
Hybridization and dissociation rates of phosphodiester or modified oligodeoxynucleotides with RNA at near-physiological conditions.

Sharon Young and Richard W. Wagner*

Gilead Sciences, Inc., 346A Lakeside Drive, Foster City, CA 94404, USA

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ABSTRACT

We have used RNase H to study both the rates of oligonucleotide hybridization and dissociation at near-physiological conditions. We have studied the Effects of oligonucleotide length, mismatch, and chemical modifications on oligonucleotide association and dissociation with RNA. Dissociation results were compared with standard thermal melting curves to compare relative stabilities evaluated by the two techniques. Although generally the two techniques correlate for the compounds evaluated, we found several instances where the thermal melting curves failed to reflect the relative stability of different oligonucleotides at 37°C using near-physiological conditions. This study suggests that direct measurement of hybridization and dissociation of an oligomer with RNA more accurately assesses the complicated kinetic scheme at 37°C using near-physiological conditions than thermal melting curves would predict.

INTRODUCTION

The development of oligonucleotides as potential therapeutics is founded on the specific interaction of a DNA molecule with its cognate nucleic acid [for review, see (1–4)]. Specifically, in the case of antisense DNA technology, the goal is to complex a target (sense) messenger RNA with a complementary (antisense) DNA oligomer. There are three central hurdles in the therapeutic development of oligonucleotides: 1) sequence specific interaction; 2) nuclease stability; and 3) cellular delivery (2). To date there has been relatively little consensus relative to the length of the oligonucleotide needed to achieve specific and stable interaction. Furthermore, most data related to oligonucleotide stability is based on melting temperature experiments (5–7). Similarly the stability of mismatched oligonucleotide/RNA duplexes at physiological conditions is not well understood. To increase the strength of hybridization, various researchers have chosen to use various intercalator appendages on oligonucleotides. On the basis of T_m data, these modified oligonucleotides form more stable duplexes than the corresponding diester control (8–11). Nuclease

stability is an issue which has been addressed in several ways. The primary serum nuclease is a 3'-exonuclease (12). Various backbone modifications including methylphosphonate, phosphorothioate, and phosphoramidate linkages block the exonuclease (12). Melting temperature experiments suggest that the hybrid duplex formed between these modified oligomers and RNA or DNA should be relatively stable at 37°C (6, 12), however the relative stability of the complexes at 37°C at near-physiological conditions has not been shown. Cellular delivery of oligonucleotides is an area which has paralleled the nuclease stability issue. Various proteins such as poly-lysine (13) or EGF (14), or lipophilic groups such as lipids (12, 15, 16) or cholesterol (17, 18), or increased hydrophobic backbone modifications such as methylphosphonate (19) or phosphoramidate (20) linkages, have been just a few of the approaches to enhance permeation. Again, most of the data supporting the relative binding efficiency of these linkages relies on the T_m measurements (6).

During the development of several novel modified oligonucleotides, we wished to compare the relative thermal melting stability of particular conjugates with an assay which would directly measure relative association and dissociation rates using hybridization conditions which were near-physiological. Oligonucleotide hybridization has previously been studied using several methods including filter hybridization (26), temperature-jump NMR experiments (27), S1 (28) and RNase H nuclease analysis (29–33), and melting temperature (T_m) measurements (5–7, 34, 35). We chose RNase H to compare oligonucleotide hybridization and stability with melting temperature measurement for the following reasons: a) a near-physiological conditions would be achieved and b) due to the selectivity of RNase H for oligodeoxynucleotide/RNA hybrid we could easily monitor site selective hybridization using gel electrophoresis.

In the oligonucleotide association assay, ^{32}P -radiolabelled RNA (labelled throughout) together with excess unlabeled calf liver RNA was incubated at time 0 with a complementary oligonucleotide in a near-physiological buffer cocktail. At various timepoints an aliquot of the hybridization mixture was incubated with RNase H to completion to specifically hydrolyze the annealed RNA. The products were then analyzed using polyacrylamide gel electrophoresis and autoradiography. For

* To whom correspondence should be addressed

measuring oligonucleotide dissociation from RNA a similar method was used. In this case the oligomer was annealed to completion with the RNA. This complex was treated as the 'time 0', at which point the complementary 'sense' oligonucleotide was added in an enormous excess to complex both unbound and dissociating 'antisense' oligomer. Aliquots were taken at various time intervals, digested with RNase H and analyzed as described above. Using this assay we demonstrate rates of hybridization and dissociation for varied-length, mismatched, and chemically-modified oligonucleotides to RNA. We also demonstrate complicated kinetic parameters for a number of oligonucleotide/RNA interactions.

MATERIALS AND METHODS

Ribonucleotides, poly(U) and poly(A) RNAs, RNase-free BSA, and RNase H were purchased from Pharmacia. T7 and SP6 polymerases, RNase inhibitor, and restriction endonucleases were purchased from Promega. Calf liver RNA, RNase-free MgCl₂, molecular biology grade Tris base, KCL and mercaptoethanol were purchased from Sigma Chemicals. Ribonucleotide cap analogues were purchased from New England Biolabs. Radionucleotides were purchased from Amersham Corp.

Oligonucleotide Synthesis

Phosphodiester oligodeoxynucleotides and phosphorothioates were prepared as described (21). Oligodeoxynucleotides containing methoxyethylphosphoramidate or C₁₂-phosphoramidate linkages were synthesized using hydrogen phosphonate chemistry (12). Anthraquinone-derivatized oligonucleotides were prepared as described (40). Oligonucleotides were purified either by gel electrophoresis or reverse phase HPLC and were precipitated, pelleted, and dissolved in sterile water.

