RNA minihelices as model substrates for ATP/CTP:tRNA nucleotidyltransferase

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Twenty-one RNA minihelices, resembling the coaxially stacked acceptor- /T-stems and T-loop found along the top of a tRNA's three-dimensional structure, were synthesized and used as substrates for ATP/CTP:tRNA nucleotidyltransferases from *Escherichia coli* and *Saccharomyces cerevisiae*. The sequence of nucleotides in the loop varied at positions corresponding to residues 56, 57 and 58 in the T-loop of a tRNA. All minihelices were substrates for both enzymes, and the identity of bases in the

loop affected the interaction. In general, RNAs with purines in the loop were better substrates than those with pyrimidines, although no single base identity absolutely determined the effectiveness of the RNA as substrate. RNAs lacking bases near the 5'-end were good substrates for the *E. coli* enzyme, but were poor substrates for that from yeast. The apparent K_m values for selected minihelices were 2–3 times that for natural tRNA, and values for apparent V_{max} were lowered 5–10-fold.

INTRODUCTION

The enzyme ATP/CTP:tRNA nucleotidyltransferase interacts specifically with tRNAs to restore all or part of the 3'-terminal sequence CCA [1]. The enzyme does not discriminate between tRNAs, and yet, in general, does not effectively recognize other types of RNA such as synthetic homopolymers or rRNA [1]. The importance of the 3'-terminal base(s) in the recognition process has been demonstrated using tRNAs [2], model substrates [3], and even 5S rRNA [2]; although in the latter case the reaction with rabbit-liver enzyme exhibited a much lower rate than with natural tRNA as substrate.

The importance of other parts of the tRNA molecule in affecting the interaction has been demonstrated using fragments of tRNA [4,5], model substrates [6], chemically modified tRNAs [7–9], and *in vitro* synthesized precursor tRNAs (p-tRNAs; [10]). Based on the chemical interference assays and mutant p-tRNAs containing mutations in the T-loop, a two-site model for the interaction was proposed [7–10] in which the enzyme ensures specificity for tRNAs by extending across the top to the tRNA's three-dimensional structure, making contact in the vicinity of the T-loop, specifically near residues 56, 57 and 58, numbered according to Steinberg et al. [11].

To further test this hypothesis and to eliminate the complication of tertiary structural effects, we describe in this report the synthesis of RNA minihelices resembling the top of a tRNA's tertiary structure, and the ability of such minihelices to act as substrates for nucleotidyltransferases from *Escherichia coli* and *Saccharomyces cerevisiae*. The minihelix RNAs vary in sequence within the loop at positions analogous to bases 56, 57 and 58 in the T-loop of a tRNA. Accordingly, experiments were carried out to determine specifically: (1) if RNA minihelices can serve as substrates for tRNA nucleotidyltransferase; (2) if the identity of bases in the T-loop of a tRNA analogue can affect recognition, independently from effects on tertiary structure; and (3) if the best minihelix substrates have kinetic parameters K_m and V_{max} that are comparable with those for intact tRNA. These experi-

ments represent an initial step in a long-range plan to design RNA molecules that can act as potent competitive inhibitors of tRNA nucleotidyltransferase, which is an essential enzyme for rapidly growing eukaryotic (e.g. tumor) cells.

EXPERIMENTAL

Cloning degenerate oligonucleotides

Two synthetic, partially complementary oligonucleotides (Genosys Biotechnologies, The Woodlands, TX, U.S.A.) were designed to code for an RNA minihelix resembling the acceptor- and T-stems of tRNA^{Phe} from *E. coli* and containing variable sequences in the loop:

SacII (sense) 5'
CGGACTTGGTTNNNTTCCGAGTCCGCCACCGCGGA
3'

(template) 3' GCCTGAACCAANNNAAGGCTCAGGCGGG
TGGCGCCTTCGA 5'

where positions designated by N were fully degenerate. A recognition site for *SacII* was included adjacent to the 3'-end of the gene, so that the construct could be linearized before *in vitro* transcription. When hybridized, the oligonucleotides contained a blunt end at one terminus and a four-base 3'-overhang compatible with ends left following cleavage by *HindIII* at the other.

The hybridized oligonucleotides were phosphorylated using polynucleotide kinase according to the supplier's instructions (New England Biolabs, Beverly, MA, U.S.A.) and joined to the vector pTZ18U (US Biochemicals, Cleveland, OH, U.S.A.) using T4 DNA ligase (New England Biolabs). The pTZ18U had previously been cleaved with *EcoRI*, treated with nuclease S1 (to remove single-stranded overhanging ends), extracted with phenol [phenol/chloroform/isoamyl alcohol (49.5:49.5:1, by vol.) buffered with 10 mM Tris/HCl (pH 8.0)/1 mM EDTA; International Biotechnologies, New Haven, CT, U.S.A.], digested with *HindIII*, and agarose-gel purified using NA45 DEAE membranes

Abbreviation used: p-tRNAs, precursor tRNAs.

