Inhibition of tRNA Aminoacylation by 2′-*O*-Methyl Oligonucleotides†

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ABSTRACT: A 2′-*O*-methyl oligonucleotide complementary to 18 nucleotides in the dihydrouridine stem-loop of *Escherichia coli* $tRNA^{Cys}$ has been shown to stably bind to the tRNA. The binding inhibits aminoacylation of the tRNA by cysteine tRNA synthetase. The same oligonucleotide sequence but with the DNA deoxy backbone does not bind to the tRNA. This provides the basis for the design and test of a series of 2′-*O*-methyl oligonucleotides for their ability to bind to *E. coli* $tRNA^{Cys}$ and inhibit aminoacylation. We show here that different regions of the tRNA have different sensitivities to oligonucleotides. A 10-mer that targets G15 forms a stable complex with the tRNA. The K_d of the complex is several orders of magnitude lower than that of the tRNA−synthetase complex. Measurements of dissociation rate constants indicate that the stronger affinity of the 10-mer to $tRNA^{Cys}$ is due to a significantly slower rate of dissociation (by a factor of 10^6) than that of the synthetase from the tRNA. Only a stoichiometric amount of the 10-mer is necessary to completely inhibit aminoacylation. Because tRNA aminoacylation is fundamental to cell growth, these results provide the rationale for the 10-mer and its derivatives as pharmaceutical agents that target specific cell growth.

Aminoacylation of tRNAs by their cognate aminoacyl tRNA synthetases is fundamental to protein synthesis. This reaction attaches an amino acid to the 3′ end of a tRNA so that the amino acid can be delivered to the growing polypeptide chain as the anticodon sequence of the tRNA reads a codon triplet in a mRNA. The specificity of aminoacylation is determined by the ability of an aminoacyl tRNA synthetase to interact with the correct amino acid and to recognize its cognate tRNA through specific nucleotides (Schimmel & Söll, 1979; Meinnel et al., 1995). In principle, interference with either the amino acid binding step or the tRNA recognition step of a synthetase can inhibit aminoacylation and arrest protein synthesis, leading to cell death.

While several amino acid analogs have proven useful as inhibitors of aminoacylation (Aldridge, 1992; Yanagisawa et al., 1994), we are interested in developing strategies that target the tRNA recognition step. These strategies focus on designing small compounds which will bind to the critical nucleotides in tRNAs that are recognized by the cognate aminoacyl tRNA synthetases. Recent studies have shown that the specific nucleotides important for synthetase recognition can vary from one organism to another (Sampson et al., 1988; Nazarenko et al., 1992). The species-specific nucleotides account for the species-selective aminoacylation of tRNA acceptor stems by the cognate synthetases (Hipps et al., 1995; Hou et al., 1995; Quinn et al., 1995). Substitutions of these nucleotides in an acceptor stem reduce aminoacylation while transfer of these nucleotides to the analogous positions of a heterologous tRNA (tRNA from a different organism) confers recognition and aminoacylation of that tRNA by the heterologous synthetase. These results highlight the possibility that species-specific tRNA nucleotides can provide the basis for drug design that would inhibit aminoacylation of a tRNA from one organism but not from others.

To explore this possibility, we synthesized oligonucleotides that were complementary to the major nucleotide determinants for aminoacylation of *Escherichia coli* $tRNA^{Cys}$. We and others previously showed that U73 and the GCA anticodon of *E. coli* $tRNA^{Cys}$ are important for aminoacylation (Figure 1) (Pallanck et al., 1992; Hou et al., 1993; Komatsoulis & Abelson, 1993). In addition, we showed that a G15:G48 tertiary hydrogen base pair that connects the dihydrouridine (D) loop with the variable loop is an important structural determinant that contributes to aminoacylation (Figure 1) (Hou et al., 1993; Hou, 1994). Substitution of G15:G48 with G15:C48 reduces the catalytic efficiency of aminoacylation by 2 orders of magnitude (Hou et al., 1993). Inspection of available tRNA genes and sequences for cysteine tRNAs shows that U73 and the GCA anticodon are conserved in evolution. In contrast, while *Bacillus subtilis*, yeast, and mouse all have a G15:C48 base pair, *E. coli* is the only organism that has G15:G48 (Steinberg et al., 1993). Aminoacylation of yeast $tRNA^{Cys}$ by the *E. coli* cysteine enzyme has a catalytic efficiency 2 orders of magnitude below that of *E. coli* $tRNA^{Cys}$ (Hou et al., 1995). Although yeast $tRNA^{Cys}$ differs from *E. coli* $tRNA^{Cys}$ in many more nucleotides than just the G15:G48 base pair, the identical catalytic efficiency of aminoacylation of yeast $tRNA^{Cys}$ and of the G15:C48 variant of *E. coli* $tRNA^{Cys}$ suggests a kinetic parallel between the two tRNAs. It is possible that the requirement for the unique G15:G48 tertiary base pair of *E. coli* $tRNA^{Cys}$ accounts for the species-specific aminoacylation against yeast $tRNA^{Cys}$ by the *E. coli* cysteine tRNA synthetase.

Earlier studies demonstrated that the secondary cloverleaf structure of tRNAs and its L shaped tertiary structure limited

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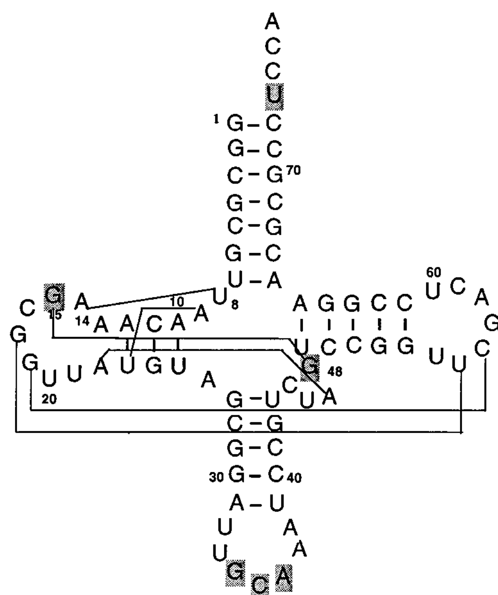

FIGURE 1: Sequence and cloverleaf structure of *E. coli* tRNA^{Cys}. U73, G15:G48, and the GCA anticodon are shaded to indicate that they are the major nucleotide determinants for recognition by cysteine tRNA synthetase. Nucleotides are numbered based on the system established for yeast tRNA^{Phe} (Steinberg et al., 1993) such that A14, G15, G18, and G19 are preserved while nucleotide 17 is missing in the D loop. Thin lines indicate tertiary hydrogen bonds that stabilize the L shaped tRNA tertiary structure.

the accessibility of complementary oligoribonucleotides to specific regions (Uhlenbeck, 1972; Schimmel et al., 1972; Freier & Tinoco, 1975). These accessible regions include the NCCA sequence at the 3′ end, the anticodon loop, a portion of the D loop, and a portion of the variable loop. None of the nucleotides in the stem regions were accessible to oligoribonucleotides. In those early studies, oligoribonucleotides of 3−4 residues in length were used. In a more recent study, a 13-mer oligoribonucleotide was used to probe the anticodon loop of *E. coli* tRNA^{fMet} (Hayase et al., 1990). However, due to the unusual conformation of the anticodon loop, the 13-mer did not gain access to the tRNA until the higher order tRNA structure was melted first. These studies emphasize the challenge of targeting tRNAs by oligonucleotides.