Preparation of RNA for Thermal Melting Temperature Determination (22)

The RNA templates and primer oligonucleotides were synthesized using hydrogen phosphonate chemistry and were HPLC purified. RNA template (1nM) and primer (1nM) were placed together in 40mM Tris (pH 7.5), 6mM MgCl₂, 2mM spermidine, 10mM

DTT and denatured in 80°C for 5 minutes and were then allowed to renature on ice for 15 minutes. The reaction volume was increased to 5ml and 4mM NTP, 20 mM MgCl₂, 30,000 U/ml T7 polymerase were added and incubated at 37°C for 4.5 hours. The reaction was terminated with 50mM EDTA, and the sample was phenol/chloroform extracted, then chloroform extracted. To remove salts and free NTPs, the RNA was batch absorbed onto a 1ml bed volume of a DEAE-anion exchange resin, fractogel, TSK-3000 (Supelco), washed twice with H₂O, then eluted with 1M NaCl, 10mM Tris (pH 7.5), and 1mM EDTA. The RNA was precipitated with 3 vol ethanol overnight at -20°C. The RNA was pelleted and washed with 70% ethanol. The RNA was then purified using polyacrylamide gel electrophoresis. Total yield was 3.5 O.D.

Thermal Melting Temperature Determination of Oligodeoxynucleotide Duplexes

Absorbance transitions of DNA/RNA or DNA/DNA hybrids v. temperature were recorded at 260nm using a Gilford Response II Temperature Controlled Spectrophotometer. All thermal denaturation solutions contained 0.1M NaCl, 1mM EDTA, 10mM Na-phosphate, pH 7.2. Each strand was present at 2μM. DNA/RNA or DNA/DNA mixtures were heated at 90°C for 5 min. and cooled to room temperature. The cell compartment was warmed from 25° to 85°C in 0.5°C increments. The reported T_m's are the temperatures at which one-half of the duplex fraction denatured. Reported T_m's are the average of two independent experiments and results have an error of ±0.5°C.

Plasmid Construction

pGEMCAT was a generous gift from Jeng-Pyng Shaw (Gilead Sciences). A 1.6 Kb fragment of the gene coding for chloramphenicol acetyl transferase (CAT) was cleaved from pSV2CAT (39) with restriction endonucleases HindIII and BamHI. The isolated DNA was next cloned into pGEM4Z (Promega) at the HindIII and BamHI sites.

pSP65Pro2 was a generous gift from Richard A. Maurer (University of Iowa). This plasmid was derived from the cDNA insert in pPRL-2 (23) which consists of portions of the rat prolactin cDNA. Poly(A) segments and the G:C tails used in the original plasmid construction were deleted, EcoRI linkers added and the DNA was cloned in the EcoRI site of the pSP65 plasmid.

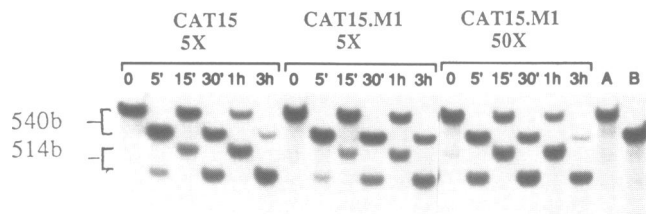


Figure 1. Detection of oligonucleotide on-rate. 50fmol CAT RNA (540 b) together with 10μg calf liver RNA and either 250fmol (5×) or 25nmol (50×) of CAT15 or the mismatched CAT15.M1 oligonucleotide were mixed at time 0 and were allowed to hybridize for the indicated times at 37°C. At each time point a 100-fold molar excess of sense oligonucleotide (to the CAT15 oligomer) was added and the mixture was digested with RNase H. EDTA was added to stop the reaction, the sample was then heated to 90°C for 5 minutes in 90% formamide. The samples were electrophoresed in a denaturing 8.3M urea-4% polyacrylamide gel. To aid cutting out the bands for quantitation, lanes were loaded in a staggered fashion where every odd lane was loaded to 30' prior to the even lanes. The resultant RNA species are approximately 514nt and 11nt in length. This gel system only resolves the 514nt band. Lane A included zero CAT15 during the 3h incubation; Lane B included CAT15 at the 50× concentration for 3h but was not RNase H digested.

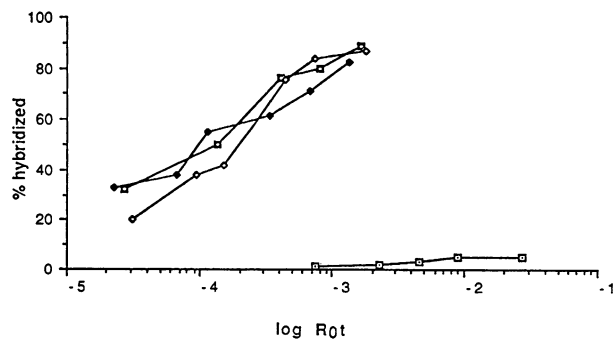


Figure 2. Hybridization of varied lengths of oligonucleotides to CAT RNA. The annealing reaction and subsequent quantitation of the hybridized complex was carried out as described in Figure 1 and the Materials and Methods. % hybridized band values represent one of two independent hybridization experiments. The error in the hybridized band values is ±5%. □, CAT10; ◆, CAT15; △, CAT18; ●, CAT21.

