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Modified Bases in RNA Reduce Secondary Structure and Enhance Hybridization†

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ABSTRACT: Secondary structure in RNA targets is a significant barrier to short DNA probes. However, when such targets are the end product of an in vitro amplification scheme, it is possible to carry out transcription in the presence of nucleoside triphosphate analogues that reduce secondary structure of the RNA without impairing subsequent hybridization. Here we show that nucleoside triphosphates of 2-aminoadenine (nA) and 2-thiouracil (sU) are taken up by T7 RNA polymerase and that the resulting RNA possesses reduced secondary structure and improved accessibility to DNA probes. The hybridization properties of short RNA transcripts were studied using a new gel mobility shift assay from which melting temperatures were determined. RNA hairpins that contained nA and sU were able to hybridize to DNA probes under conditions where the unmodified hairpins did not. DNA−RNA hybrids that contained nA and sU in the RNA strand exhibited enhanced specificity, increased stability, and greater equality of base pairing strength than the same hybrids without modifications. Substitution of guanine (G) with inosine (I) further reduced secondary structure, but RNA with this base hybridized nonselectively. The high stability of nA-T and A-sU base pairs in DNA−RNA hybrids, combined with the destabilizing effect of the nA-sU couple in RNA targets, accounts for the improved hybridization properties. These results suggest that incorporation of nA and sU during in vitro transcription is a promising strategy for enhancing the performance of oligomeric DNA probes with an RNA target.

The biological functions of RNA require substantial folding into secondary and tertiary structures, which can be a significant barrier to the hybridization of complementary DNA probes. Under conditions that allow short probes to form stable hybrids, many sequences in RNA are inaccessible due to intramolecular base pairings (*1*). Consequently, the strength with which different DNA probes hybridize to the same RNA target can vary by as much as 10^6-fold (*2*, *3*). Detection of single nucleotide polymorphisms and point mutations is difficult and sometimes impossible if the underlying sequence is inaccessible to hybridization. Since RNA folding programs based on nearest-neighbor thermodynamic parameters are not totally reliable for long transcripts (*4*−*9*), sequences that are available for hybridization must be determined experimentally for each RNA. Several strategies have been described that lessen the interference due to secondary structures. For example, increasing the stringency of hybridization can reduce secondary structures in RNA, although this approach requires longer probes than would otherwise be necessary, thus increasing the possibility that mismatches will be tolerated in the hybrid. Also, while fragmentation of the RNA target lessens secondary structures, it also reduces the target signal. Additional strategies include modifications that enhance the base pairing strength of short probes. Such modifications promote strand invasion of the probe into RNA structure and permit the use of more stringent hybridization conditions. Peptide and locked nucleic acid probes are also attractive, because they form highly stable hybrids that are better able to overcome secondary structures (*10*, *11*).

An alternative strategy that has not been seriously investigated is reduction of intramolecular base pairings in an RNA target under conditions that support hybridization with short probes. We propose that pseudocomplementary bases hold the promise for generating nucleic acid targets that lack intramolecular structures but are freely available for interacting with short probes. Pseudocomplementary bases are analogues that do not form hydrogen bonds to one another but are able to pair with regular base complements. For example, 2-aminoadenine (nA;[1] also known as 2,6-diaminopurine) and 2-thiothymine (sT) are base analogues that act as a pseudocomplementary couple (*12*−*14*). While the regular adenine (A) and thymine (T) form a stable Watson−Crick base pair (Figure 1A), the two analogues cannot, due to steric clashes between the 2-amino group of nA and the 2-thiol group of sT (Figure 1B). However, nA can form a stable base pair with T (Figure 1C), while sT can form a stable pair with A (Figure 1D), without steric clashes. Indeed, complementary DNA oligomers substituted with nA and sT do not hybridize to one another but readily hybridize to each

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[1] Abbreviations: A, adenine; nA, 2-aminoadenine (2,6-diaminopurine); T, thymine; sT, 2-thiothymine; U, uracil; sU, 2-thiouracil; G, guanine; cG, 7-deazaguanine; sG, 6-thioguanine; I, inosine; C, cytidine; P, 2-pyrimidinone; HPLC, high-performance liquid chromatography; NTP, nucleoside triphosphate; T_m, melting temperature.

