Unrestricted accessibility of short oligonucleotides to RNA

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ABSTRACT

The propensity of RNA to fold into higher-order structures poses a major barrier to the use of short probes (<15 nucleotides) by preventing their accessibility. Introduction of the pseudo-complementary bases 2-aminoadenine (nA) and 2-thiouracil (sU) and the destabilizing base 7-deazaguanine (cG) into RNA provides a partial solution to this problem. While complementary in hydrogen bonding groups, nA and sU cannot form a stable base pair due to steric hindrance, and are thus pseudo-complementary. Each, however, recognizes the regular T/U and A complements, allowing pairing with oligonucleotides. Short pseudo-complementary RNAs can be prepared by *in vitro* transcription. Relative to standard transcripts, the modified transcripts possess reduced secondary structure and increased accessibility to short (8-mer) probes in the locked nucleic acid (LNA) configuration. They also hybridize to complementary probes with increased specificity and thermostability. Practical application of this strategy to oligonucleotide-based hybridization assays will require engineering of RNA polymerase for more efficient utilization of pseudo-complementary nucleoside triphosphates.

Keywords: RNA; LNA; pseudo-complementary; secondary structure; hybridization; 2-aminoadenine; 2-thiouracil; 7-deazaguanine

INTRODUCTION

Higher-order structures of RNA (and to a lesser extent single-stranded DNA) have severely impeded sequence detection by short probes of 8–10 nucleotides (nt) in length (Doty et al. 1959; Lima et al. 1992; Hacia et al. 1998). This limitation has prevented their general use as specific agents for detection of single nucleotide polymorphisms and point mutations (Wallace et al. 1979; Zoller and Smith 1983), and has posed a serious block to the development of universal arrays for application in the resequencing of genes or the profiling of genetic samples. Even high-affinity probes, such as those with an LNA backbone (Jepsen et al. 2004), lack a general and dependable accessibility to RNA.

We have recently shown (Gamper et al. 2004) that accessibility of short (12-mer) DNA probes to an RNA target can be improved if the RNA is substituted with the pseudo-complementary bases nA (also known as 2,6-diaminopu-

rine) and sU and the destabilizing base hX (hypoxanthine, inosine) (Fig. 1). While these substitutions eliminated intramolecular A-U pairing (between nA and sU) and G-U pairing (between G and sU) and attenuated the strength of G-C pairing (between hX and C), they did not completely remove RNA structures to allow unrestricted accessibility to short probes. Furthermore, because base-pairing with hX is degenerate, this modification reduced the specificity of hybridization and was not desirable. In pursuit of a more dependable hybridization technology, we investigated here the possibility that the accessibility problem of RNA could be overcome by substituting the RNA target with the pseudo-complementary bases nA and sU (nA + sU), and the more specific and yet destabilizing base cG (7-deazaguanine). Our results demonstrate that RNA substituted in this way, together with short probes (8-mers) in the LNA configuration, can produce a powerful combination that permits hybridization at essentially all sequences.

RESULTS

A perfect RNA hairpin (Fig. 2; HP25) of 25 bp, high in G/C content, was tested as a model substrate for the accessibility of probes. This RNA, previously designed and studied by us

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FIGURE 1. Pairing properties of 2-aminoadenine (nA), 2-thiouracil (sU), 7-deazaguanine (cG), and hypoxanthine (hX).

(Gamper et al. 2004), was prepared by *in vitro* transcription with T7 RNA polymerase, in the presence of the pseudo-complementary nA + sU and/or the destabilizing hX or cG nucleoside triphosphates (Table 1). These modified nucleoside triphosphates attenuated the transcription of radiolabeled hairpin under standard reaction conditions. Replacement of ATP with nATP and UTP with sUTP reduced the yield of full-length hairpin by 30%–40% with one substitution and by 60%–70% with both substitutions. Replacement of GTP by cGTP had no effect on transcription, but substitution with hXTP reduced the yield of hairpin by almost 75%. Transcription in the presence of all three NTP analogs reduced hairpin synthesis to only 30% of the control reaction, regardless of whether cGTP or hXTP was used. While these levels of transcription efficiency were adequate for this study, the preparation of pseudo-complementary transcripts longer than 200 nt was problematic (Gamper et al. 2004).