RNA Synthesis

Capped sense RNAs were transcribed *in vitro* using SP6 or T7 polymerase according to the method described previously (24, 25). The reaction contained 40mM Tris (pH 7.4), 6mM MgCl₂, 2mM spermidine, 0.5mM each of ATP, CTP, GTP, and UTP, 2.5mM GpppG, 40 U of SP6 or 200 U of T7 polymerase, 60 U of RNasin and 3μg of plasmid DNA linearized either with NcoI (pGEMCAT) or SalI (pSP65Pro2). 100μCi (α-³²P)UTP was included to label the RNA to a specific activity of (8×10⁵ CPM)/μg.

Hybridization Assay

50fmol of labelled CAT RNA together with 10μg calf liver RNA were heated at 100°C in 5μl H₂O. Hybridization buffer (50mM Tris, pH 8.0, 9mM MgCl₂, 20mM KCl, 1mM β-mercaptoethanol) was added and the reaction was equilibrated for 10 minutes at 37°C. Oligodeoxynucleotide was added to the mixture at the indicated concentration (see figure legends) at time 0 and hybridized at 37°C. At various time points, a 100-fold molar excess of sense oligonucleotide (complementary to antisense oligonucleotide) was added together with 25μg BSA, and 0.1 U RNase H and the mixture was reacted for 30 min at 37°C. The sense oligonucleotide was added to prevent re-hybridization of the antisense oligomer which is released during the RNase H hydrolysis reaction. EDTA was added to a final concentration of 50mM, the sample was heated to 90°C for 5 minutes in 90% formamide and the samples were electrophoresed in a denaturing 8.3M urea-4% (40: 1.6 acrylamide to bis-acrylamide ratio) polyacrylamide gel. The digestion products were visualized by autoradiography. As a control for all oligonucleotides tested, we hybridized a 1000-fold molar excess of oligomer to the RNA and digested the duplex as described above. In this way we confirmed that the RNase H digestion went to completion. The samples were quantitated by cutting out each band and counting them by liquid scintillation spectroscopy. %RNA hybridized = [(cpm of hybridized band) ÷

(cpm of hybridized band + cpm of unhybridized RNA)] × 100. All values were background subtracted. Because the RNA was labelled throughout the polymer, RNA which was digested away or which was too short for efficient recovery was corrected in the 'hybridized band' value. % Hybridization vs. Log [moles of oligodeoxynucleotide/L × seconds] was plotted for hybridization rates.

Hybrid Stability Assay

Oligonucleotides and RNA were hybridized as described above. At time 0, a 100-fold molar excess of sense oligodeoxynucleotide was added, and the mixture was incubated at 37°C for the indicated time points. Aliquots were taken and RNase H digestion, gel electrophoresis and quantitation were done as described above. Stability was plotted as % RNA hybridized vs. time (hours).

RESULTS

Oligonucleotide Hybridization to RNA

Figure 1 shows a typical hybridization experiment which detects the annealing of a 15 nucleotide-long phosphodiester oligodeoxynucleotide to complementary CAT RNA. The oligomer overlaps the AUG initiation codon (see Table 1). The hybridization was carried out at 37°C using a 50-fold molar excess of unlabeled competitor calf liver RNA, which in pilot experiments was shown not to interfere with the 15-mer oligonucleotide hybridization (data not shown). We compared the rate of hybridization for a 10, 15, 18, or 21 nucleotide fragment to CAT RNA. Oligonucleotide hybridization was quantitated by digesting the RNA/DNA hybrid with RNase H and analyzing the products by electrophoresing them on a polyacrylamide gel. RNase H selectively hydrolyzes the bound RNA. In the case of CAT RNA (540 b) the resultant products are approximately 510 and 15nt in length when the CAT15, CAT18, or CAT21 oligomers are bound and the duplex is

CAT RNA (Total length=540nt)	
5'...(6)-GCT AAG GAA GCT AAA ATG GAG AAA AAA ATC-(36)... 3'	
	————— CAT10
	————— CAT15
	————— CAT18
	————— CAT21
CAT OLIGOMERS	
CAT10	5'-CTC CAT TTT A
CAT15	5'-CTC CAT TTT AGC TTC-3'
CAT15.M1	5'-CTC CAT TTT AGG TTC-3'
CAT15.M2	5'-CTG CAT TTT AGG TTC-3'
CAT18	5'-CTC CAT TTT AGC TTC CTT-3'
CAT18.M1	5'-CTC CAT TTT AGG TTC CTT-3'
CAT18.M2	5'-CTG CAT TTT AGG TTC CTT-3'
CAT21	5'-CTC CAT TTT AGC TTC CTT AGC-3'
CAT21.M1	5'-CTC CAT TTT AGG TTC CTT AGC-3'
CAT21.M2	5'-CTG CAT TTT AGG TTC CTT AGC-3'
PROLACTIN RNA (TOTAL LENGTH=823nt)	
5' (37)- CCA GTG GTC ATC ACC ATG AAC AGC CAG GTG-(66) 3'	
	————— PRO15
PROLACTIN OLIGOMER	
PRO15	5'-GCT GTT CAT GTT GAT-3'

Table 1. Portions of target RNA and sequences of oligonucleotides.

