control pads that were washed as usual (22). Whereas a 5'-³²P-end-labeled minihelix^{Ala} (a 35-mer) reproducibly precipitated with 100% efficiency, experiments using an end-labeled 13-mer revealed that under assay conditions and in the presence or absence of 5' oligomer, 55 ± 13% (16 trials) remained on the filter pad. A maximum of 68% remained bound. On the basis of these results, a factor of 1.5 was used to scale all of the yields of aminoacylation to give what is believed to represent a lower bound for charging.

Melting temperature of RNA duplexes were determined from melting curves constructed from absorbance data obtained on a Beckman DU-64 spectrophotometer. Absorbance measurements (260 nm) were made using a water-regulated cell holder connected to a circulating water/ethylene glycol bath (RM6 Lauda, Brinkmann). Complementary strands were placed in 50 mM sodium phosphate (pH 5.5) and heated to 90°C. After equilibration to 5°C (15 min), MgCl₂ and KCl were added to 10 mM and 20 mM, respectively. For each melting curve, data were obtained every 2 degrees from 5°C to 80°C with a 5- to 10-min equilibration time at each temperature.

The extinction coefficients (ϵ_{260}) and hyperchromicity of minihelix^{Ala} and the single-stranded RNA oligomers were experimentally determined by comparing the A_{260} of the native structures to that of the nucleotides after T2 ribonuclease digestion. Total hydrolysis was carried out at 37°C in silane-treated, autoclaved Eppendorf tubes in 10 mM ammonium acetate (pH 4.5), using 1 unit of T2 ribonuclease and a final reaction volume of 20 μ l. Before absorbance measurements, reaction mixtures were diluted to 120 μ l with 50 mM sodium phosphate (pH 7.0) so that the final A_{260} of the RNA was between 0.3 and 0.7. Hyperchromicity values were as follows: 4-mer, 1.06; 6-mer, 1.19; 8-mer, 1.23; 9-mer, 1.23; 13-mer, 1.26; and minihelix^{Ala}, 1.44. Using these values and the extinction coefficients for monoribonucleotides (23), we estimated the extinction coefficients (ϵ_{260}) for the intact RNAs to be as follows: 4-mer, $4.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$; 6-mer, $5.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$; 8-mer, $7.8 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$; 9-mer, $8.9 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$; 13-mer, $10.7 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$; and minihelix^{Ala}, $26.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Enzyme Preparation and Assays. *E. coli* alanine-tRNA synthetase was purified by using a modification of the procedure described by Regan (24). The concentration of active alanine-tRNA synthetase was determined by the adenylate burst assay (25). Unless otherwise indicated, aminoacylation assays (22, 26) were conducted at 37°C, 25°C, or 15°C in a reaction mixture containing 50 mM sodium phosphate (pH 5.5 or 7.5), 20 mM KCl, 10 mM MgCl₂, bovine serum albumin at 0.1 mg/ml, 20 mM 2-mercaptoethanol, 21.7 μ M [³H]alanine, 4 mM ATP, and concentrations of alanine-tRNA synthetase and nucleic acid substrate as indicated in the figure legends. Prior to aminoacylation of duplex substrates, complementary RNA oligomers were annealed by heating to 75°C

for 4 min in 20 mM sodium phosphate (pH 7.0) and then slowly cooled to 4°C.

RESULTS

Aminoacylation of RNA Duplexes Composed of Complementary Single Strands. The minihelix^{Ala} hairpin structure is derived from the acceptor stem and T Ψ C loop of tRNA^{Ala} and can be fully aminoacylated by alanine-tRNA synthetase (14, 15). This minihelix is shown in Fig. 1A along with duplex variants composed of a 13-mer corresponding to the 3' CCA stem of tRNA^{Ala} and a complementary 5' oligomer of variable length. The duplexes, however, no longer contain the T Ψ C loop and lack the hairpin structure. Recent improvements in the techniques and strategies employed for the chemical synthesis of RNA by using fully protected diisopropylamino- β -cyanoethyl ribonucleoside phosphoramidites and automated solid-phase methods have enabled the synthesis in high yields of biologically active RNA molecules (19, 20, 27). In this work, the RNA duplexes shown in Fig. 1A were chemically synthesized and found to be substrates for alanine-tRNA synthetase.