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(Schleicher and Schuell, Keene, NH, U.S.A.) to recover the DNA. All restriction enzymes were obtained from New England Biolabs.

Relevant portions of the resulting recombinant vector and resulting transcript are shown below, including the start-site for transcription by T7 RNA polymerase (*), the bases derived from the synthetic oligonucleotides (underlined), and the cut site (†) on the template strand for the unique *Sac*II site introduced by the synthetic oligonucleotides.

Sense: TAATACGACTCACTATAGGGCGGACTTGGTTN
NNTTCCGAGTCCGCCACCGCGGAAGCTT

Template: ATTATGCTGAGTGATATCCCGCTGAACCA-
ANNNAAGGCTCAGGCGGGTGGCGCCTTCGAA

↑ (*Sac*II)

RNA transcript: GGGCGGACTTGGTTNNTTCCGAGT-
CCGCCACC

The resulting RNA minihelix lacked a 3'-terminal adenosine residue, corresponding to the A of the CCA sequence normally found at the 3'-terminus of a tRNA.

Recombinant plasmids were used to transform competent *E. coli* JM109 cells and were isolated from white colonies on plates containing ampicillin and X-Gal using mini-plasmid preparation kits from Promega (Madison, WI, U.S.A.). Plasmids were screened using *Eco*RI, *Sac*II and *Hind*III. Those lacking the *Eco*RI site, and containing *Sac*II and *Hind*III sites, were sequenced using Sequenase 2.0 DNA sequencing kits from US Biochemicals.

In vitro transcription

Following complete cleavage of the plasmid template with *Sac*II and extraction with phenol, RNAs were transcribed *in vitro* using the Ribomax system (Promega) and T7 RNA polymerase purified according to Zawadzki and Gross [12] from *E. coli* BL21/pAR1219 [13]. RNA products were extracted with phenol, precipitated with ethanol, and purified by eluting from 20% (w/v) polyacrylamide gels or by spun-column chromatography using Sephadex G50 [14]. Concentrations of the minihelices were determined using absorption at 260 nm, assuming an absorbance of 1.0 corresponds to 40 µg/ml of RNA, and were verified using 20% (w/v) denaturing polyacrylamide gels [100 mM Tris/borate, 8 M urea; acrylamide/*N,N'*-methylenebisacrylamide (19:1, w/w)] stained with 0.05% (w/v) Stains-all.

Enzyme purification and assay

E. coli and yeast nucleotidyltransferases were purified as described previously [10]. The final concentration of enzyme in the assay was approx. 0.025 units/ml (one unit of activity results in the attachment of 1 µmol of AMP to tRNA per h at 37 °C). The assay contained 50 mM Tris/glycine (pH 9.4), 10 mM MgCl₂, 5 mM dithiothreitol, 500 µM [α -³²P]ATP (0.7 mCi/ml) and substrate, in a volume of 7.5–15 µl. Following incubation for 30–60 min, deionized formamide containing 0.025% (w/v) Bromophenol Blue and 0.025% (w/v) Xylene Cyanole was added; and the entire sample was loaded on to a 20% denaturing polyacrylamide gel. Incorporation of label remained linear with time over the duration of the assay. Autoradiograms were exposed at –70 °C for 1–3 days. These were used as guides to slice out radioactive bands, which were placed in a Packard Tri-Carb 1900 CA liquid-scintillation counter to determine radioactivity. Densitometry scans of the autoradiograms were performed using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

Collection of analogues

The collection of minihelices generated by cloning degenerate synthetic oligonucleotides is depicted in Figure 1. The helix resembles the coaxially stacked acceptor- and T-stems of tRNA^{Phe} from *E. coli* and the variable bases correspond to positions 56, 57 and 58 in the T-loop. Over 100 recombinant plasmids were selected at random and analysed. Nineteen of the possible 64 different complete genes coding for minihelices were identified by sequencing, transcribed and used as substrate for nucleotidyltransferase. In this collection, all four nucleotide bases are present at each of the variable positions. In addition, two constructs (AAA* and CGG*) were isolated that lacked the three 5'-bases (GGG). These last three bases were originally present in pTZ18U, adjacent to the site of cleavage by *Eco*RI; they may have been removed during treatment with nuclease S1.