Oligonucleotide	T _m (°C) with RNA	T _m with DNA
CAT10	ND	28.5
CAT15	51.5	50.0
CAT15.M1	38.0	ND
CAT15.M2	----*	ND
C12-CAT15	ND	45.5
S-CAT15	ND	38.0
3'ANQ-CAT15	ND	49.0
CAT18	59.0	55.0
CAT18.M1	55.0	ND
3'MEA-CAT18	57.0	ND
3'ANQ-CAT18	61.0	ND
S-CAT18	43.0	ND
C12-CAT18	55.5	ND
CAT18.M1	49.5	ND
CAT18.M2	35.0	ND
CAT18.S3	ND	54.5
CAT21	62.5	ND
CAT21.M1	56.0	ND
CAT21.M2	44.5	ND
PRO15	ND	55.0
3'ANQ-PRO15	ND	59.0
C12-PRO15	ND	53.0
MEA-PRO15	ND	54.5

Table 2. Thermal melting temperature (T_m) of selected oligonucleotides hybridized to either RNA or DNA. "C12-" = two 3'-terminal dodecylamide backbone linkages in an otherwise phosphodiester oligonucleotide; "S-" = phosphorothioate backbone linkages at every phosphate position; "MEA-" = two 3'-terminal methoxyethylamide backbone linkages in an otherwise phosphodiester oligonucleotide; "3'ANQ-" = a single anthraquinone attached to the 3'-terminal end of a phosphodiester oligonucleotide. ND = not determined; ----* = not detectable.

digested. These sizes are approximate since RNase H does not hydrolyze the RNA/DNA complex at every bound RNA base (data not shown). Using our gel system only the larger (~ 510 nt) product is observed. We found that the 10-nucleotide-long fragment has a rate of hybridization at least three orders of magnitude less than the longer oligomers (Figure 2A). The CAT15, CAT18, and CAT21 oligonucleotides however each bound to their target sequence with relatively similar rates (Figure 2A). Therefore we could conclude that the hybridization of the 10-mer probably did not bind either because its off-rate was so fast that the on-rate was not detectable using this assay or because the 10-mer was binding to other sites. However, above a critical length the 15–21 nucleotide-long oligomers bound at similar rates in a time-dependent manner, demonstrating that for these lengths the on-rate is detectable.

To compare hybridization rates of oligomers targeted to a different RNA, we chose a 15-mer directed against the AUG initiation codon of rat prolactin RNA. This oligonucleotide had a similar base composition and T_m relative to CAT15 (Table 1 and 2). Surprisingly, we found that the hybridization rate for this oligonucleotide was about four-fold slower than CAT15 (results not shown). We hypothesized that nucleic acid secondary structure might be the hindrance to hybridization. Therefore we heated the mixture of oligonucleotide together with the prolactin RNA to 95°C and allowed the mixture to react. We found that the Pro15 and CAT15 oligos hybridized at nearly identical rates (data not shown). Therefore we could conclude that nucleic acid secondary structure may influence the rate of hybridization at 37°C.

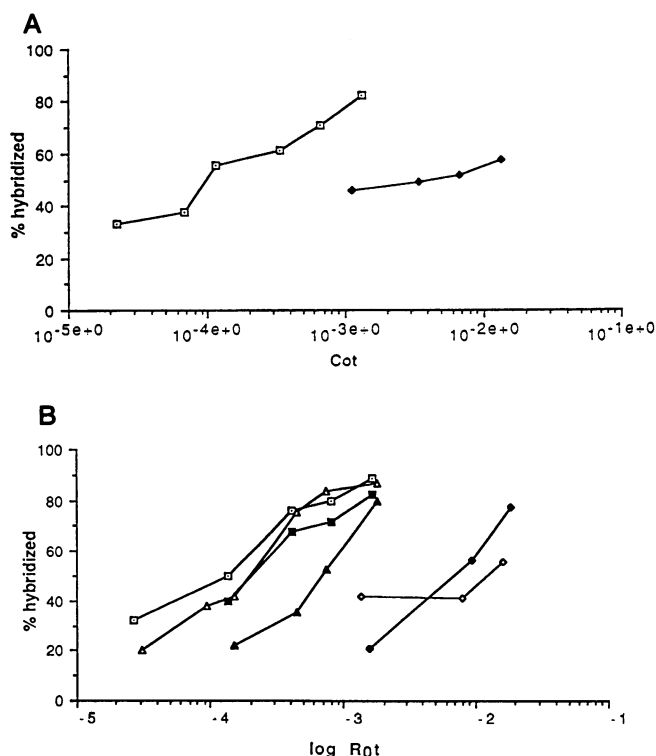


Figure 3. Hybridization of oligonucleotides containing mismatches. The annealing reaction and quantitation were carried out as described in Figure 1 and in Materials and Methods. (A.) Association rates of CAT15 and CAT15 containing a single mismatch. \square , CAT15; \blacklozenge , CAT15.M1. (B.) Association rates of CAT18 and CAT21 containing mismatches. \square , CAT18; \blacksquare , CAT18.M1; \blacklozenge , CAT18.M2; \triangle , CAT21; \blacktriangle , CAT21.M1; \blacklozenge , CAT21.M2.