To achieve the maximum possible levels for charging, aminoacylation assays were performed at 15°C. This avoided

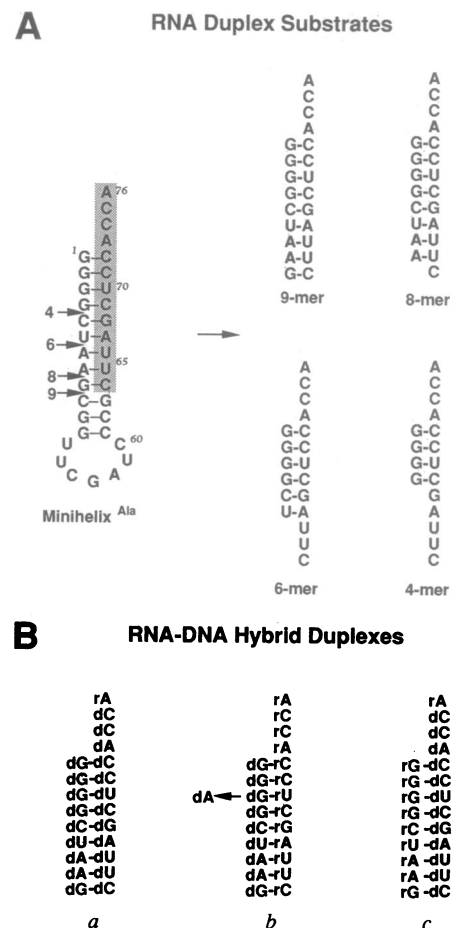


FIG. 1. (A) Sequences of synthetic RNA duplex substrates and of minihelix^{Ala} from which they are derived. The sequence of minihelix^{Ala} is based on the acceptor-T Ψ C helix of *E. coli* tRNA^{Ala} (cf. ref. 14). Numbering is based on that of the full-length tRNA. The shaded region is a ribotridecamer whose sequence corresponds to one strand of each RNA duplex molecule shown on the right. The second strand of each RNA duplex is derived from the 5' end of the minihelix as indicated by the numbered arrows. (B) Sequences of RNA-DNA hybrid duplexes used in this work: a, deoxyribonamer + deoxyribotridecamer (3'-rA); b, deoxyribonamer (dG3 or dA3) + ribotridecamer; c, ribonamer + deoxyribotridecamer (3'-rA).

the possibility of partial dissociation of the shorter duplexes and minimized chemical deacylation which occurs at higher temperatures with prolonged incubation times. While the alanine-tRNA synthetase does not charge the 13-mer alone (Fig. 2A), in the presence of equimolar amounts (1 μ M) of complementary RNA (9-mer, 8-mer, or 6-mer) and 250 nM enzyme, complete aminoacylation of the 13-mer was achieved (6750, 6115, and 7450 pmol/ A_{260} of duplex for the 9-mer, 8-mer, and 6-mer, respectively). This corresponds to 102%, 90%, and 104% of the estimated theoretical maximum charging of one amino acid per duplex. Even in the presence of the GpGpGpG tetramer (Fig. 2A), a reproducible and significant amount of aminoacylation of the 13-mer occurs (1100 pmol/ A_{260} of duplex, corresponding to 15% of the theoretical maximum value).

To further characterize the RNA duplex substrates and evaluate the effect of the hairpin loop structure, the initial rates of aminoacylation with alanine of minihelix^{Ala} and of the ribonamer-ribotridecamer duplex were determined. As shown in Fig. 2B, the initial rate of aminoacylation of the duplex is about equal to that of minihelix^{Ala}, which has an additional three base pairs and a hairpin loop. A comparison of the kinetic parameters for aminoacylation of mini- and microhelix^{Ala} and tRNA^{Ala} has been previously published (14, 15); the aminoacylation efficiency of the duplex substrates requires more detailed study.