The G15:G48 tertiary base pair of *E. coli* tRNA^{Cys} is located within the core structure of the tRNA, which consists of multiple tertiary hydrogen and stacking interactions. This raised the question of whether oligonucleotides that target G15 in *E. coli* tRNA^{Cys} could gain access to this core structure. Because oligonucleotides with 2′-*O*-alkyl modification form far more stable duplexes with RNA strands than their deoxy counterparts (Inoue et al., 1987), we synthesized all of the oligonucleotides with a uniform 2′-*O*-methyl (2′-*O*-Me) modification. We show here that a 2′-*O*-Me oligonucleotide complementary to an 18-base long sequence which includes G15 stably binds to *E. coli* tRNA^{Cys} and discriminates against yeast tRNA^{Cys}. The corresponding oligodeoxynucleotide fails to bind to *E. coli* tRNA^{Cys}, and this provides the evidence that the 2′-*O*-Me modification is critical for binding. Additional experiments show that 2′-*O*-Me oligonucleotides as short as a 10-mer can bind to G15 of *E. coli* tRNA^{Cys}. The stoichiometric binding of the 10-mer to the tRNA completely inhibits aminoacylation. Because the 10-mer dissociates from *E. coli* tRNA^{Cys} significantly slower than that of cysteine tRNA synthetase, it is an effective competitor against the enzyme for tRNA^{Cys}. This 10-mer, within the statistical boundaries that define unique RNA sequences in bacteria, provides an example of sequence-specific inhibitor of tRNA aminoacylation.

MATERIALS AND METHODS

Synthesis and Radiolabeling of 2′-O-Me Oligonucleotides. Synthesis of oligonucleotides with 2′-*O*-Me phosphoramidites (Glen Research) was performed on a Pharmacia automated synthesizer. Each oligonucleotide was synthesized with an acridine-derivatized controlled-pore glass (CPG) support so that the 3′ end of the oligonucleotide would contain an acridine group. Oligonucleotides were purified by HPLC on a reverse-phase column with an acetonitrile gradient (usually 0−45%) in 100 mM triethylamine acetate (pH 7.5) buffer (Gamper et al., 1993). All oligonucleotides were at least 95% pure when evaluated by electrophoresis on a 20% denaturing polyacrylamide gel, followed by silver staining for visualization.

Oligonucleotides were labeled at the 5′ end by T4 polynucleotide kinase with [γ-^{32}P]ATP (NEN, >3000 Ci/mmol). The labeled oligonucleotides were separated from free ATP by electrophoresis in a 20% polyacrylamide/7 M urea gel and were eluted from the gel in 0.125 M ammonium acetate, 0.125 mM EDTA (ethylenediaminetetraacetic acid), and 0.025% SDS (sodium dodecyl sulfate). The eluted oligonucleotides were precipitated by ethanol with carrier and resuspended. Specific activities of the labeled oligonucleotides were 3600−4200 cpm/fmol.

Preparation of tRNA Transcripts and Aminoacylation Assays. All tRNA transcripts were made by T7 RNA polymerase from tRNA genes that were constructed in plasmid pTFMa (Hou et al., 1993). Transcription was performed for 4 h at 37 °C in a reaction that contained 40 mM Tris-HCl, pH 8.0, 24 mM $MgCl_2$, 1 mM spermidine, 5 mM dithiothreitol, 0.01% Triton X-100, 0.24 U/μL RNasin, 4 mM NTP, and 16 mM GMP. Full-length transcripts were purified by electrophoresis (12% polyacrylamide/7 M urea) and ethanol precipitated. Concentrations of tRNAs were determined by absorption at 260 nm (1 OD_{260} = 0.04 μg/μL RNA).

Aminoacylation of tRNAs with cysteine was assayed at 37 °C as previously published (Hou et al., 1993; Hamann & Hou, 1995). To determine the effect of oligonucleotides on tRNA aminoacylation, tRNAs were preannealed (80 °C, 2 min, 37 °C, 30 min in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 20 mM $MgCl_2$) and then incubated with an oligonucleotide at 37 °C for 15 min prior to the addition of the assay buffer and the enzyme (1 nM). Aminoacylation was monitored for 10 min at 37 °C while aliquots at different time points were removed and precipitated by 5% trichloroacetic acid on filter pads. The filter pads were washed with ethanol and ether, dried, and counted in scintillation fluor. For determination of k_{cat}/K_m of aminoacylation, tRNA concentrations ranged from 0.2 to 16 μM.

Gel Shift Assays. Oligonucleotides labeled at the 5′ end were used for the tRNA gel shift assay (Pyle et al., 1990; Bhattachryya et al., 1990; Lima et al., 1992). Assays were performed by incubating a preannealed tRNA transcript (2 μM) with a labeled oligonucleotide (1000 cpm, 4 μM) in 24 μL of annealing buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 20 mM $MgCl_2$) for 30 min at 37 °C. A 10×

Table 1: Ten 2′-*O*-Methyl Oligonucleotides[a] That Target *E. coli* and Yeast $tRNA^{Cys}$

name	targeted sequence and structure		length	rel k_{cat}/K_m of aminoacylation	% inhibition
E. coli $tRNA^{Cys}$				1.00	
01	5′-U^{8}AACAAAGCGGUUAUGUA^{26}-3′	(e•D stem-loop)	18		100
02	5′-U^{8}GGCGCAGUGGUAGCGCA^{26}-3′	(y•D stem-loop)	18	1.00	0
03	5′-U^{8}AACA**U**A**C**CGGU**A**AUGUA^{26}-3′	(e•D stem-loop)	18	1.00	0
04	5′-A^{10}CAAAGCGGUUAUG^{24}-3′	(e•D stem-loop)	14		100
05	5′-A^{12}AAGCGGUUA^{22}-3′	(e•D stem-loop)	10		100
06	5′-A^{13}AGCGGUUAU^{21}-3′	(e•D loop)	8	0.03	97
07	5′-A^{13}AGCG^{18}-3′	(e•D loop)	5	0.80	20
08	5′-C^{69}GCCUCCA^{76}-3′	(e•acceptor stem)	8	0.85	15
09	5′-A^{37}AUCCGUCUA^{46}-3′	(e•AC stem-loop)	10	0.04	96
10	5′-C^{51}GGUUCGACUCCG^{64}-3′	(e•TΨC stem-loop)	14	0.40	60

[a] All oligonucleotides contain the uniform 2′-*O*-Me modification and a 3′-terminal acridine group. Underlined bases in the target sequence are the major determinants for aminoacylation for *E. coli* $tRNA^{Cys}$. While the target sequence for 03 is the same as that for 01, 03 contains 3 mismatches with the target. Here the complement of 03 is shown, which contains U13, C15, and A21 (boldfaced) that differ from the wild-type A13, G15, and U21. Symbols: e = *E. coli*; y = yeast; AC = anticodon.

loading buffer containing 40% glycerol and 0.5% each xylene cyanol and bromophenol blue was added, and 5 μL of the sample was loaded onto a 12% polyacrylamide gel (43 cm × 30 cm × 0.4 mm, 89 mM Tris-HCl, pH8.3, 89 mM borate, 2 mM EDTA, and 5 mM $MgCl_2$). The complex of tRNA and oligonucleotide was separated from free species by nondenaturing electrophoresis at room temperature and at 750 V until bromophenol blue reached the bottom. The gel was analyzed by autoradiography to determine the fraction of bound oligonucleotide.