Effects of Mismatches on Hybridization

We next looked at the effects of mismatches on oligonucleotide hybridization. For this study we continued to use the CAT RNA sequence. We examined the effect of a single or double mismatch at a common site in the 15, 18, and 21-mer series, previously evaluated. A single mismatch (.M1) was at the center of the 15-mer oligomer in which we changed a C to a G causing a G-G mismatch; a double mismatch (.M2) also included a G-G mismatch at the 5' end of the DNA fragment. As shown in Figure 1 and 3A we found that a single mismatch in the center of an oligonucleotide impedes the hybridization rate of a 15-mer greater than an order of magnitude compared to the perfectly matched control. The same mismatch in the 18-mer (CAT18.M1; Figure 3B) and 21-mer (CAT21.M1; Figure 3B) also had decreased hybridization rates, but the effect was less than 10-fold. We next looked at the effects of two internal mismatches. A 15-mer with the two internal mismatches would not hybridize at all using the present hybridization conditions. The longer 18- and 21-mers with two mismatches hybridized at rates 5–10 fold slower than their perfectly matched controls (Figure 3B). Therefore we conclude that the mismatches we have chosen can significantly affect the hybridization of oligonucleotides to RNA at 37°C. Furthermore this effect is length-dependent: a 15-mer with one base mismatch is slower to hybridize than a 18-mer with the same mismatch. The fact that mismatches actually influenced the 'on-rate' (rate of collision of an oligomer with a given RNA strand) at first glance seemed to be a puzzle. If one compares the T_m 's for these oligos (Table 2), one would predict that an increase in size affects the T_m of mismatched oligonucleotides through the 'off-rate', approximately 10 degrees per mismatch given these particular oligonucleotides. However we are observing an effect on the 'on-rate.' In the 10–15-mer range, the effect we see is probably due to the limitation of the assay to distinguish on-rate vs. off-rate for events that happen very rapidly, that is when both the on-rate and off-rate occur within less than 5 min. As will be demonstrated, for the longer-length oligomers, 18–21nt, the complexes formed between the DNA and RNA are quite stable. An explanation for the observed result is that insertion of a mismatch actually causes an interference so that even if a proper alignment is achieved during a DNA/RNA collision, the complex is not always successfully zippered.

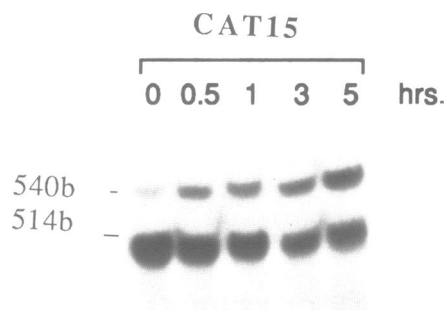


Figure 4. Detection of oligonucleotide/RNA dissociation. CAT15 was hybridized to CAT RNA for 3h using a 20-fold excess of oligomer to RNA at time 0 a 100-fold excess of sense oligomer (to antisense oligomer) was added, and the mixture was incubated at 37°C for the indicated time points. Aliquots were taken and the mixture was digested with RNase H. Electrophoresis and band quantitation were carried out as described in Figure 1.

Stability of the RNA/DNA Hybrid

In studying the rate of hybridization of oligonucleotides to RNA, we additionally wished to study the stability of the duplex once formed. To do this we developed a technique to monitor oligo dissociation. Oligonucleotide and RNA were hybridized using an excess of the oligomer. At Time 0, a 100-fold molar excess (relative to antisense) of sense oligo was added to anneal with unhybridized and dissociating oligonucleotide. RNase H was added at the indicated times and the products were analyzed. As a control, the preformed duplex of CAT15 and CAT RNA was heated to 90°C for 5 min in the presence of a 100-fold excess of sense oligomer prior to an 18h incubation of the mixture at 37°C. Subsequent RNase H digestion of the mixture revealed zero binding of antisense oligomer to RNA (data not shown). Therefore, we concluded that the sense oligonucleotide effectively complexes antisense oligomer within detectable limits. Figure 4 shows a typical dissociation rate experiment and Figure 5 plots the percentage RNA hybridized vs. time in hours using CAT RNA and the 15-mer oligonucleotide. We found that the stability of the 15-mer was approximately 5 to 10-fold less than the 18-mer, having a dissociation of $T_{1/2} = \sim 5$ h for the 15-mer vs. > 30 h for the 18-mer (Figure 5). Surprisingly, the 21-mer was less stable than the 18-mer, having a $T_{1/2}$ of about 25 hours (Figure 5). Since the 21-mer has a T_m of 62.5°C vs. 59°C for the 18-mer (Table 2), it was expected that the 21-mer should be more stable than the 18-mer. To investigate this finding we made a second 18 nucleotide oligomer which shifted the CAT18 sequence three nucleotides downstream (CAT18.S3). This shift, therefore, also covered the same binding site as the CAT21 sequence. The hybridization rate of CAT18.S3 was similar to CAT18 and CAT21 (data not shown). However, the off-rate of CAT18.S3 (Figure 5) was faster than CAT21 and CAT18. Taken together, the off-rate of CAT18 versus CAT21 was the first instance in which we failed to draw a direct correlation between melting temperature and 37°C dissociation measurements. By shifting the 18-mer three nucleotides, we were then able to restore the expected relative stability for an 18-mer versus a 21-mer. Therefore we conclude that other factors may influence the off-rate of oligonucleotides. One explanation may be that dissociation of the complex may go through a reversible intermediate, such as a hairpin, which affects CAT18 less than CAT18.S3 or CAT21.