Aminoacylation of RNA·DNA Hybrid Duplexes. Fig. 1B shows duplex variants which consist of different combinations of ribo- and deoxyribonucleotides. While a duplex (9-mer + 13-mer) consisting of complementary oligodeoxyribonucleotides except for a 3'-rA (Fig. 1B, duplex *a*) is not

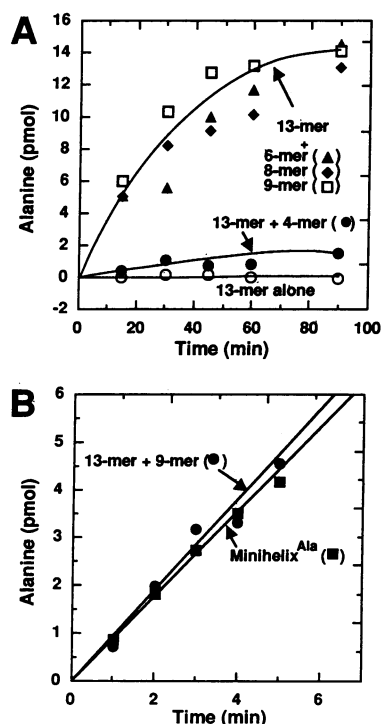


FIG. 2. (A) Aminoacylation with alanine of RNA duplex substrates at 15°C, pH 7.5. Before each assay, the ribotridecamer was annealed with an equimolar amount of complementary oligomer containing 4, 6, 8, or 9 nucleotides. Assays were carried out with a final concentration of 1 μ M duplex substrate (or ribotridecamer alone) and 0.25 μ M alanine-tRNA synthetase. Each time point represents the incorporation of alanine per 10- μ l reaction aliquot. (B) Initial rates of aminoacylation with alanine of minihelix^{Ala} and the ribonamer-ribotridecamer duplex. The assays were carried out at 25°C, pH 7.5, in a final concentration of 5 μ M RNA, 97.5 μ M [¹⁴C]alanine, and 25 nM alanine-tRNA synthetase.

charged (data not shown), a mixed duplex consisting of a deoxyribonamer and a ribotridecamer (Fig. 1B, duplex *b*) is aminoacylated (Fig. 3). However, the efficiency of aminoacylation is reduced significantly (Fig. 3 *Inset*). Interestingly, the opposite mixed duplex consisting of a ribonamer annealed to a deoxyribotridecamer with rA substituted at the 3' end (Fig. 1B, duplex *c*) is not recognized by the enzyme (data not shown).

The specificity of recognition of the one RNA·DNA hybrid was tested by attempting to aminoacylate a deoxyribonamer-ribotridecamer duplex containing dA3 so as to form a dA3·U70 base pair (Fig. 1B, duplex *b*). In tRNA^{Ala}, a G3 to A3 substitution eliminates aminoacylation with alanine *in vivo* and *in vitro* (4). As shown in Fig. 3, aminoacylation of the dA3·U70 duplex is not detected. Thus, the weak aminoacylation of this RNA·DNA hybrid duplex requires the same major determinant (dG3·U70) as alanine tRNA (G3·U70). The intermediate electrophoretic migration of specifically designed RNA·DNA duplexes relative to DNA and RNA duplexes on polyacrylamide gels strongly suggests a unique helix geometry for each type of duplex (28). The reduced charging of the hybrid duplex and the lack of charging of the DNA duplex suggest that the enzyme is extremely sensitive to the exact geometry of the helical groove that provides access to the guanine-uracil base pair.

Aminoacylation with Limiting Amounts of the Strand Complementary to the Acceptor Strand. Melting temperatures were determined under aminoacylation assay conditions as described in *Materials and Methods*. While the melting temperature for the 6-mer duplex was relatively high (48°C at 2.5 μ M and 52°C at 40 μ M), the melting profile was broad and there was significant dissociation at 25°C (12.5%). The 9-mer duplex had a significantly higher melting temperature (59°C at 2.5 μ M and 64°C at 40 μ M) with a much sharper transition profile. At 25°C, however, no melting of the duplex was detected; the first indication of dissociation appeared at 45°C. At 50°C, the temperature used to melt the duplex in the cycling experiment (described below), about 11% of the duplex was dissociated.

Fig. 4A shows the aminoacylation with alanine (25°C) of an RNA 13-mer alone (lower curve) or when present in 10-fold excess over either a complementary 9-mer (middle curve) or 6-mer (upper curve). Due to the length of the experiment (5 hr), fresh enzyme was added each hour, and this small