Polyacrylamide gel assay

All RNA minihelices were substrates for both enzymes. Labelling of 15 minihelices by the *E. coli* enzyme is shown in Figure 2;

Figure 1 Sequence of RNA minihelices synthesized *in vitro* and used as substrates for nucleotidyltransferase

The RNA minihelices comprised the above sequence with different bases in the loop (NNN), corresponding to positions 56, 57 and 58 in the T-loop of a tRNA. The 19 sets of variable bases are listed on the left. The remaining sequence resembles that of the acceptor- and T-stems of tRNA^{Phe} from *E. coli*.

Figure 2 Autoradiogram of RNA minihelices labelled with ³²P by nucleotidyltransferase from *E. coli*

An unfractionated mixture of *E. coli* tRNAs from which the 3'-terminal A residue had been removed was used as reference (lane labelled 'tRNA'). The minihelices are depicted by the triplet of variable bases in the loop. Those indicated by an * lack the 5'-bases GGG. All RNAs were present at a concentration of 33 µM. The arrow indicates the position of the labelled minihelix.

Figure 3 Autoradiogram of RNA minihelices labelled with ^{32}P by nucleotidyltransferase from yeast

An unfractionated mixture of *E. coli* tRNAs from which the 3'-terminal A residue had been removed was used as reference (lane labelled tRNA). The minihelices are depicted as described in the legend to Figure 2. All RNAs were present at a concentration of $3.3\ \mu\text{M}$. The arrow indicates the position of the labelled minihelix.

Table 1 Extents of labelling of 14 minihelix RNAs relative to that for the minihelix AAA

Each minihelix RNA is depicted according to the variable triplet sequence in the loop; that marked with an * lacks the 5' three bases GGG. Labelled bands were sliced from the gel (see Figures 2 and 3) containing samples incubated at a substrate concentration of $3.3\ \mu\text{M}$ and counted. Densitometry scans were also recorded. The ratios of extents of labelling for 14 minihelices relative to that for the minihelix AAA were calculated using both radioactivity and densitometry data; the average of the two values is presented.

Analogue	<i>E. coli</i>	Yeast
AAA	1.00	1.00
AAA*	1.51	0.09
AGA	1.39	0.79
GAG	1.06	0.45
AAU	0.71	0.70
AGU	1.00	0.54
AUA	0.34	0.38
AUU	0.66	0.49
UAA	1.10	0.56
CAG	1.13	0.67
CAU	0.62	0.44
CUA	0.68	0.49
UCC	0.36	0.38
GCU	0.92	0.50
CGG*	0.75	0.26

labelling of the same set of 15 minihelices by the yeast enzyme is shown in Figure 3. The labelled bands were sliced from the gel and radioactivity was measured for each. In addition, densitometer scans of autoradiograms were performed. The minihelix AAA was a good substrate for both enzymes and was selected as a reference, so that the relative effectiveness of RNA minihelices as substrates could be assessed. As a specific example, ratios of band intensities (using both radioactivity and densitometry data) at the lowest concentration of substrate ($3.3\ \mu\text{M}$) for the other 14 RNAs relative to that for AAA were calculated and are presented in Table 1. These ratios reproducibly depended on the identity of the variable triplet sequence in the loop over the entire range of substrate concentrations ($3.3\text{--}33.3\ \mu\text{M}$). In a separate experiment, six additional minihelix RNAs were tested using only the yeast enzyme. The extents of labelling in this particular experiment were not quantified; however, visual estimations of band intensities suggest that GAU and UUA RNAs were

Figure 4 Autoradiogram of RNA minihelices labelled with ^{32}P by nucleotidyltransferase from yeast (a) or *E. coli* (b) in the presence of an equimolar amount of wild-type or mutant p-tRNAs

The sequence of the wild-type p-tRNA is G57A58. The single mutant (A57A58) was used with that from yeast (a); both single (A57A58) and double (A57U58) mutant p-tRNAs were used as competing substrates with the *E. coli* enzyme (b). Minihelices are depicted as described in the legend to Figure 2. All RNAs were present at equimolar concentrations of $17\ \mu\text{M}$ (a) or $7\ \mu\text{M}$ (b). The arrow indicates the position of the labelled minihelix; the labelled p-tRNAs (top band) and its two or three degradation products migrate well above the minihelices.

comparable with AAA; GCA and CUU were labelled to a lesser extent; and UCU and UUG were poor substrates.