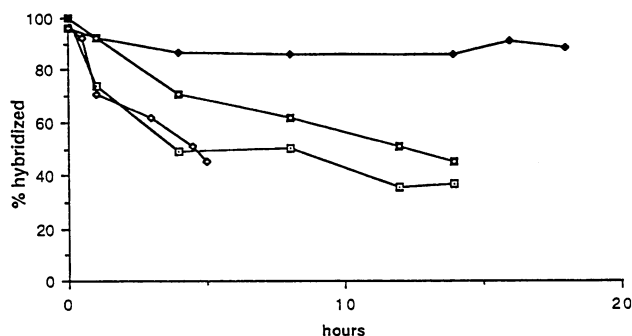


Figure 5. Dissociation rates of oligonucleotides of varied lengths. Oligonucleotides were hybridized to CAT RNA and allowed to dissociate as described in Figure 3. Quantitation of hybridized bands were carried out as described in Materials and Methods. Dissociation rates represent one of two independent reactions. % hybridized band values have an error of $\pm 5\%$. \square , CAT15; \blacklozenge , CAT18; \circ , CAT18.S3; \triangle , CAT21; \blacksquare , CAT18.M1.

Effects of Mismatch on Oligo Stability

Similar to the finding that mismatches reduce the rate of oligo association to target RNA, we found that mismatches decreased the stability of oligomer/RNA hybrids. Furthermore the effect of mismatch on off-rate was length-dependent (Figure 6A). That is, the rate of dissociation of a 15-mer with one mismatch was approximately 40-fold less stable than the perfect complement; however, the singly mismatched 21-mer was only about 6-fold less stable (Figure 6A). We analyzed a series of 16, 17, and 18-mer oligonucleotides with the same mismatch, and these followed the same trend (data not shown). The effect of two mismatches was even more drastic. The 18-mer and 21-mer oligonucleotides dissociated from the RNA at a $T_{1/2}$ of about 5 minutes, and the 15-mer was not detectable (Figure 6B). We concluded that the effect of adding a mismatch to an oligonucleotide/RNA hybrid significantly destabilizes the duplex at 37°C and the degree of destabilization is length dependent. The instability of these complexes seem to be influenced at both the level of on-rate and off-rate.

Effect of Conjugates on Oligonucleotide Hybridization to RNA

To use oligonucleotides as potential therapeutics there has been an enormous amount of effort to modify oligonucleotides to increase their serum and general nuclease stability, lipophilicity, and hybridization efficiency. We have found that two 3'-terminal methoxyethylphosphoramidate linkages increase the serum stability while only lowering the T_m by about 1 to 2°C (see Table 2, (12)). To increase the lipophilicity of oligonucleotides, we have also found that by attaching two dodecylamidate linkages

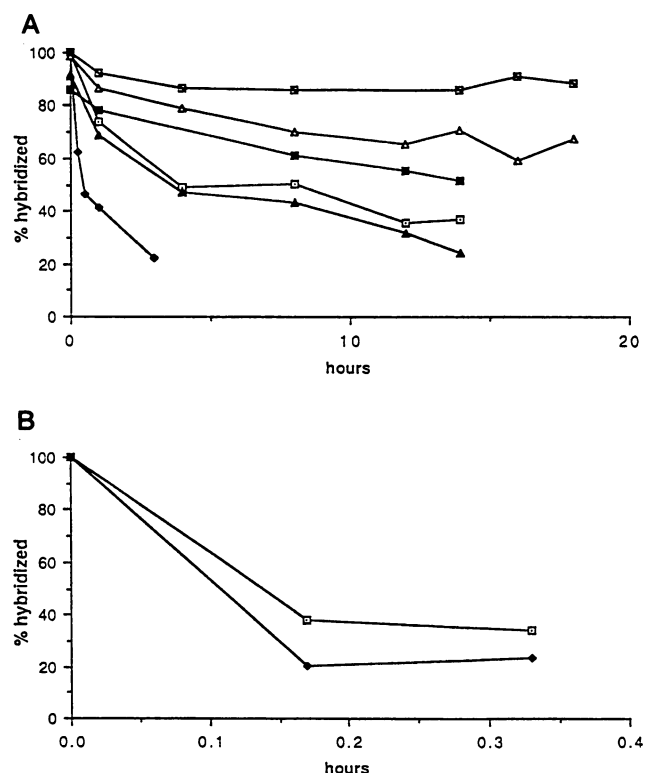


Figure 6. Dissociation rates of oligonucleotides containing a single mismatch. See Figures 4 and 5 and Methods for details. (A.) \square , CAT15.M1; \blacksquare , CAT18.M1; \triangle , CAT21.M1; \blacklozenge , CAT18.M2. (B.) Note time scale change. \square , CAT18.M2; \blacklozenge , CAT21.M2.

in place of the methoxyethylphosphoramidate linkages, the cell association of the oligonucleotide increases 10–20 fold (12). The C₁₂-conjugate shows a decrease in T_m by 2° to 4°C compared to diester. Thirdly, we have found that the addition of anthraquinone to the terminal 3'-end of an oligonucleotide both aids in serum stability (40) and also increases the T_m by 2° to 4°C (Table 2).

We next addressed the question whether modified oligonucleotides affect either the rate of hybridization or duplex stability using these different conjugates. For our studies we chose the CAT15 and CAT18 oligonucleotides, derivatized with all phosphorothioate linkages, two 3'-terminal methoxyethylphosphoramidate (3'-MEA) or C₁₂-phosphoramidate (3'-C₁₂) linkages, and 3'-anthraquinone (3'-ANQ) oligonucleotides.