The strength of intramolecular base-pairing was evaluated by the melting temperature (T_m) of each modified hairpin. If the hairpin became destabilized because of the presence of modified bases, the T_m of the hairpin should decrease relative to that of the unmodified RNA. The T_m was determined by a recently developed gel mobility shift assay (Gamper et al. 2004), where the accessibility of a 25-mer RNA probe to the RNA hairpin was monitored by gel electrophoresis under non-denaturing conditions. Determination of the accessibility over a range of temperatures (10°C–90°C) yielded a binding curve from which the T_m was calculated (Table 2). Notably, the hairpin composed of regular bases was not accessible to the probe under any conditions, demonstrating the stability of the hairpin, which was predicted by Mfold to have a T_m of 105°C in 25 mM NaCl and 5 mM MgCl₂ (Zuker 2003). In contrast, each of the modified hairpins had an experimentally determined T_m that was much decreased, indicating destabilization of the hairpin structure. Specifically, substitution with hX, cG, or nA + sU each lowered the T_m to 56°C, 77°C, or 81°C, respectively, while substitution with nA + sU together with hX or cG lowered the T_m further to 39°C or 63°C. Thus, all of the modified bases reduced the hairpin stability. The denaturing effects of these modified bases were relatively the same in all of the solutions conditions tested, including those lacking MgCl₂ or containing formamide. Although the combination of nA + sU and hX appeared to be most effective in T_m reduction, hX is not favorable due to its lack of specificity in base-pairing (see below). Focus is therefore placed on nA + sU and cG to evaluate the ability of this combination to improve the accessibility and specificity of target–probe hybridization.

Probes with an LNA backbone, as short as 7–8 bases, have been shown to hybridize to their target sequences with exquisite stability and specificity (Jepsen et al. 2004). We have confirmed that these properties are retained upon hybridization of LNA probes to a pseudo-complementary RNA. Specifically, the stability of a 7-mer LNA probe (5'-

FIGURE 2. RNA targets used in this study. SS14 was chemically synthesized. SS32 and HP25 were transcribed off synthetic DNA templates using different combinations of NTPs. One arm of the HP25 hairpin is recapitulated in SS32.

TABLE 1. Incorporation of NTP analogs by T7 RNA polymerase

NTPs	Relative amount of transcript
ATP,UTP,GTP,CTP	1.00
nATP,UTP,GTP,CTP	0.59
ATP,sUTP,GTP,CTP	0.70
nATP,sUTP,GTP,CTP	0.44
ATP,UTP,hXTP,CTP	0.45
ATP,UTP,cGTP,CTP	0.98
nATP,sUTP,hXTP,CTP	0.27
nATP,sUTP,cGTP,CTP	0.35

A 59-mer RNA transcript (HP25) was generated overnight under standard conditions using a synthetic DNA template that contained the consensus promoter for T7 RNA polymerase. NTPs were present at 1.0 mM except for α -³²P-CTP, which was at 0.5 mM. (nA) 2-aminoadenosine, (sU) 2-thiouridine, (hX) hypoxanthine (inosine), (cG) 7-deazaguanosine.

GAAGTCT) hybridized to a pseudo-complementary RNA (Fig. 2; SS32) was assessed by the T_m of the hybrid in three different solutions (Table 3). Relative to the hybrid with the unmodified RNA ($T_m = 54^\circ\text{C}$ in 20% formamide), the introduction of nA + sU increased the stability of the probe–RNA hybrid by raising the T_m to 84°C . This was expected because nA–T/U and sU–A base pairs are known to stabilize the A-form duplex (Vormbrock et al. 1974; Howard and Miles 1984; Kumar and Davis 1997; Testa et al. 1999). Hybrids in which the RNA was substituted with nA + sU, together with hX or cG, had T_m s of 67°C and 80°C , respectively. The reduced T_m s, compared to that with nA + sU only, reflect the destabilizing effects of hX and cG (Martin et al. 1985; Seela and Driller 1985), as evidenced when each was separately substituted into the RNA strand of the hybrid ($T_m = 29^\circ\text{C}$ and 45°C , respectively). Importantly, despite the destabilizing effect of cG, its combination with nA + sU significantly improved the T_m of the hybrid (80°C) relative to the unmodified control (54°C). Remarkably, these elevated T_m values were observed even under stringent conditions (20 mM HEPES at pH 7.5, 25 mM NaCl, 20% formamide), reflecting the ability of pseudo-complementary RNA to form extremely stable hybrids with LNA probes.