Competition gels

The RNA minihelices were not detectably labelled in the presence of an equimolar amount of unfractionated tRNA (from which the 3'-terminal A residue had been removed), except at very low concentrations where the tRNA substrate may have been depleted before the end of the incubation. However, using nucleotidyltransferase from yeast, the minihelix AGA effectively competed with a mutant p-tRNA (p-A57), containing a G-to-A substitution at position 57 (Figure 4a). In contrast, labelling of the minihelix UCC by the yeast enzyme in the presence of p-A57 was barely detectable (Figure 4a). Using the *E. coli* enzyme, the minihelices AAA and AAA* (lacking three bases on the 5'-end) were labelled poorly in the presence of wild-type p-tRNA, somewhat better in the presence of the single mutant p-A57, and better still in the presence of a double mutant p-tRNA (p-A57U58) (Figure 4b).

Kinetic parameters

To assess whether some of the best minihelix substrates (AAA, AAA* and AGA) were comparable with intact tRNA in terms of kinetic parameters, an additional set of substrate titration experiments was carried out on a single polyacrylamide gel using *E. coli* nucleotidyltransferase. Apparent Michaelis-Menten constants (K_m) and V_{max} values were calculated using Eadie-Hofstee plots of radioactivity recovered from gel slices. In addition, densitometry data from the initial set of four gels in which 15 analogues were screened for activity over a range of substrate concentrations (see Figures 2 and 3) were analysed using initial rates of incorporation into intact tRNA as an internal reference. The kinetic parameters for each selected minihelix were calculated

Table 2 Apparent K_m and V_{max} values for selected minihelices using nucleotidyltransferase from *E. coli*

Values for kinetic parameters represent an average using both radioactivity and densitometry data. Apparent K_m and V_{max} values for *E. coli* tRNA^{Phe} and *in vitro* transcribed p-tRNAs have been described previously [10] and are included for reference.

RNA	K_m (μ M)	V_{max} (μ mol $\cdot$ h ⁻¹)
AAA	13	0.023
AAA*	15	0.031
AGA	8	0.015
tRNA ^{Phe}	5	0.14
p-G57A58	8	0.2
p-A57A58	10	0.2
p-A57U58	14	0.08

using both densitometry and radioactivity data and the results are presented in Table 2. For all three types of substrate (tRNA, p-tRNA and selected RNA minihelices), the apparent K_m values are similar; they differ at most by a factor of 3. Values of V_{max} were reduced by a factor of 5–10, relative to that for intact tRNA (Table 2).

DISCUSSION

Analogues are substrates

All RNA minihelices were detectably labelled by either enzyme at substrate concentrations in the 3–33 μ M range. The RNA minihelices contained the sequence CpC at their 3'-end, and thereby resembled a tRNA lacking its 3'-terminal adenosine. They also conformed to the optimal 3'-terminal recognition sequence determined using tRNA and model substrates [2,3,6]. That is, small molecules such as CpC and cytidine have been shown to be substrates for the enzyme from rabbit liver. However, the apparent K_m for CpC was 5 mM (compared with 4 μ M for tRNA) and the V_{max} value was reduced nearly 20-fold [3]. Some of the minihelix RNAs have apparent K_m values much closer to that for tRNA (Table 2). It is therefore apparent that structural aspects of the RNA minihelices enhance substrate binding, relative to simple mono- or di-nucleosides.

None of the RNA minihelices were able to effectively inhibit labelling of natural tRNAs; the best substrates had apparent K_m values roughly 2–3 times that for tRNA^{Phe} (Table 2). Accordingly, additional structural aspects of a tRNA, not present in the RNA minihelices, may be required for optimal binding of substrate and/or full activation of catalytic activity. We note that the *E. coli* enzyme requires a chemically intact G at position 19 in the D-loop for full activity [7]. Perhaps additional interactions take place at the corner of the tRNA that involve tertiary base-paired residues such as G19–C56.

However, some minihelices were able to effectively compete at equimolar concentrations with mutant p-tRNAs, whose apparent K_m values were somewhat higher than those for wild-type p-tRNAs (Figure 4). Thus the possibility still remains that an RNA minihelix with a slightly different structure (i.e. a different sequence in the loop or smaller overall length) might serve as an effective inhibitor of tRNA nucleotidyltransferases.

Identity of nucleotides in the loop affects recognition

Several experiments using chemical modification and mutant p-tRNAs have suggested the importance of nucleotides in the T-

loop of a tRNA to the recognition process [7–10]. To test the hypothesis that nucleotides at positions analogous to 56, 57 and 58 in the T-loop of a tRNA directly affect recognition, we constructed RNA minihelices with different bases at these positions. The identity of bases in the loop reproducibly affected the extent of labelling by both enzymes (Table 1). As a general rule, RNA substrates with all purines at the three variable positions were better substrates than those with all pyrimidines. However, there was no unambiguous correlation between the identity of bases at any one site with the level of activity.