Determination of the Hybridization Rates and Equilibrium Dissociation Constants of tRNA−Oligonucleotide Complexes. To measure the dissociation rate of a tRNA−oligonucleotide complex, a ^{32}P-labeled oligonucleotide (40 nM) and preannealed $tRNA^{Cys}$ (200 nM) were incubated together in the annealing buffer for 1 h at 37 °C; then unlabeled oligonucleotide (20 μM) was added so that the final concentration of the oligonucleotide was 500-fold greater than that of the labeled oligonucleotide. A reaction of 50 μL was incubated at 37 °C, and aliquots of 5 μL were removed at specific time intervals and immediately snap-frozen on dry ice. Reactions were individually thawed and loaded onto a nondenaturing gel as described above. The relative distribution of label between the free and bound oligonucleotide was quantified by a phosphorimager (Molecular Dynamics).

To determine the equilibrium constant of a tRNA−oligonucleotide complex, 15 pM of a 5′-labeled oligonucleotide (1000 cpm) was incubated with a concentration series of preannealed *E. coli* $tRNA^{Cys}$ (20 pM−5 nM) in annealing buffer at 37 °C for 24 h. After addition of the 10× loading buffer, reactions were analyzed by nondenaturing gel electrophoresis as described above.

Protection of $tRNA^{Cys}$ from Chemical Modification by Oligonucleotides. *E. coli* $tRNA^{Cys}$ transcript was labeled at the 3′ end by T4 RNA ligase and [5′-^{32}P]pCp (NEN, 3000 Ci/mmol). Approximately 1 pmol of the preannealed labeled tRNA was incubated with 140 pmol of an oligonucleotide in the annealing buffer for 30 min at 37 °C. The complex of tRNA−oligonucleotide was treated with diethyl pyrocarbonate (DEPC) as previously described (Peattie & Gilbert, 1980; Ehresmann et al., 1987). Aniline-induced cleavage sites were detected by electrophoresis of the RNA fragments on a 12% denaturing polyacrylamide gel.

Nitrocellulose Filter Binding Assay. The nitrocellulose filter binding assay was adapted from previously published procedures (Yarus & Berg, 1967). The T7 transcript of *E. coli* $tRNA^{Cys}$ was labeled at the 5′ end by T4 polynucleotide kinase and [γ-^{32}P]ATP and purified by denaturing gel electrophoresis. The labeled tRNA (2 nM, 5000 cpm) was preannealed and added to a binding mixture of 100 μL that contained 60 mM sodium acetate, pH 5.0, 10 mM $MgCl_2$, and 50 mM NaCl with varying amounts of cysteine tRNA synthetase (0−320 nM). After incubation at room temperature for 15 s, the binding mixture was passed through a nitrocellulose filter paper (25 mm, Schleicher & Schuell) that had been previously washed with 0.4 M KOH for 40 min, rinsed with water twice, and equilibrated in wash buffer (60 mM sodium acetate, pH 5.0, 5 mM $MgCl_2$). The filter paper was washed with 2 mL of wash buffer, air-dried, and counted in 10 mL of scintillation fluor. The concentration of the enzyme at which 50% binding was observed is the apparent K_d.

RESULTS

Synthesis of 2′-O-Me Oligonucleotides. Table 1 lists all the oligonucleotides that were used in this study. They were synthesized with uniform 2′-*O*-Me substitution and a 3′ terminal acridine because these modifications have been shown to increase affinity for complementary RNA compared to unmodified DNA oligonucleotides (Asseline et al., 1984; Inoue et al., 1987; Sproat et al., 1993). We believed that this increased affinity would be necessary to compete with the secondary and tertiary hydrogen bond interactions in $tRNA^{Cys}$. Our goal was to test if these oligonucleotides can act as effective inhibitors that could bind to *E. coli* $tRNA^{Cys}$ in its native structure and inhibit the ability of the tRNA to be aminoacylated by the cognate cysteine tRNA synthetase.

Specific Inhibition of tRNA Aminoacylation by a 2′-O-Me Oligonucleotide That Binds to E. coli $tRNA^{Cys}$. We first synthesized oligonucleotide 01 to target G15 of *E. coli* $tRNA^{Cys}$ (Figure 1). The target sequence is from U8 to A26 and is symmetrical with regard to the D stem-loop of the tRNA (Table 1). The target sequence consists of 18 nucleotides and includes the critical G15. Except for C16 and U20, every nucleotide in the target is involved in a Watson−Crick or tertiary hydrogen base pair or base triple. We tested if oligonucleotide 01 could bind to *E. coli* $tRNA^{Cys}$ and inhibit aminoacylation. Figure 2 shows that, with 6 μM of the oligonucleotide to 3 μM of the tRNA, 01 formed a stable complex with *E. coli* $tRNA^{Cys}$ that could be detected