Our results showed that the 3'-MEA-CAT18 and 3'-ANQ-CAT18 oligodeoxynucleotides each hybridized at rates 2 to 3-fold less than the CAT18 phosphodiester (Figure 7A). The S-CAT18 hybridized 4-fold slower and the C₁₂-CAT18 hybridized 8-fold slower than the diester control (Figure 7A). We next checked the rates of hybridization for the 3'-ANQ-CAT15, C₁₂-CAT15, and S-CAT15 oligomers. For this series, the 3'-ANQ-CAT15 hybridized at a similar rate compared to diester, the thioate sequence was 6–8-fold slower, and the C₁₂ sequence was about 5-fold slower (Figure 7B). The 15-mer series was repeated with prolactin conjugates and similar results were obtained (data not shown). It is likely that with the 3'-modified oligonucleotides, the decreased on-rate reflects an altered secondary structure of the oligonucleotide. For backbone effects, this could be due to alignment problems, secondary structure, steric hindrance, or

hydration differences between the modified backbone and the phosphodiester backbone.

We next checked the stability of the conjugate/RNA hybrids. We found that the conjugates had the following rank order of stability for CAT18: diester > MEA = anthraquinone > C₁₂ ≥ thioate (Figure 8A). Similar results were obtained for a series of Pro15 conjugates (data not shown). Paralleling the results with the phosphodiester oligonucleotides, 15-mer conjugates were 10-fold less stable than their corresponding 18-mer conjugates (Figure 8B). Surprisingly, the 3'-anthraquinone did not seem to affect the hybrid stability for both the 15 and 18 nucleotide-linked fragments (Figure 8A and 8B). On the basis of T_m data, it was expected that the hybrid would be more stable. This was another example where near-physiological, 37°C hybridization did not parallel the expected relative stability indicated by T_m measurement. Interestingly, the dissociation rate of the C₁₂-phosphoramidate-linked 15-mer and 18-mer oligonucleotides dissociated only about 2-fold faster than the diester even though the association rate of these conjugates was impeded (Figure 8A and 8B). Therefore it seems that once formed, the C₁₂-oligomer/RNA duplex is nearly as stable as a diester/RNA-hybrid at 37°C.

We conclude that altering the chemical composition of an oligonucleotide may change the hybridization efficiency and stability of the oligonucleotide. In certain instances, the stability does not seem to directly parallel that predicted by T_m measurements. At 37°C using near-physiological salt conditions, an oligonucleotide with an intercalating reagent attached should have an increased relative stability to an underivatized oligomer

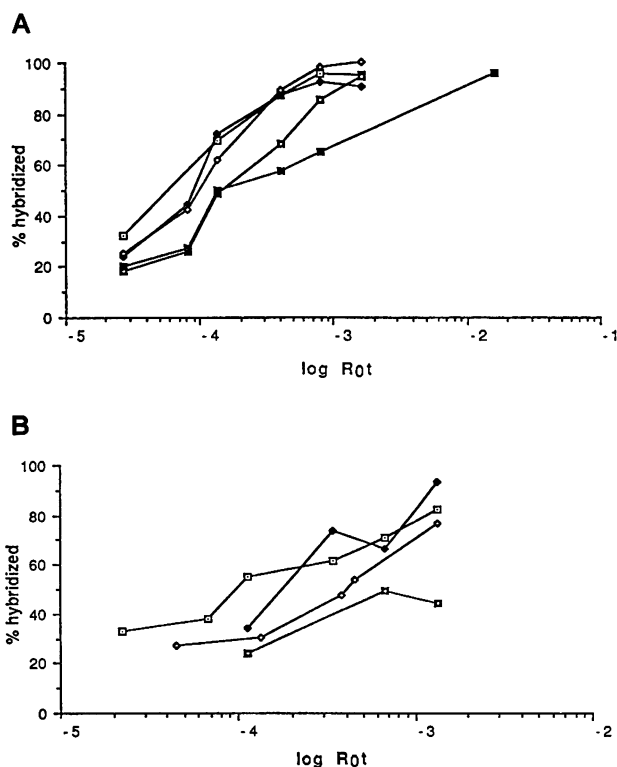


Figure 7. Association rates of chemically modified oligonucleotides. See Figures 1, 2, and methods for experimental detail. (A.) \square , CAT18; \blacklozenge , 3'-MEA-CAT18; \circ , S-CAT18; \blacktriangle , 3'-ANQ-CAT18; \blacksquare , C₁₂-CAT18. (B.) \square , CAT15; \blacklozenge , 3'-ANQ-CAT15; \circ , S-CAT15; \blacklozenge , C₁₂-CAT15.