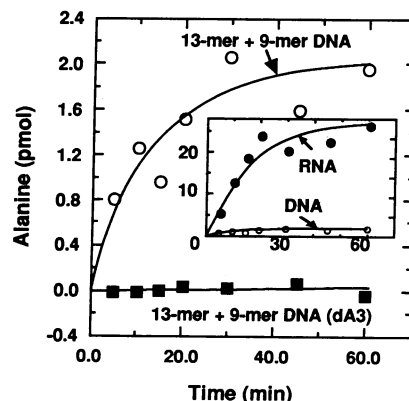


FIG. 3. Aminoacylation with alanine of RNA·DNA duplex substrates at 25°C, pH 7.5. Before each assay, the ribotridecamer was annealed with an equimolar amount of complementary deoxyribonamer containing either dG3 ("wild-type") or dA3. Assays were carried out with a final concentration of 5 μ M duplex substrate and 1.25 μ M alanine-tRNA synthetase. Each time point represents the incorporation of alanine per 10- μ l reaction aliquot. (*Inset*) Replot of the data for the RNA·DNA (dG3) duplex (labeled "DNA") compared to aminoacylation of the corresponding RNA·RNA duplex (labeled "RNA").

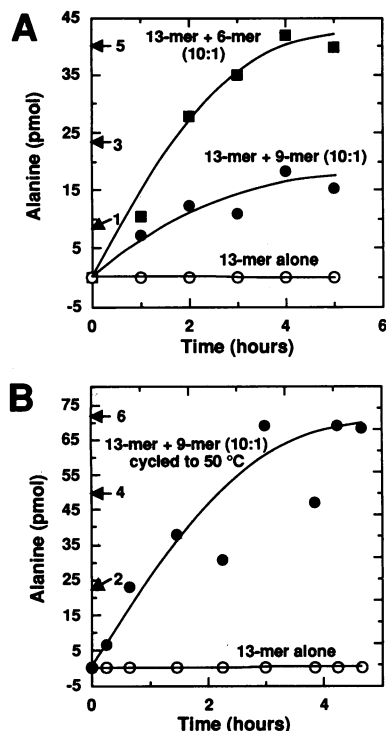


FIG. 4. Aminoacylation with alanine of a single-stranded ribotridecamer in the presence of a complementary ribohexamer or ribononamer at pH 5.5. Before each assay a 10-fold excess of the ribotridecamer was annealed with the complementary 5' oligomer. Final starting concentrations were 10 μ M ribotridecamer, 1 μ M ribohexamer or ribononamer, and 0.25 μ M alanine-tRNA synthetase. Each time point represents the incorporation of alanine per 10- μ l reaction aliquot. Numbered arrows indicate the stoichiometry of charging of the 3' oligomer with alanine. Assays shown in A were carried out at 25°C and fresh alanine-tRNA synthetase was added to a final concentration of 0.25 μ M every hour. After initiating the assay shown in B by addition of alanine-tRNA synthetase to 0.25 μ M, the reaction mixture was incubated at 25°C for 15 min. A 10- μ l aliquot was then removed to determine alanine incorporation and the reaction was brought to 50°C for 4 min. After cooling to 25°C (5 min), fresh alanine-tRNA synthetase was added to 0.25 μ M and the cycle was repeated.

dilution was taken into account in all calculations. Numbered arrows on the ordinate axis indicate the stoichiometry of charging of the 13-mer. The results suggest that at 25°C the 6-mer can form a duplex with a 13-mer which is then recognized by alanine-tRNA synthetase and charged with alanine. This transient duplex then may dissociate, allowing reassociation of the 6-mer with an uncharged 13-mer. In this way, the 6-mer acts as a necessary cofactor for charging with alanine-tRNA synthetase, and a 5-fold amplification of charging is seen after 5 hr.

Longer incubations did not result in more charging, possibly due to gradual hydrolysis of the RNA. In the presence of stoichiometric amounts of 13-mer, no "overcharging" is detected (data not shown). In the presence of the 9-mer, however, significantly less amplification of 13-mer charging at 25°C (only about 2-fold after 5 hr) is seen. The presence of 3 additional base pairs apparently provides enough incremental stability to prevent significant dissociation of the initial duplex.

To explore this possibility further and to achieve charging amplification with the 9-mer cofactor, the 9-mer and 13-mer (10-fold excess) were incubated at 25°C in the presence of enzyme for 15 min, cycled to 50°C for 4 min, and cooled again to 25°C. All assays longer than 1.5 hr or involving cycling to elevated temperatures were performed at pH 5.5 to minimize

chemical deacylation, which occurs much more rapidly and to a greater extent at higher pH. Fresh enzyme was then added and the cycle was repeated. Aliquots were taken periodically to check for alanine incorporation. As Fig. 4B indicates, a 6-fold amplification of 13-mer aminoacylation was achieved in about 4 hr.