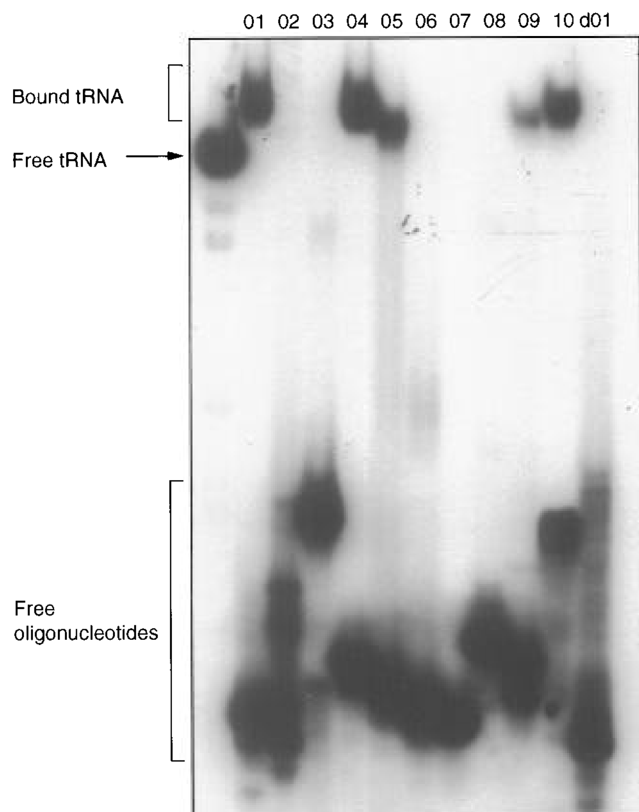

FIGURE 2: The binding of 2′-*O*-Me RNA oligonucleotides to *E. coli* $tRNA^{Cys}$ by gel shift analysis on a 12% native polyacrylamide gel. Oligonucleotides 01 to 10 were each labeled with ^{32}P and incubated with *E. coli* $tRNA^{Cys}$ transcript at a molar ratio of 2.4:1 in the annealing buffer at 37 °C for 30 min. The mixture of a given oligonucleotide−tRNA complex was applied to the gel, as indicated by the oligonucleotide number on the top. The last lane on the right (d01) shows the interaction between *E. coli* $tRNA^{Cys}$ and the deoxy base version of 01. The mobility of free oligonucleotide ranged widely due to various secondary structures under the nondenaturing conditions of the gel. The free and bound tRNA are indicated by arrows.

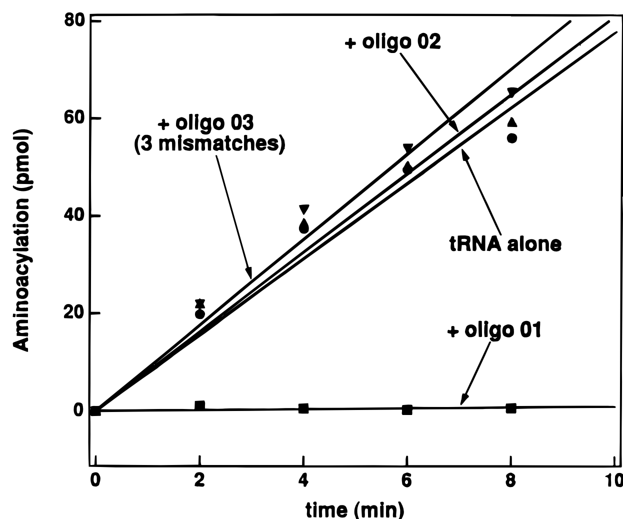

FIGURE 3: Specific inhibition of aminoacylation of *E. coli* $tRNA^{Cys}$ by 2′-*O*-Me oligonucleotides. Oligonucleotides 01−03 were each incubated with 3 μM *E. coli* $tRNA^{Cys}$ transcript at a 2:1 molar ratio in annealing buffer at 37 °C for 5 min. Aminoacylation was initiated by adding the assay mixture and the enzyme at a final concentration of 4 nM. Symbols: circles (tRNA alone); squares (tRNA + 01); triangles (tRNA + 02); drops (tRNA + 03).

in a gel shift assay. To form this complex, we incubated a previously denatured and properly reannealed T7 transcript of *E. coli* $tRNA^{Cys}$ with the oligonucleotide. Prior to the addition of the oligonucleotide, the preannealed $tRNA^{Cys}$ was an efficient substrate for *E. coli* cysteine tRNA synthetase. Its kinetic parameters of aminoacylation are $K_m = 3.0$ μM, $k_{cat} = 0.5$ s^{-1}, and $k_{cat}/K_m = 1.67 \times 10^5$ M^{-1} s^{-1} (Hamann & Hou, 1995). These kinetic properties of the preannealed $tRNA^{Cys}$ are independent of the heat/cool conditions and are similar to those of the native $tRNA^{Cys}$ isolated from *E. coli* (Hou et al., 1993). This suggests that the preannealed tRNA retained the proper tRNA tertiary structure. The minor kinetic difference between the preannealed tRNA and the native tRNA reflects the lack of modified nucleotides in the T7 transcript. The formation of the tRNA−oligonucleotide complex therefore indicates that the oligonucleotide was able to invade the tertiary structure of the tRNA. Thus, although G15 is in the core region of $tRNA^{Cys}$, a 2′-*O*-Me oligonucleotide that targets G15 had access to the tRNA in the native tRNA structure. However, while oligonucleotide 01 bound to *E. coli* $tRNA^{Cys}$, the same oligonucleotide with a deoxy backbone did not (Figure 2). This demonstrates that the 2′-*O*-Me modification of oligonucleotide 01 is necessary for binding to the tRNA.

To establish the specificity of oligonucleotide binding, we showed that an analogous oligonucleotide (02) complementary to the sequence from U8 to A26 in yeast $tRNA^{Cys}$ did not bind to *E. coli* $tRNA^{Cys}$ (Figure 2). This oligonucleotide, however, was capable of forming a stable complex with yeast $tRNA^{Cys}$ in a gel shift assay (data not shown). Between U8 and A26, yeast $tRNA^{Cys}$ differs from *E. coli* $tRNA^{Cys}$ in 9 nucleotides. This suggests that 9 mismatches between an oligonucleotide and its target abolished binding. Additional experiments showed that an oligonucleotide that contained three mismatches with the *E. coli* target sequence from U8 to A26 failed to bind to *E. coli* $tRNA^{Cys}$. This oligonucleotide, 03 (Table 1 and Figure 2), differs from the correct sequence (01) at positions 12, 15, and 21. Thus, as few as 3 mismatches between the 18-mer oligonucleotide and *E. coli* $tRNA^{Cys}$ eliminated binding.

We then tested if the binding of oligonucleotide 01 to *E. coli* $tRNA^{Cys}$ inhibited aminoacylation. By incubating 12 μM of 01 with 6 μM of the preannealed tRNA, we showed that 01 completely inhibited aminoacylation (Figure 3). Higher ratios of the oligonucleotide to tRNA (3:1, 4:1) also elicited this complete inhibition, whereas ratios below 2:1 conferred only partial inhibition. This indicates that, within the precision of the concentrations of the oligonucleotide and $tRNA^{Cys}$, a stoichiometric amount of the oligonucleotide is sufficient to completely block aminoacylation. Even when the tRNA (6 μM) was first allowed to form a stable complex with the synthetase (12 μM), the addition of the oligonucleotide (12 μM) to the tRNA−synthetase complex abolished aminoacylation (data not shown). In contrast, oligonucleotides 02 and 03 failed to inhibit aminoacylation under the same conditions (Figure 3). This established that the ability of an 18-mer 2′-*O*-Me oligonucleotide to bind to the tRNA and to inhibit aminoacylation are well correlated.

To quantitatively assess the inhibitory effect of 01, we determined the k_{cat}/K_m parameter of aminoacylation as a function of tRNA concentration in the presence of a 2-fold molar excess of 01. The k_{cat}/K_m parameter is the second-order rate constant, which determines the catalytic efficiency

of aminoacylation whether the substrate concentrations are saturating or subsaturating. The effect of 01 on aminoacylation can therefore be measured as the ratio of k_{cat}/K_m in the presence of 01 divided by k_{cat}/K_m in the absence of 01. This analysis showed that, under a range of tRNA concentrations from 0.2 to 16 μM, the addition of 01 at a 2:1 molar ratio to $tRNA^{Cys}$ completely abolished aminoacylation. This is indicated as 100% inhibition in Table 1.