Specificity of the LNA probes was confirmed by testing their ability to discriminate against mismatches with the pseudo-complementary RNA. One perfect-match and 12 single-mismatch 8-mer LNA probes were each hybridized to single-stranded RNA (SS14 or SS32) that was prepared with different combinations of bases (Fig. 3). The equilibrium between the free RNA and RNA–LNA hybrid was fixed at 8°C below the T_m of each perfect-match hybrid and then determined by gel shift assay. The fraction of the RNA in the hybrid was analyzed and compared. Here, while the unmodified RNA was permissive for U–G formation, promoting 81% hybridization (panel 1), substitution of the RNA with sU prevented this mismatch, promoting only 9%–12% hybridization (panels 3–5). This is due to the

fact that the sU–G pairing is less stable than U–G (Vormbrock et al. 1974; Testa et al. 1999). Thus, the presence of sU in the RNA increased the ability of the LNA probe to discriminate against the U–G mismatch. Relative to the unmodified target, suppression of the U–T mismatch was much improved for all four modified transcripts, an effect attributable to substitution with sU or cG. In contrast, presence of nA in the RNA strand reduced specificity by accommodating an nA–C mismatch, as has been observed in other LNA-containing hybrids (Koshkin 2004; Rosenbohm et al. 2004). Importantly, while substitution with hX decreased the specificity of hybrid formation (panel 5), as expected due to the degeneracy of hX in base-pairing (Martin et al. 1985; Case-Green and Southern 1994), introduction of cG retained the specificity (panels 2 and 4), allowing only the cG–C base-pairing. Overall, despite the reduced specificity by nA, the RNA that contained nA + sU, alone or in combination with cG, exhibited the greatest selectivity in hybridization.

We also verified that substitution of nA + sU and cG into a structured RNA indeed increases its accessibility to a short LNA probe. An RNA transcript (Fig. 2; SS32) that embodied one arm of the 25-bp-long hairpin described earlier (Gamper et al. 2004) was used as a target. This RNA, in the absence of modification, contained a hairpin at one end that was inaccessible to 12-mer DNA probes at 10°C in the presence of 5 mM MgCl_2 and 25 mM NaCl (Gamper et al. 2004). Here, under identical conditions, an 8-mer LNA probe targeted to the same end was shown to slowly hybridize to the unmodified transcript (Fig. 4). The time course of the hybridization was compared among RNAs substituted with nA + sU and/or hX/cG, to determine the hierarchy of accessibility. Clearly, substitution with nA + sU + cG generated the greatest burst of hybrid, followed by substitution with nA + sU + hX. This comparison indicates that stability of the intermolecular LNA–RNA hybrid can sometimes be more important than destabilization of RNA secondary structure in driving hybrid-

TABLE 2. Melting temperatures of a 25-bp RNA hairpin

Base composition of RNA hairpin	Solution			
	1	2	3	4
A,U,G,C ¹	>100°	>100°	—	—
A,U,hX,C ²	56°	46°	32°	24°
A,U,cG,C ²	77°	71°	60°	53°
nA,sU,G,C ²	81°	80°	70°	62°
nA,sU,hX,C ²	39°	41°	27°	16°
nA,sU,cG,C ²	63°	65°	52°	45°

The 25-bp RNA hairpin (HP25) was melted in 25 mM NaCl, 20 mM HEPES buffer (pH 7.5) with (1) 5 mM MgCl_2 , (2) no additions, (3) 20% formamide, or (4) 35% formamide.

¹Predicted T_m s from Mfold.