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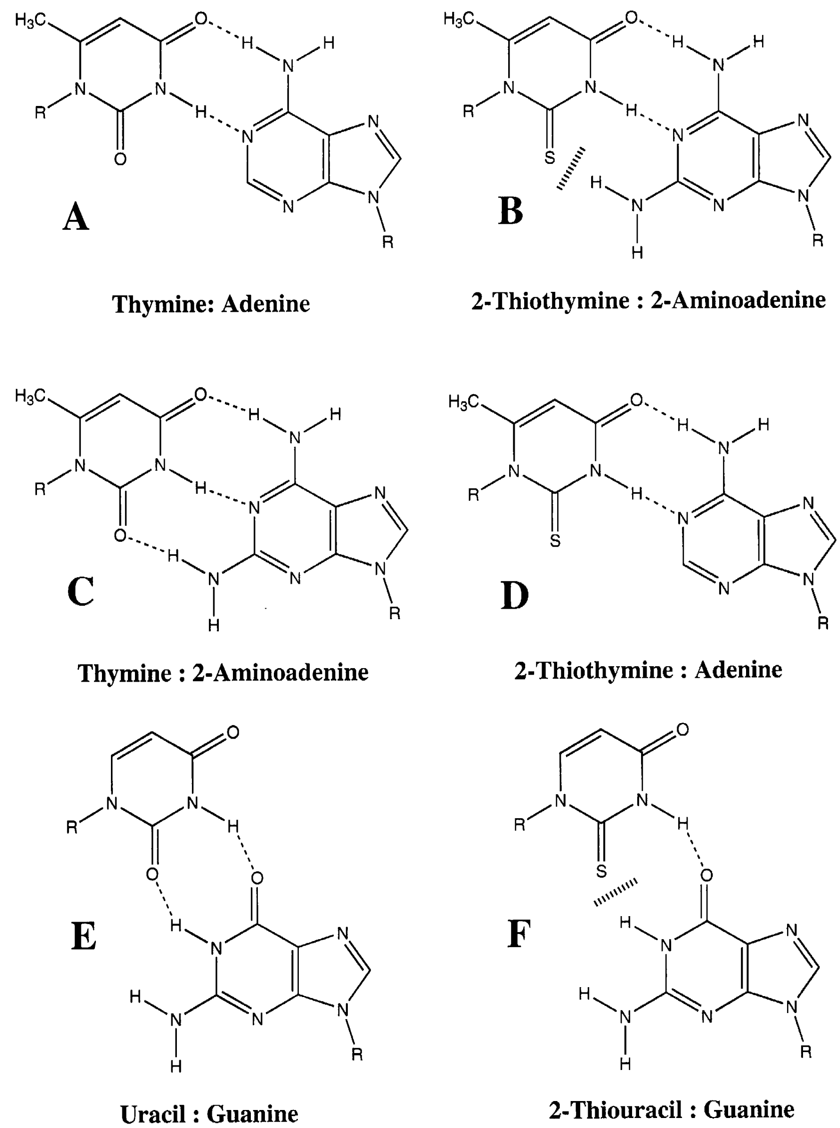


FIGURE 1: Base pairing properties of thymine/2-thiothymine, uracil/2-thiouracil, and adenine/2-aminoadenine. Hydrogen bonding in the nA-sT and sU-G couples is weakened by steric repulsion between the 2-thiocarbonyl group of sT and the 2-amino group of nA or between the 2-thiocarbonyl group of sU and the N1 hydrogen of G, respectively. This study shows that the nA-sU couple in RNA is also weakened by the same steric clashes.

of their complements that are composed of regular bases (*12*). This has provided the basis for our proposal that secondary structure in DNA or RNA from any genetic sample can be eliminated by enzymatic amplification of the nucleic acid in the presence of pseudocomplementary nucleoside triphosphates. The availability of such targets would facilitate the detection of single nucleotide polymorphisms and point mutations by short probes.

To expand the pseudocomplementary technology, the properties of the analogous nA-sU couple in RNA need to be tested. When introduced separately into RNA, nA and sU (2-thiouracil) each stabilize duplex structure (*15*−*17*) by forming the nA-U and A-sU pairs where the bulky 2-amino and 2-thiocarbonyl groups are well accommodated in the wide minor groove of an RNA duplex (*17*−*20*). However, the question of whether the nA-sU couple in RNA is destabilizing remains to be tested, although intriguingly it has already been shown that the wobble pairing between U and G (Figure 1E) is reduced when U contains a 2-thiocarbonyl group (Figure 1F) (*15*, *21*). A further question is whether nA and sU can be incorporated into the same RNA to test the nA-sU couple. While the triphosphate form of nA and sU can be individually substituted for ATP and UTP in one transcription reaction (*22*), there has been no report on substitution of both into one RNA sequence.

Effective pseudocomplementary analogues of the G-C base pair have not been described. Reduction of RNA structure that results from G-C base pairing is primarily achieved by using analogues of G or C that pair with reduced strength. Inosine (I) is commonly used for this purpose. Compression effects in sequencing are eliminated when ITP is substituted for GTP (*23*). Without a C2 amino group, inosine self-associates to a lesser extent than guanine (*24*) and forms a base pair with cytidine that is less stable than a G-C base pair (by 1.4 kcal/mol) (*25*). We have utilized inosine to determine the extent to which reduced G-C pairing in nA- and sU-substituted RNA improves the accessibility of short probes. Other base analogues that could be used for the same purpose include 7-deazaguanine (*23*) and N^4-methyl- or N^4-ethylcytidine (*26*, *27*).