The Minimal Length of a 2′-O-Me Oligonucleotide That Targets G15. To determine the minimal length of a 2′-*O*-Me oligonucleotide that targeted G15 of *E. coli* $tRNA^{Cys}$, we synthesized oligonucleotides 04 to 07 that were progressively shorter in length from 14 to 5 residues. We showed that the 5-mer (07) and 8-mer (06) did not bind to the tRNA, whereas the 10-mer (05) and 14-mer (04) did (Figure 2, Table 1). Each of these oligonucleotides was tested for its ability to inhibit aminoacylation at a 2:1 molar ratio to the tRNA. In Table 1, the k_{cat}/K_m value of aminoacylation is compared to that in the absence of the oligonucleotide. Both the 10-mer and 14-mer conferred 100% inhibition of aminoacylation, and thus their ability to inhibit aminoacylation is correlated with their ability to bind to the tRNA. Interestingly, although the 8-mer did not bind to the tRNA, it was still an effective inhibitor of tRNA aminoacylation (97% inhibition, Table 1). The ability of the 8-mer to almost completely inhibit aminoacylation suggests that it was able to form a complex with the tRNA but that this complex was not stable enough to survive through the gel shift assay. The ability of the 5-mer to confer 20% inhibition suggests that the interaction between the 5-mer and the tRNA is even weaker. Under modified gel shift conditions (such as 4 °C), it is possible that both 8-mer and 5-mer can bind to the tRNA. However, at ambient temperature, the minimal length of a 2′-*O*-Me oligonucleotide that targeted G15 of *E. coli* $tRNA^{Cys}$ and that achieved stable binding and complete inhibition was 10.

We also surveyed oligonucleotides that targeted other regions of *E. coli* $tRNA^{Cys}$. A 10-mer (09) complementary to the anticodon stem-loop and a 14-mer (10) complementary to the TΨC stem-loop both bound to the tRNA (Figure 2). However, while the 10-mer completely inhibited aminoacylation, the 14-mer only showed 60% inhibition (Table 1). This illustrates that the ability of an oligonucleotide to bind to the tRNA does not necessarily abolish aminoacylation. The binding of the 10-mer to the anticodon stem-loop of *E. coli* $tRNA^{Cys}$ will form a duplex with nucleotides from A37 to A46. This duplex will likely interfere with the ability of cysteine tRNA synthetase to interact with the adjacent A36 and G48 nucleotides that are critical for aminoacylation. In contrast, the TΨC stem-loop of *E. coli* $tRNA^{Cys}$ does not contain major nucleotide determinants for cysteine tRNA synthetase, and therefore its binding with the 14-mer has a less profound effect on aminoacylation. Interestingly, an 8-mer (08) which was designed to hybridize with the U73 major determinant in the acceptor stem did not bind to the tRNA and had only a 15% inhibitory effect on aminoacylation (Table 1, Figure 2). This is in contrast to the other 8-mer (06) which targeted G15 and almost completely inhibited aminoacylation. Thus, although G15 is in the most structured region of *E. coli* $tRNA^{Cys}$, and although U73 is adjacent to the single-stranded CCA end, G15 is a better target than U73 for inhibition of aminoacylation by oligonucleotides. These results, together with the fact that G15:G48 is unique to *E. coli* $tRNA^{Cys}$ while U73 and the GCA anticodon are conserved, further highlight the potential of G15 as a species-specific target for inhibition of tRNA aminoacylation. Additional experiments were carried out to characterize oligonucleotides that target G15.

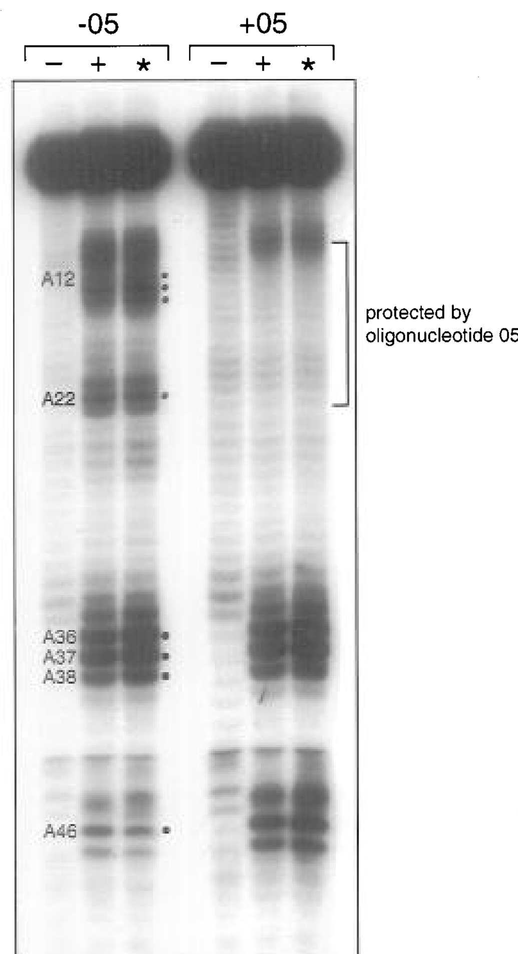

FIGURE 4: Protection of *E. coli* $tRNA^{Cys}$ from reaction with DEPC by oligonucleotide 05. ^{32}P-Labeled *E. coli* $tRNA^{Cys}$, free or bound with 05, was treated with no DEPC (−), with DEPC in 10 mM $MgCl_2$ (+), or with DEPC in 1 mM EDTA (*) for 10 min at room temperature. The chemical treatment was followed by aniline scission, ethanol precipitation of the tRNA, and electrophoresis on a 12% polyacrylamide/7 M urea gel at 2200 V until bromophenol blue ran to the bottom of the gel.