DISCUSSION

The experiments described here were facilitated by implementation of chemical synthesis of oligoribonucleotides. While template-directed RNA synthesis with T7 RNA polymerase is convenient for substrates such as the 24-nucleotide microhelix and 35-nucleotide minihelix, short oligomers (of length less than 15 nucleotides) are usually not efficiently made by this method (18). Conversely, the chemical synthesis of oligoribonucleotides that are greater than 20 bases is not yet routine. However, as demonstrated in this work, short complementary single strands can be specifically aminoacylated by alanine-tRNA synthetase. A variety of modified bases and base analogs can now be easily substituted at specific locations in the reconstructed acceptor helix.

According to the UV absorbance melting curves determined for the stoichiometric mixtures of the ribotridecamer with the ribohexamer or ribononamer, the duplexes are completely annealed under the conditions of the assay in Fig. 2A. Thus, the high efficiency of aminoacylation of the six-, eight-, and nine-base-pair duplexes probably reflects the preponderance of duplex helix that is formed in each case and the lack of sensitivity of the enzyme to the number of base pairs in this range. However, the tetramer-tridecamer duplex is more unstable, with an estimated calculated melting temperature (see ref. 29) that is lower than the temperature used in the assay. A low melting temperature may explain in part why the tetramer-tridecamer combination is less efficiently aminoacylated.

The results presented here extend further the delineation of minimum sequence elements required for aminoacylation with alanine-tRNA synthetase. In previous work, it was demonstrated that a 12-base-pair hairpin minihelix and 7-base-pair hairpin microhelix were aminoacylated efficiently with alanine (14, 15). In addition to establishing here the dispensability of the hairpin structure, the efficient charging of complementary single strands with just 6 base pairs and the less efficient but significant charging of the 4-base-pair duplex (Fig. 2A) emphasize the strong concentration of recognition at the G3·U70 base pair. The 4-base-pair duplex constitutes little more than $\frac{1}{3}$ turn of an RNA A-form helix. Nonetheless, the lack of charging of the single-stranded ribotridecamer, the reduced charging of the deoxyribononamer-ribotridecamer duplex, and the lack of aminoacylation of the ribononamer-deoxyribotridecamer (3'-rA) duplex emphasize the strong dependence of recognition on the RNA- in contrast to the DNA-helix geometry.

It is noteworthy that three of the duplexes have "ragged" 5' overhangs caused by incomplete base pairing of the ribotridecamer with the complementary strand (Fig. 1A). These ragged ends do not interfere with aminoacylation, and they extend the earlier observation that the sequence or location (relative to the 3' acceptor end) of the single-strand loop of duplex hairpins is inconsequential to charging (ref. 14; cf. also ref. 16).

When limiting amounts of the complementary strand are used, the dissociation and rebinding of the limiting strand to the tridecamer acceptor strand facilitates an extent of aminoacylation of the acceptor well beyond the stoichiometric amount of limiting strand. Thus, the limiting complementary RNA serves as a template on which the substrate is positioned for catalysis. In this sense, it bears analogy to the guide sequences that are used in RNA splicing and processing

(30–33). The data obtained under the conditions used in this study suggest that the rate of aminoacylation (beyond that attained with a 1:1 complex) is controlled by the rate of dissociation of the complementary guide strand from a charged acceptor strand and rebinding to an uncharged acceptor strand. This provides a mechanism to regulate aminoacylation by an RNA cofactor. The results shown in Fig. 4A indicate that the more easily dissociated ribohexamer facilitates a more rapid charging of the acceptor strand (after the stoichiometric ratio is reached) than does the more tightly bound ribononamer.

The RNA cofactor-dependent aminoacylation of a single-stranded oligonucleotide expands possibilities for the development of early coding systems. In particular, the results demonstrate at least the feasibility of sequence-specific recognition of one RNA by another that subsequently results in an aminoacylation event. In this example, the critical sequence for recognition is proximal to the amino acid attachment site. Because of their greater flexibility compared to double-stranded molecules, however, charged single-stranded oligonucleotides might more freely form complexes with each other (or on templates) through base pairing and stacking interactions, utilizing sequences that may or may not be proximal to the attached amino acid. Were the activated aminoacyl linkages brought close in these complexes, peptide bond formation could occur spontaneously.

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