Here we describe the enzymatic synthesis of RNA substituted individually or in combination with nA, sU, and

I, using T7 RNA polymerase. To determine if modifications reduce the capacity of RNA to form intramolecular structures, we have developed a gel shift assay in which the accessibility of the RNA to a short DNA probe was measured. In this gel shift assay, the accessibility of the RNA to the probe was determined as a function of temperature, such that the temperature that unfolds the RNA to allow hybridization could be determined. With this assay melting profiles could be determined for RNA duplexes or DNA−RNA hybrids present at nanomolar concentration. The results established that nA and sU function as a pseudocomplementary pair in RNA and that transcripts substituted with these analogues had reduced secondary structure and improved accessibility to complementary DNA probes. The resulting DNA−RNA hybrids exhibited greater stability and specificity relative to hybrids formed with unmodified RNA. Additionally, incorporation of I together with nA and sU further enhanced the single-stranded character of the modified RNA. The ability of nA and sU to reduce RNA structure will have important application in the research and biotechnology of RNA.

MATERIALS AND METHODS

Materials. Integrated DNA Technologies (IDT, Coralville, IA) synthesized the oligodeoxynucleotides. Probes for hybridization were used directly while templates for transcription were purified by gel electrophoresis or reverse-phase HPLC. Dharmacon (Boulder, CO) synthesized the oligoribonucleotides. Standard NTPs were purchased from Roche Molecular Biochemicals (Indianapolis, IN), nATP and sUTP were from TriLink Biotechnologies (San Diego, CA), ITP was from Sigma (St. Louis, MO), and [α-^{32}P]CTP was from Amersham Biosciences (Piscataway, NJ). T7 RNA polymerase (fortified with an RNase inhibitor) and RNase T2 were obtained from Ambion (Austin, TX) and Life Technologies (Rockville, MD), respectively.

Transcription. Single-stranded and hairpin oligoribonucleotides were transcribed from synthetic double-stranded DNA templates that contained the consensus promoter and transcription initiation sequences for T7 RNA polymerase. Longer transcripts were prepared from linearized plasmids available from Ambion. Transcription was carried out at 37 °C for at least 5 h in 10−20 μL volumes that contained T7 RNA polymerase (20 units), DNA template (65 nM linearized plasmid or 200−800 nM double-stranded oligodeoxynucleotide), 1 mM ATP or nATP, 1 mM UTP or sUTP, 1 mM GTP or ITP, and 60 μM [α-^{32}P]CTP in transcription buffer (6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 0.5% Tween 20, and 40 mM Tris-HCl, pH 7.9). Reactions conducted in the presence of ITP were supplemented with 1 mM GMP since T7 RNA polymerase cannot initiate transcription with ITP. Prior to use, transcripts were desalted by gel filtration through CentriSpin 10 or 20 columns (Princeton Separations, Adelphia, NJ) preequilibrated in water. Cerenkov counting of the flow-through provided an indication of the efficiency of transcription. Radiolabeled RNA stocks were stored at −20 °C for up to 3 weeks.

Transcripts were analyzed by electrophoresis in 0.2 mm thick 7 M urea/12% polyacrylamide gels. Dried gels were visualized by autoradiography and phosphorimaging. Relative yields of full-length transcripts were determined using ImageQuant. Transcription reactions were also spotted onto Whatman DE81 filter disks. After being washed four times in 100 mL of phosphate buffer and once in 100 mL of 70% ethanol, the dried membranes were counted in scintillation fluid. The ratio of nucleotides adjacent to the 5′-side of labeled CMP residues in specific transcripts was confirmed by thin-layer chromatography of RNase T2 hydrolysates (*28*). This technique established that nA, sU, and I were incorporated into RNA at the expected frequencies.

Melting Point Determinations. A new assay was developed to determine the melting profiles of short radiolabeled hybrids between 10 and 90 °C. This method, which required a separate aliquot of hybrid for each temperature in the profile, accommodated hybrid concentrations as low as 1 nM and as high as 2 μM. Melting profiles of hybrids that contained ^{32}P-labeled RNA were acquired in buffer 1 (25 mM NaCl, 20 mM Hepes, pH 7.5) or buffer 2 (5 mM $MgCl_2$, 25 mM NaCl, 20 mM Hepes, pH 7.5). Prior to analysis a short DNA−RNA hybrid (≤50 nM in RNA and 1 μM in DNA) or an RNA hairpin (≤50 nM) was subjected to a heat−cool cycle (2 min at 90 °C followed by rapid cooling in an ice bath). For processing, 10 μL aliquots of the stock duplex were placed into thin-walled PCR tubes and kept in an ice bath. One by one the tubes were inserted into the sample block of a thermocycler preset to a given temperature. By utilizing two or more thermocyclers additional aliquots could be processed at the same time. After 5 min incubation each aliquot was removed from the block and quenched by adding 1 μL of competing oligonucleotide, mixing, and placing the tube in an ice bath. The added oligonucleotide (final concentration 5−10 μM) was complementary to the DNA strand of a DNA−RNA hybrid or to one of the arms of an RNA hairpin. By hybridizing within seconds to any free complement, this oligonucleotide fixed the ratio of annealed to melted RNA that was present at the temperature of addition. Alteration of that temperature was minimized by keeping the volume of added probe to 10% or less of the sample volume. For same day analysis, the samples were stored in an ice bath; otherwise they were kept in a −20 °C freezer.