Binding of Oligonucleotides Protects E. coli $tRNA^{Cys}$ from a Chemical Probe. To prove that an oligonucleotide inhibits aminoacylation of *E. coli* $tRNA^{Cys}$ by binding specifically to the target sequence, we tested if the binding of the oligonucleotide protected the target sequence of the tRNA from a chemical probe. We characterized oligonucleotides 01, 04, and 05 that stably bound to G15 of $tRNA^{Cys}$ and inhibited aminoacylation. For this experiment, ^{32}P-labeled *E. coli* $tRNA^{Cys}$, either free or bound with an oligonucleotide, was treated with the chemical probe diethyl pyrocarbonate (DEPC) at a concentration that would generate statistically only one hit per tRNA molecule. DEPC attacks the N7 of A to form an aniline-sensitive adduct (Peattie & Gilbert, 1980). After aniline scission, the DEPC adduct on the tRNA can be identified by electrophoresis on a denaturing gel. By this method, nucleotides in the target sequence that are hydrogen bonded in a stable structure are expected to be protected from DEPC modification, whereas those in the single-stranded regions should be accessible. Figure 4 shows

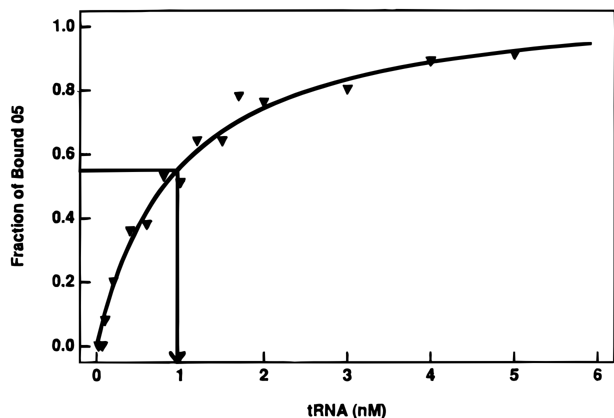

FIGURE 5: Determination of K_d for the tRNA^{Cys}−05 complex. Varying amounts of *E. coli* tRNA^{Cys} were incubated with 15 pM of ^{32}P-labeled 05 in the annealing buffer at 37 °C for 12 h. Each complex was analyzed by gel shift assay. The amount of bound oligonucleotide in each complex was determined by phosphorimage analysis.

the results of protection by oligonucleotide 05, which is a 10-mer that targets A12 to A22 in the D loop. In the native *E. coli* tRNA^{Cys}, A12−A14 and A22 were accessible to DEPC, as well as A36−A38 that are outside the target sequence. However, upon binding oligonucleotide 05 to the tRNA, A12−A14 and A22 were completely protected from DEPC whereas A36−A38 remained accessible. Additional nucleotides (such as A46) in the variable loop were also modified (the DEPC hits at the bottom of the gel when 05 was bound, Figure 4). This suggests that the binding of 05 to the tRNA denatured the tRNA structure somewhat to allow DEPC modifications of the variable loop. With oligonucleotides 01 and 04 that target longer sequences around G15, the site-specific protection of tRNA^{Cys} was also evident (data not shown). In all cases, protection was observed whether the tRNA−oligonucleotide complex was incubated in 10 mM $MgCl_2$ or 1 mM EDTA (e.g., Figure 4). This confirmed that these oligonucleotides specifically bound to the target sequence and that their binding protected the target sequence from DEPC modification.

Equilibrium Dissociation Constants and the Kinetics of Hybridization. To assess the stability of the tRNA−oligonucleotide complex, we determined the equilibrium dissociation constant for the complex formed by 01, 04, or 05. A concentration series of *E. coli* tRNA^{Cys} was incubated with a constant but limiting amount of the labeled oligonucleotide at 37 °C, and the distribution of free versus bound oligonucleotide was analyzed by a gel shift assay at pH 8.0. The conditions of the gel shift assay were such that a mismatched oligonucleotide and the tRNA did not form a complex by this analysis and that the detection of binding was unaffected by experimental variables such as the percentage of polyacrylamide in the gel (6−12%), the volume of sample loaded on the gel, or the electrophoresis time. To ensure that binding was at equilibrium, we established that the extent of binding was independent of incubation time (3−24 h) even at the lowest tRNA concentrations. Figure 5 shows the results for oligonucleotide 05 (at 15 pM). The fraction of the bound oligonucleotide increased as the concentration of the tRNA (from 0.02 to 5 nM) increased. This relationship can be fit to a theoretical binding curve that gives a K_d of about 1 nM (Table 2). This K_d corresponds to a binding free energy of −12.2 kcal/mol according to the equation $\Delta G° = -RT \ln(1/K_d)$, where R is the universal gas constant and T is the absolute temperature.

Table 2: Equilibrium Dissociation Constants and Rate Constants for Oligonucleotides 01, 04, and 05 Binding to Preannealed *E. coli* tRNA^{Cys}

oligonucleotide	length	K_d (nM)	k_{off} (s^{-1})	k_{on} (M^{-1} s^{-1})
01	18	nd	6.1×10^{-5}	nd
04	14	0.1	1.3×10^{-4}	1.3×10^{6}
05	10	1.0	3.7×10^{-4}	3.7×10^{5}

[a] Hybridization conditions are given in the text. Sequences within *E. coli* tRNA^{Cys} that hybridize with oligonucleotides are described in Table 1. Estimated errors are by a factor of 2. Association rate constants were calculated from the measured dissociation rates and the measured equilibrium constants.

The K_d of approximately 1 nM indicates a strong association between the oligonucleotide and the tRNA. This K_d is much smaller than those previously reported for tri- or tetraoligoribonucleotides that bind to a portion of the D loop of *E. coli* tRNA^{Tyr} (Uhlenbeck, 1972; Schimmel et al., 1972; Freier & Tinoco, 1975). The latter K_d's were determined by equilibrium dialysis and ranged from 25 μM to 2 mM. A more recent study of a series of oligoribonucleotides of 10-mer used the gel shift assay to determine K_d (Lima et al., 1992). These 10-mers were complementary to various portions of the stem-loop region of a structured Ha-ras RNA. The study showed that those complementary to the stem have K_d's in the range of 1 μM, whereas those complementary to the 5′ side of the loop have K_d's between 0.5 nM and 0.03 nM. Thus, depending on the structure of the RNA target sequence, K_d can differ by as much as 10^5-fold. In our study, the K_d of 1 nM of the tRNA−05 complex reflects that the binding of the 10-mer to the tRNA must compete against the secondary and tertiary structure that encompasses the target sequence in the D loop.

The K_d of the tRNA−04 complex was determined as 0.1 nM (Table 2). Thus, as the length of the oligonucleotide increased from a 10-mer to a 14-mer, the K_d decreased by 10-fold, indicating enhanced affinity for the tRNA. The gain of binding free energy from 10-mer (05) to 14-mer (04) is 1.4 kcal/mol. We were unable to determine the K_d of the tRNA−01 complex due to limiting specific activity of the labeled oligonucleotide.