² T_m s determined by gel mobility shift assay using a 25-mer RNA probe (5'-ACCUGACUCCUGAGGAGAAGUCUGC).

TABLE 3. Melting temperatures of a 7-bp RNA–LNA hybrid

Base composition of RNA strand	Solution		
	1	2	3
A,U,G,C	64°	58°	54°
A,U,hX,C	47°	37°	29°
A,U,cG,C	63°	55°	45°
nA,sU,G,C	>90°	>90°	84°
nA,sU,hX,C	80°	75°	67°
nA,sU,cG,C	>90°	~90°	80°

The RNA–LNA hybrids consisted of a 7-mer LNA probe (5'-GAAGTCT) annealed to the unmodified 14-mer RNA target (SS14) or to the 32-mer RNA target (SS32) that was substituted with one or more base analogs. The hybrids were melted in 25 mM NaCl, 20 mM HEPES buffer (pH 7.5) with (1) 5 mM MgCl₂, (2) no additions, or (3) 20% formamide. T_m s were determined by gel mobility shift assay using an 8-mer LNA competitor (5'-CAGACTTC).

ization. Further support is provided by the observation that, while substitution of the RNA with nA + sU or cG alone resulted in improved rates of hybridization, substitution with hX alone was insufficient. The LNA 8-mer used here was part of a set of LNAs that was tiled across the RNA target. All members of this set readily hybridized to the unmodified RNA under more stringent conditions (data not shown).

Additional experiments established that pseudo-complementary RNA is fully accessible to short high-affinity probes. Here we used the RNA hairpin presented in Figure 2 (HP25) as the target for a set of tiled 8-mer LNA probes (Fig. 5A). The hairpin was substituted with nA + sU and/or hX/cG, and hybridization was carried out at a temperature 13°C above the T_m for each hairpin, after which the RNA–LNA hybrid was kept in an ice bath until examined by gel electrophoresis. These analyses showed that hairpins substituted with nA + sU and either cG or hX had the ability to hybridize to every member of the LNA probe set, which included a total of 11 oligomers encompassing one arm of the hairpin stem. Hairpin substituted with just nA + sU performed nearly as well, with hybridization observed for nine of the 11 probes. In contrast, hairpin substituted with the highly destabilizing hX base only hybridized effectively to two probes. Under no conditions did the unmodified hairpin or the hairpin substituted with just cG hybridize to any of the probes (data not shown). The high T_m of these hairpins precluded access to LNA probes.

Many of the hybridization reactions analyzed by gel mobility shift assay in Figure 5A generated two or more shifted bands, especially when the hairpin target contained nA + sU substitutions. We surmise that multiple bands observed in a single lane may reflect different secondary structures of the same probe–target complex. Since gel electrophoresis was conducted under conditions where each modified hairpin existed in a collapsed state, it is plausible that any one of

the hairpins could fold into alternative structures influenced by the position of the LNA–RNA hybrid on the RNA sequence. Formation of mismatched LNA–RNA hybrids might also account for some of the bands, since the hybridization conditions were not that stringent.

Besides substitution of the RNA hairpin with nA + sU + cG, successful and readily accessible hybridization required probes with an LNA backbone. For example, a set of 12-mer DNA probes performed poorly due to their low affinity for the target hairpins (Fig. 5B). Only six of the 14 DNA probes formed a stable hybrid when the hairpin contained nA + sU + hX, and none hybridized when it contained nA + sU + cG. The DNA–RNA hybrids formed by these probes were stable at 40°C but not at 65°C, thus

FIGURE 3. Mismatch discrimination in RNA–LNA hybrids. Perfect-match and mismatch 8-mer LNA probes (1 μM) were hybridized to regular (SS14) and modified (SS32) versions of an RNA target (<50 nM) in 25 mM NaCl, 20 mM HEPES (pH 7.5) at the designated temperatures and formamide concentrations. After 5 min, a complementary LNA oligomer (5 μM) was added to sequester free probe and reactions were placed in an ice bath. The fraction of radiolabeled RNA engaged in an RNA–LNA hybrid (listed at the bottom of each lane) was determined by gel mobility shift assay. (C) No probe control; (PM) perfect-match hybrid.