It is interesting to note that the best substrates (e.g. AAA, AGA, GAG, UAA, CAG) contained a purine at position '57' and the worst substrates (CUA, UCC, AUU and AUA) contained pyrimidines at this site. These data correlate well with the observations that substituting pyrimidines at position 57 in mutant p-tRNAs dramatically inhibited the interaction [10]. Despite the fact that the purine at position 57 is not base paired in the crystal structure of a tRNA [15], it is not clear whether base substitutions in p-tRNAs had disrupted tertiary structural elements that were essential for recognition. Results of the present set of experiments suggest that the identity of bases within the T-loop can directly affect recognition by nucleotidyltransferase, because there can be no long-range tertiary interactions within the RNA minihelix comparable with those in a tRNA.

However, stacking or base pairing within the loop may also be important. For example, purines would be more likely to form base pairs with other bases in the loop, which are all uridines (Figure 1). The nature of base pairing within the loop would directly affect the pattern of hydrogen-bond donors/acceptors and hydrophobic/hydrophilic surfaces available for interaction with the enzyme. Future molecular modelling studies on possible loop conformations may help to characterize such patterns.

The identity of the base at the site analogous to position 56 in a tRNA did not appear to be decisive in determining whether the RNA could act as substrate for either enzyme. That is, at least one minihelix that contained each of the four possible nucleotides at the site analogous to position 56 was nearly comparable in activity (i.e. 75 % or greater) with the AAA minihelix (e.g. AGA, GAG, UAA and CAG; Table 1).

From crystal data, it is known that the universally conserved cytidine at position 56 is involved in a tertiary base pair with G19 [15]. Previous results of chemical interference assays indicated the importance of chemically intact residues at positions 56 [8] and 19 [7]. These data are consistent with the results reported here if formation of the tertiary C56–G19 base pair in a tRNA is one of several structural features that contribute to interaction with nucleotidyltransferase. Such tertiary structure would not be found in the RNA minihelices; hence the identity of the base at the site analogous to position 56 should not greatly affect recognition. In addition, the RNA minihelices would not be expected to compete effectively with the natural tRNA substrate, because they lack the tertiary structural element that contributes to recognition. Even the best minihelix substrates did not inhibit labelling of a wild-type p-tRNA (Figure 4).

Missing bases at the 5'-end affect yeast but not *E. coli* enzyme

Relatively large fragments of tRNA have been shown to re-anneal to form substrate for the yeast enzyme, provided that each fragment contained either the 5'- or the 3'-terminus of the tRNA [4]. Synthetic oligonucleotides and small fragments of tRNA have also been shown to be substrates for the yeast enzyme, provided that there was some base pairing of the oligonucleotides that resembled the terminus of the acceptor

stem of a tRNA [5]. Accordingly, the importance of secondary structure to substrate recognition was proposed. Our results support the notion that base pairing near the site of catalysis is important in the case of the enzyme from yeast, because constructs lacking the 5' three bases (AAA* and CCG*), which were unable to form base pairs near the site of addition of AMP, were very poor substrates for the yeast enzyme.

In contrast, the same substrates were labelled very effectively by the enzyme from *E. coli*, suggesting that the bacterial enzyme is much less stringent in its requirement for base pairing near the site of catalysis. These data are in accord with previous damage-selection experiments in which chemically modified bases near the 3'-end (positions 71–73) affected recognition of substrate by the yeast enzyme far more frequently than when the *E. coli* enzyme was used [7]. They also correlate well with the observation that the *E. coli* initiator tRNA^{Met} is unique in that it does not contain base-paired residues at the end of the acceptor stem (i.e. 1–72). Thus the *E. coli* enzyme must be able to tolerate the lack of base pairs near the site of catalysis if it is to repair the initiator tRNA^{Met}.

CONCLUSIONS

RNA minihelices resembling the top of a tRNA's three-dimensional structure are effective substrates for tRNA nucleotidyltransferases from *E. coli* and yeast; their apparent K_m values are only 2–3 times that of a natural tRNA and their V_{max} values are reduced 5–10-fold. Variations in sequence within the loop, corresponding to positions 56, 57 and 58 of a tRNA, affect the

ability of the minihelices to act as substrates. The lack of base-paired nucleotides near the site of catalysis inhibits interaction with the yeast enzyme, but not with that from *E. coli*.

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