The rate constants for dissociation (k_{off}) of tRNA complexes with 01, 04, and 05 were measured by competition experiments. The tRNA−^{32}P-labeled oligonucleotide complex was first formed by incubating 40 nM of labeled oligonucleotide with 200 nM tRNA and then was mixed with a large excess of unlabeled oligonucleotide (20 μM) that competed for the binding site. The decrease of labeled complex versus time was monitored by gel shift assays, and the fraction of bound oligonucleotide was fitted to the following first-order exponential decay equation.

$$\text{fraction of bound} = (e^{-k_{off}t})$$

The results showed that the dissociation rate constants for tRNA complexes with 01, 04, and 05 were, respectively, 0.61×10^{-4} s^{-1}, 1.3×10^{-4} s^{-1}, and 3.7×10^{-4} s^{-1} (Table 2). These rate constants are within the low end of the values measured by Lima et al. (1992), which ranged in k_{off} from 10^{-2} to 10^{-4} s^{-1}. Thus, in parallel with the trend observed for K_d, k_{off} decreased as the length of the oligonucleotide

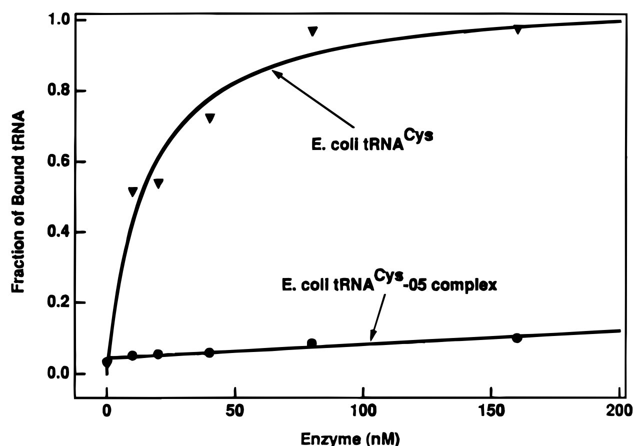

FIGURE 6: Retention of *E. coli* $tRNA^{Cys}$ and tRNA−05 complex on nitrocellulose filter pads by cysteine tRNA synthetase. ^{32}P-Labeled *E. coli* $tRNA^{Cys}$, free or bound with 30-fold molar excess of 05, was briefly incubated with a concentration series of cysteine tRNA synthetase (10−320 nM) in 60 mM sodium acetate, pH 5.0, 10 mM $MgCl_2$, and 50 mM NaCl. Incubation proceeded for 15 s to achieve equilibrium at room temperature, and the reaction mixture was filtered through a nitrocellulose membrane under mild vacuum. The bound tRNA retained on the filter pads was quantitated by scintillation counting.

increased. From these dissociation rate constants, the half-lives of tRNA−oligonucleotide complexes were calculated as 190, 92, and 31 min, respectively, for 01, 04, and 05. The bimolecular association rate constants (k_{on}) were calculated from the measured dissociation rate constants and the measured equilibrium constants according to the equation $K_d = k_{off}/k_{on}$. These constants are summarized in Table 2.

Mechanism of Inhibition of tRNA Aminoacylation by an Oligonucleotide. The tight affinity of 01, 04, and 05 with *E. coli* $tRNA^{Cys}$ raised the possibility that these oligonucleotides inhibited tRNA aminoacylation by preventing the tRNA from interacting with cysteine tRNA synthetase. To test this possibility, we asked if these oligonucleotides, when complexed with $tRNA^{Cys}$, eliminated the ability of the tRNA to bind to the cysteine enzyme in a nitrocellulose filter binding assay. We focused on 05, which is the shortest oligonucleotide (10-mer) of the three. We show in Figure 6 that free $tRNA^{Cys}$ could be quantitatively retained by the enzyme on nitrocellulose filter pads under the binding conditions (60 mM sodium acetate, pH 5.0, 10 mM $MgCl_2$, and 50 mM NaCl). Because the tRNA−synthetase complex is more stable at acidic than at neutral pH, we performed the binding assay at pH 5.0. We previously showed that, under the conditions of the assay, cysteine tRNA synthetase does not bind to the noncognate $tRNA^{Gly}$ and that $tRNA^{Cys}$ does not interact with the noncognate alanine tRNA synthetase. This established the specificity of the binding interaction. Curve fitting of the tRNA−synthetase interaction in Figure 6 shows that the apparent K_d of the tRNA−synthetase interaction was 15 nM, which is in accordance with the previously reported value of 12 nM (Ohannesian et al., 1996). However, the presence of a 2-fold molar excess of oligonucleotide 05 to $tRNA^{Cys}$ abolished the ability of the tRNA to be retained by the enzyme on filter pads. Even when $tRNA^{Cys}$ (0.5 μM) preformed a complex with the enzyme (0.5 μM), the addition of the oligonucleotide (1 μM) disrupted this complex. Thus, the mechanism of inhibition of tRNA aminoacylation by the oligonucleotide is attributed to the tRNA−05 hybrid that prevents the tRNA from making contact with the synthetase. The mechanism of inhibition by oligonucleotides 01 and 04 is most likely similar to that described for 05.

DISCUSSION

Although the nitrocellulose filter binding assay showed that the interaction between $tRNA^{Cys}$ and cysteine tRNA synthetase has a K_d of 15 nM, this does not reflect the interaction of the two at physiological pH. We estimate that the interaction between $tRNA^{Cys}$ and cysteine tRNA synthetase has a K_d on the order of 3 μM at pH 7.5. This is based on the Michaelis−Menten constant (K_m) of tRNA aminoacylation (Hamann & Hou, 1995). Because tRNA aminoacylation obeys Michaelis−Menten kinetics, the chemistry of aminoacylation is rate-limiting and K_m is an approximation of K_d (Schimmel & Söll, 1979). This estimation indicates a relatively weak interaction between $tRNA^{Cys}$ and cysteine tRNA synthetase at neutral pH. As a result, we could not directly measure the K_d for the $tRNA^{Cys}$−synthetase complex by the gel shift assay at pH 8.0 (data not shown). In contrast, we were able to use gel shift assays to determine the K_d for the tRNA−05 or tRNA−04 complex as 1 nM, or 0.1 nM respectively. This establishes that the stability of $tRNA^{Cys}$ binding to 04 and 05 is about 3−4 orders of magnitude stronger than the stability of $tRNA^{Cys}$ binding to the synthetase at physiological pH.

Earlier studies indicated that the bimolecular association rate constant (k_{on}) of a tRNA binding to its cognate synthetase is on the order of 10^8 M^{-1} s^{-1} (Schimmel & Söll, 1979), which is near the diffusion controlled limit. The initial tRNA−synthetase complex then undergoes a unimolecular conformational change that allows for a more precise reading of the bimolecular interaction. The dissociation rate constant (k_{off}) and the rate constant of the unimolecular conformational change are in the range of 10^2−10^3 s^{-1}. The overall rapid rate of dissociation of a tRNA−synthetase complex in principle is to facilitate tRNA turnover during protein synthesis. We show here that k_{on} of $tRNA^{Cys}$ binding to oligonucleotides 04 and 05 is on the order of 10^6 M^{-1} s^{-1}, whereas k_{off} is on the order of 10^{-4} s^{-1}. While k_{on} differs from that of tRNA−synthetase interaction by 100-fold, k_{off} differs by 10^6-fold. We conclude that the differences in k_{off} are largely responsible for the enhanced stability of tRNA−oligonucleotide complexes compared to the tRNA−synthetase complex.