FIGURE 4. Hybridization of an 8-mer LNA probe to a short RNA hairpin element. Radiolabeled SS32 RNA target substituted with the indicated bases was hybridized at 10°C to an 8-mer LNA probe (5'-GAAGTCTG; 1 μ M) in 5 mM MgCl₂, 25 mM NaCl, 20 mM HEPES (pH 7.5). The time course of annealing was determined by adding a competitor LNA oligomer (10 μ M; 5'-CAGACTTC) to replicate reactions at the indicated times. Probe–target hybrid was resolved from target by gel mobility shift assay (Gamper et al. 2004).

precluding hybridization to the cG-containing RNA target. In the gel corresponding to this target, neither full-length hairpin nor prematurely terminated “half-hairpin” were shifted to slower moving bands. The single-stranded half-hairpin target was homologous to the DNA probes and functioned as a control for nonspecific hybridization. Hybrids formed with the hX-containing RNA target, particularly those located near the 3'-end of the RNA, were probably lost upon cooling and subsequent collapse of the RNA hairpin by a strand displacement mechanism. Upstream hybrids escaped this fate by blocking association of the RNA arms that flanked the hairpin loop.

LNA probes formed sufficiently stable hybrids with the pseudo-complementary RNA hairpins to resist strand displacement at temperatures below the melting point of the hairpin. We confirmed this behavior by showing that a 25-mer DNA was unable to displace a 9-mer LNA from a complementary 32-mer RNA (Fig. 6). Gel analysis indicated that a ternary LNA–RNA–DNA complex formed when DNA probe was added after LNA probe to the target RNA. Although the longer DNA probe hybridized to the same sequence as the LNA probe on the RNA target, the single-stranded DNA overhang of the 25-mer probe was unable to displace the LNA. To our knowledge, this is the first example of a short hybrid resistant to branch migration. In this model experiment the weakly hybridizing DNA probe mimicked the inability of the pseudo-complementary RNA hairpin to displace LNA probes. As expected, the 9-mer LNA was displaced from the RNA target by a 25-mer RNA probe. In the same experiment, short DNA and RNA

probes were also displaced from the RNA target by longer overlapping DNA and RNA probes.

DISCUSSION

Unrestricted accessibility of short probes to RNA requires that the probes be in the LNA configuration and that the RNA target contain the pseudo-complementary nA + sU and the destabilizing cG bases. Key to this success was the exceptionally high affinity of the LNA probes to the modified RNA. The RNA–LNA hybrids described here exhibit high thermostability, even under the stringent conditions that denature the pseudo-complementary RNA target. Furthermore, once formed, the stability of these hybrids is such that they are resistant to strand displacement, even at

FIGURE 5. Accessibility of a 25-bp RNA hairpin to short LNA and DNA probes. (A) Hybridization of tiled 8-mer LNA probes to radiolabeled HP25. The probes (5 μ M) were hybridized at the designated temperatures in 25 mM NaCl, 20 mM HEPES (pH 7.5), 20% formamide to HP25 (<50 nM) that was substituted with different combinations of bases. After 10 min the reactions were placed on ice and analyzed by gel mobility shift assay. (T) Target only. (B) Hybridization of tiled 12-mer DNA probes to HP25. The protocol was identical to that described for the tiled LNA probes.

FIGURE 6. LNA in an RNA–LNA hybrid is displaced by RNA but not DNA. Hybridizations were conducted in 5 mM MgCl₂, 25 mM NaCl, 20 mM HEPES (pH 7.5). The first probe (5 μM) was annealed to the radiolabeled SS32 RNA target for 5 min at 60°C. A second probe (5 μM) was then added and annealing was continued for an additional 30 min at 37°C. Reactions were kept on ice until analyzed by gel mobility shift assay.

lower temperatures, where the modified RNA might form secondary structures.