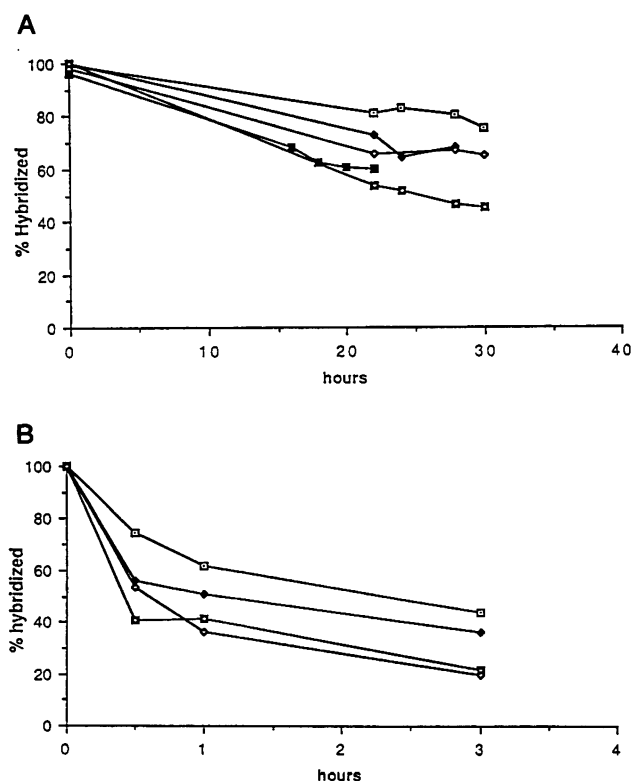


Figure 8. Dissociation rates of chemically modified oligonucleotides. (A.) \square , CAT18; \blacklozenge , 3'-MEA-CAT18; \circ , S-CAT18; \blacktriangle , 3'-ANQ-CAT18; \blacksquare , C₁₂-CAT18. (B.) \square , CAT15; \blacklozenge , 3'-ANQ-CAT15; \circ , S-CAT15; \blacklozenge , C₁₂-CAT15.

as suggested by T_m measurements (36–38). We have shown that this is not necessarily the case for off-rate measurements at 37°C. It is likely that for shorter oligonucleotides (less than 15nt) that the intercalator may stabilize the duplex. This remains to be investigated. In the case of the C_{12} -oligonucleotide, hybridization efficiency was found to be impaired while T_m was not. We checked this phenomenon for the prolactin oligonucleotides and found the exact same result. Therefore it seems that complicated kinetic mechanisms accompany modified oligonucleotides for association and dissociation to RNA.

CONCLUSION

We have used RNase H to study both the rate of oligonucleotide hybridization to RNA and the rate of hybrid dissociation at near-physiological conditions. The effects of the oligomer length, mismatches, chemical modifications and RNA target site have been evaluated for two different RNAs, chloramphenicol acetyl transferase (CAT) and rat prolactin.

We found that the rate of association of an oligomer to RNA can be influenced by several factors. For oligonucleotides 15–21nt (CAT15, CAT18, and CAT21), the on-rate is approximately the same for the oligomers when they hybridize to the same RNA target site. If the RNA target site is different, as in the case of prolactin RNA and the Pro15 oligonucleotide, we have found that the on-rate can be significantly affected compared to CAT15. This effect can be explained as the result of nucleic acid secondary structure impedance to association of the Pro15 oligomer.

For hybridization of mismatched oligonucleotides to RNA we observed a significantly decreased rate of association compared to the perfectly matched parent molecules. We hypothesize that this is a result of unsuccessful duplex nucleation (41, 42) and zippering between the oligomer and its target during a correctly aligned collision. Therefore we believe nucleation efficiency impedes the on-rate for mismatched oligonucleotides. A similar effect has been demonstrated for association of a duplex antisense RNA complex containing a single mismatch (43). We also studied the hybridization rates of modified oligonucleotides. We found two cases, phosphorothioate and 3'- C_{12} -phosphoroamidate backbone-modified oligomers in which the on-rate was 4–8-fold slower than the corresponding unmodified oligomer. There are a number of explanations for this effect such as steric hindrance, hydration differences or alignment problems of the modified oligonucleotides. We can therefore conclude from our study of rates of oligonucleotide association to RNA that oligomer hybridization can be dramatically altered by each of the factors outlined above.

To extrapolate these findings to how they might affect the design of antisense oligomers as gene inhibitors we feel that the following insights are made. We have shown that association rates can be affected by secondary structure. In the single comparison that we made between two sites, Pro15 and CAT15, we observed a four-fold difference in association rate. It is envisioned that effects on association rates of greater than an order of magnitude can be caused by secondary structure. If this effect is combined with that caused by a modified oligonucleotide (as in the case of a phosphorothioate), the overall change in association could be greater than two orders of magnitude. Therefore we predict that the biological efficacy of oligonucleotides will vary dramatically on the basis of association rate. Furthermore, target

sites and oligomer modifications should be carefully chosen to optimize the potency of the antisense effect.

We have also developed an assay to measure the rate of dissociation of an oligomer/RNA complex. We feel that this assay more accurately depicts the relative rate of dissociation than that predicted by thermal melting temperature measurements. A clear example of the occurred when we compared the dissociation rates of CAT18, CAT18.S3, and CAT21. Whereas the T_m data would suggest the following stability: CAT21 > CAT18 = CAT18.S3, the dissociation rate derived from the RNase H assay revealed the following stability: CAT18 > CAT21 > CAT18.S3. We predict that this effect is due to secondary structure adopted either by the RNA or possibly DNA. When we compared the relative dissociation rates of modified oligomer/RNA complexes, one interesting result occurred. We found that the 3'-anthraquinone oligonucleotide, which has a 2–4°C increase for thermal melting temperature over the unmodified oligomer, dissociated faster than the unmodified oligomer. We predict that the effect of the intercalator on dissociation rate may become more apparent for oligomers smaller than that tested in this study. Our conclusions for other modifications, such as phosphorothioate, are that the rate of dissociation can increase when backbone changes are made. This effect may be the result of hybridization or secondary structure differences along the hybrid complex.

The choice of which oligonucleotide to use for antisense gene inhibition is one which is weighted on several factors: ability to hybridize, ability to discriminate a mismatch, site recognition, and RNA/DNA hybrid stability. Based on the results outlined above, the assay system detailed in this paper provides informative data concerning the complicated kinetic parameters for each of these particular points. However, the ultimate decision must be determined by biological efficacy.

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