The kinetic parameters of the tRNA−05 complex formation provide a useful framework for understanding the basis of inhibition. Whether $tRNA^{Cys}$ is free or in complex with cysteine tRNA synthetase, nitrocellulose filter binding assays showed that 05 is able to bind to $tRNA^{Cys}$ and inhibits the ability of the tRNA to interact with cysteine tRNA synthetase. Because the tRNA−05 complex dissociates slowly ($t_{1/2}$ = 31 min) relative to the time of the assay (10 min), inhibition of aminoacylation is achieved essentially by removing $tRNA^{Cys}$ from the pool that is available to cysteine tRNA synthetase. This was supported by experiments. At a defined tRNA concentration (e.g., a concentration equal to K_m), and with 05 at a series of concentrations below that of $tRNA^{Cys}$, we observed that the initial rate of aminoacylation is inversely proportional to the increase in the concentration of 05. This inverse relationship held until stoichiometric oligonucleotide was reached (data not shown).

Thus, to achieve complete inhibition of aminoacylation of $tRNA^{Cys}$, only a stoichiometric amount of 05 is needed. It is not necessary to use a large excess of the oligonucleotide. The intracellular concentrations of tRNAs and synthetases are both in the micromolar range (e.g., Putney & Schimmel, 1981; Swanson et al., 1988; Hou & Schimmel, 1989). This suggests that micromolar concentration of oligonucleotide 01, 04, or 05 inside a cell should be sufficient to achieve efficient inhibition of tRNA aminoacylation.

Increasing the length of the oligonucleotide increases the affinity for the target tRNA. For example, the K_d for the tRNA−04 complex is 10-fold lower than the K_d for the tRNA−05 complex. Although we were unable to determine the K_d for the tRNA−01 (18-mer) complex, we believe that it would be smaller than that of the tRNA−04 (14-mer) complex. However, higher affinity does not mean better specificity. Longer tRNA−oligonucleotide complexes would have a slower off rate, so mismatched hybrids formed with other RNA targets might not dissociate in time to allow discrimination (Hershlag, 1991). We show here that, despite the high affinity of 01 with the tRNA, 3 mismatches in 01 completely eliminate binding. This indicates good specificity even for the 18-mer. The 10-mer (05) has less binding free energy than the 18-mer and therefore will be more sensitive to mismatches. In addition, the 10-mer dissociates from the tRNA more rapidly than the 18-mer (Table 2) so that it will have an overall better discrimination than the 18-mer. This is supported by theoretical analysis of ribozyme kinetics (Hershlag, 1991). In eucaryotic cells, a stretch of 11−15 nucleotides is believed to define unique sequences for cellular RNAs (Hélène & Toulmé, 1990; Freier, 1993). The number of nucleotides that define unique sequences for bacterial RNAs should be even smaller. Thus, the 10-mer (05) is well within the kinetic, thermodynamic, and statistical boundaries that will ensure sufficient discrimination for targeting tRNAs in bacteria.

The strong affinities of oligonucleotides 01, 04, and 05 to *E. coli* $tRNA^{Cys}$ are largely due to the 2′-*O*-Me modification. The deoxyribose version of 01 did not even bind to $tRNA^{Cys}$ in the gel shift assay. Numerous studies have shown that the 2′-*O*-Me modification favors duplexes with an A-form structure (Sproat et al., 1993). Hence, 2′-*O*-Me oligonucleotides form highly stable hybrids with complementary RNA targets. As discussed above, one advantage of these stable duplexes is that inhibition of tRNA aminoacylation by oligonucleotides 01, 04, and 05 will be almost irreversible compared to the doubling time of *E. coli*. A second advantage of the 2′-*O*-Me modification is that it confers inhibition of aminoacylation independent of RNase H activity. Traditional antisense inhibition is achieved by RNase H hydrolysis of the RNA strand when it is in an RNA−DNA heteroduplex (Berkower et al., 1973; Crouch & Dinksen, 1982; Wagner, 1994). While 2′-*O*-Me oligonucleotides form more stable hybrids with RNAs, these hybrids are not substrates for RNase H (Inoue et al., 1987). As a result, the uniform 2′-*O*-Me modification of oligonucleotides reduces or eliminates the antisense activities of these compounds. In contrast, the hybrid formed between *E. coli* $tRNA^{Cys}$ and oligonucleotide 01, 04, or 05 is itself defective for aminoacylation. Therefore, RNase H should have no role in this application of antisense inhibition.

Native tRNAs in a cell contain modified bases. In *E. coli* $tRNA^{Cys}$, the target sequence of the 10-mer contains a dihyrouridine at position 21 (Steinberg et al., 1993). This modification is not at the Watson−Crick base pairing position and therefore is not expected to interefere with the ability of the 10-mer to bind to the tRNA. In general, only a subset of base modifications (such as N^2,N^2-dimethylguanosine) are at the Watson−Crick base pairing positions. The ability of a 2′-*O*-Me oligonucleotide to target a modified nucleotide in a tRNA remains to be tested.

Several 2′-*O*-Me oligonucleotides have been targeted to an RNA template to prevent reverse transcription by the avian myeloblastosis virus reverse transcriptase in vitro (Boiziau et al., 1995). The duplex between an oligonucleotide and the RNA template serves as a stable block that prevents cDNA synthesis. Additionally, 2′-*O*-Me oligonucleotides that bind to the splice junction of U2 snRNA inhibit splicing in the HeLa cell nuclear extract (Lamond et al., 1989). The ability of 2′-*O*-Me oligonucleotides to interfere with a biological function demonstrates the potential of these oligonucleotides as a class of pharmaceutical agents that specifically target RNAs. Recently, 2′-*O*-Me oligonucleotides have been successfully used to probe the in vivo structure of the splice leader RNA sequence of *Trypanosoma brucei* and *Leptomonas collosoma* (Harris et al., 1995). The oligonucleotides were delivered to the lysolecithin permeabilized cells.

The 2′-*O*-Me oligonucleotides described here can serve as a model for the design of antibiotics that target infectious pathogens which possess their own tRNAs. The strategy is to exploit species-specific nucleotide sequences of a pathogen so that the oligonucleotides will only bind to the pathogenic tRNAs but not to the host tRNAs. By inhibiting aminoacylation of a specific pathogenic tRNA, these oligonucleotides should be able to kill the infectious organisms. The 2′-*O*-Me modification enhances the nucelase resistance of oligonucleotides and provides the basis for the pharmaceutical sector to develop other related modifications (such as 2′-*O*-methoxyethyl) that confer complete resistance of oligonucleotides to either RNA- or DNA-specific nucleases (Sproat et al., 1993). These modified oligonucleotides therefore offer attractive potential for use as inhibitors of tRNA aminoacylation in vivo. Given these considerations, we attempted to introduce oligonucleotide 05 into *E. coli* to examine if it inhibited aminoacylation in vivo. *E. coli* cells were treated with $CaCl_2$ and incubated with the oligonucleotide under conditions that facilitated the uptake of plasmid DNA. However, while plasmid DNA migrated across the cell wall and cell membrane, the oligonucleotide remained in the periplasm (Hou, unpublished results). Thus, the primary challenge of the antisense strategy in bacteria is the efficient uptake of oligonucleotides. We are pursuing structural analysis of the tRNA−05 complex in order to elucidate its three-dimensional structure. This insight will facilitate the development of antibiotics that are potent and specific and can be delivered to bacteria.

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