The combination of pseudo-complementary RNA targets and LNA probes could enable new technological advances in biomedical research. For example, due to the enhanced discrimination ability against mismatches, it should facilitate the detection of single nucleotide polymorphisms and point mutations. This combination could also promote the development of universal microarrays by improving the efficiency, specificity, and reliability of short probes. With every sequence in a target accessible, the sensitivity of such probes could increase by one or two orders of magnitude (Chou et al. 2004; Ramdas et al. 2004). The base analogs we have described should also help equalize the stability of hybrids that differ in G-C content (Gamper et al. 2004). In virtually all applications, specificity would require using cG in place of hX. We have demonstrated that all three base analogs are incorporated into RNA by T7 RNA polymerase. However, while this enzymatic synthesis of pseudo-complementary RNA is convenient, it is currently limited to transcripts up to 200 nt. Further optimization of reaction conditions, together with engineering of T7 RNA polymerase for a broader range of nucleotide triphosphates, may remove this limitation. It is worth mentioning that we have used DNA polymerase to successfully incorporate nA, sT, and hX/cG into long primer extension products (>10³ nt in length; H.B. Gamper, A. Gewirtz, and Y.-M. Hou, unpubl. results), thus demonstrating the feasibility of using pseudo-complementary DNA as a target for short probes. Additional research is also needed to develop a pseudo-comple-

mentary G-C base pair to allow hybridizations to be conducted under a broad range of conditions with short DNA probes.

MATERIALS AND METHODS

Nucleic acids

Oligonucleotides were purchased from Integrated DNA Technologies. Regular NTPs were from Roche Molecular Biochemicals, ITP was from Sigma, nATP, sUTP, and cGTP were from TriLink Biotechnologies, and [α-³²P]CTP was from Amersham Biosciences. Transcription reactions contained 200–800 nM gel-purified dsDNA template with a consensus T7 promoter, 1 mM ATP or nATP, 1 mM UTP or sUTP, 1 mM GTP, ITP, or hXTP, 60 μM [α-³²P]CTP, 1 mM GMP (only in the presence of a GTP analog), 6 mM MgCl₂, 2 mM spermidine, 10 mM dithiothreitol, 0.5% Tween 20, 40 mM Tris-HCl at pH 7.9, and T7 RNA polymerase (20 units; Ambion) in a total volume of 10–20 μL. After at least 5 h incubation at 37°C, reactions were diluted to 50 μL and centrifuged through a gel filtration column (CentriSpin 20, Princeton Separations). Radiolabeled transcripts were stored up to 6 wk at –20°C. Chemically synthesized RNA oligomers were end labeled by T4 polynucleotide kinase reaction in the presence of [γ-³²P]ATP, after which free counts were removed using a CentriSpin 10 column.

Analysis of transcription reactions

An aliquot of each transcription reaction was analyzed by electrophoresis in a 7 M urea/12% polyacrylamide gel. Full-length product was visualized and counted using a phosphorimager.

Analysis of hybridization reactions

The apparent melting temperature of each LNA–RNA hybrid was determined using a new gel mobility shift assay previously described (Gamper et al. 2004). Briefly, the hybrid of interest (containing a radiolabeled RNA) was subdivided into 10 μL aliquots, each of which was incubated for 5 min at a different temperature within the range of 10°C–90°C. The hybridization reactions were quenched by adding a 10-fold molar amount of a competing oligonucleotide (which was identical in backbone composition and complementary to the probe) followed by rapid cooling in an ice bath. After the addition of a weighting solution, the reactions were loaded onto a nondenaturing 12% polyacrylamide gel prepared with 5 mM MgCl₂ and electrophoresed in the cold room. After drying, gels were analyzed by phosphorimaging. Plotting the fraction of hybrid as a function of temperature yielded a melt curve and a *T_m*. A similar protocol was followed for determining the melting temperature of RNA hairpins. In this case the competing oligonucleotide (added in 100-fold molar excess) had an RNA backbone and was complementary to the entire length of one arm of the hairpin. Hybridization time courses were monitored by adding a competing oligonucleotide to reaction aliquots as a function of time. Tiling experiments, in which multiple probes were separately hybridized to RNA hairpin, were quenched in an ice bath with no prior addition of oligonucleotide competitor.

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