Electrophoretic Analysis. The hybridization state of radiolabeled transcripts was determined by nondenaturing gel electrophoresis. Samples from a melting analysis or other hybridization experiment were mixed with 2 μL of tracking dyes in 20% glycerol, and 10 μL aliquots were electrophoresed at 8 W for 1−3 h in a 0.8 mm thick 12% polyacrylamide gel prepared in Tris−borate buffer with 5 mM $MgCl_2$ and set up in a cold room. Dried gels were subjected to autoradiography and/or phosphorimaging. Bands were quantified using ImageQuant, and the percent of hybridized RNA was determined for each lane. These values were plotted as a function of temperature, and the midpoint between low- and high-temperature plateaus was taken as the melting temperature.

Optical Melting. Absorption versus temperature profiles were recorded at 260 nm in a Cary Model 3E spectrophotometer by increasing the temperature at 0.5 °C/min from 10 to 60 °C. Solutions of 1 mL contained 2 μM hybrid in buffer 1. Complementary strands were annealed by heating at 90 °C for 2 min followed by slow cooling to 10 °C. Melting temperatures were extracted from first derivative plots of the absorption curves.

RESULTS

Incorporation of nA, sU, and I into RNA. Although nATP, sUTP, and ITP can each be separately incorporated into RNA (*22*, *23*), there are no reports on whether they can be incorporated as a group. To address this possibility, we tested T7 RNA polymerase with different combinations of the NTP analogues under standard reaction conditions. Synthesis of a 95 nucleotide long runoff RNA in the presence of one or more NTP analogues was compared to the same synthesis using regular NTPs. The template for transcription was a linearized plasmid that contained a T7 promoter followed by a consensus transcription initiation sequence. When added separately, nATP, sUTP, or ITP reduced the RNA yield to 59%, 70%, or 45% of the control, respectively. Addition of both nATP and sUTP reduced the yield to 44%, and addition of all three analogues reduced the yield to 27% of the control. Relative yields of modified RNAs were higher when total transcripts were bound to DE81 filter disks and counted. By this measure the yield of RNA was reduced to 52% of the control when nA and sU were present and to 50% when I was present. The reduced yield relative to the control can be attributed to extended pausing or premature termination of transcription by the polymerase at specific sites of base analogue incorporation.

The various modified RNA transcripts had different mobilities upon electrophoresis in a denaturing polyacrylamide gel (Figure 2A). Compared to the control, the RNA with nA and sU exhibited reduced mobility while that with I exhibited increased mobility. Introduction of all three analogues yielded an intermediate mobility. The successful incorporation of analogues into RNA was confirmed by subjecting the RNA (internally labeled with [^{32}P]CTP) to RNase T2 digestion, followed by two-dimensional thin-layer chromatography of the digestion products. Nucleotide bases that are 5′ to C residues were released as radiolabeled 3′-NMPs. In the chromatograms each analogue was converted to a labeled nucleoside monophosphate, which migrated to a specific position at the expected frequency (data not shown).

The yield of the modified RNA on a mass basis decreased significantly as the length increased (Figure 2B). For RNA transcripts that were 500 nucleotides or longer, substitution with nA and sU decreased the efficiency of transcription to 10% of the control. When all three analogues were present, transcription efficiency steadily decreased with length until yields were less than 1% of the control. In this experiment, runoff transcripts of different length were prepared from a mixture of linearized plasmids that contained the same T7 promoter and initiation region. This assured that transcription efficiency would be solely dependent upon transcript length (and associated sequence) for any given combination of NTPs.

We chose transcripts less than 60 nucleotides long for hybridization studies (Figure 3). The transcripts were prepared from synthetic double-stranded DNA templates that contained the T7 promoter upstream from G/A-rich initiation sequences. Transcription that yielded RNA hairpins, with stems of 15, 20, and 25 bp (G/C content of 55−60%), was performed using different combinations of regular and modified NTPs. These hairpins shared a common sequence and were used to evaluate the effects of nA, sU, and I on intramolecular base pairing. Transcription that yielded single-

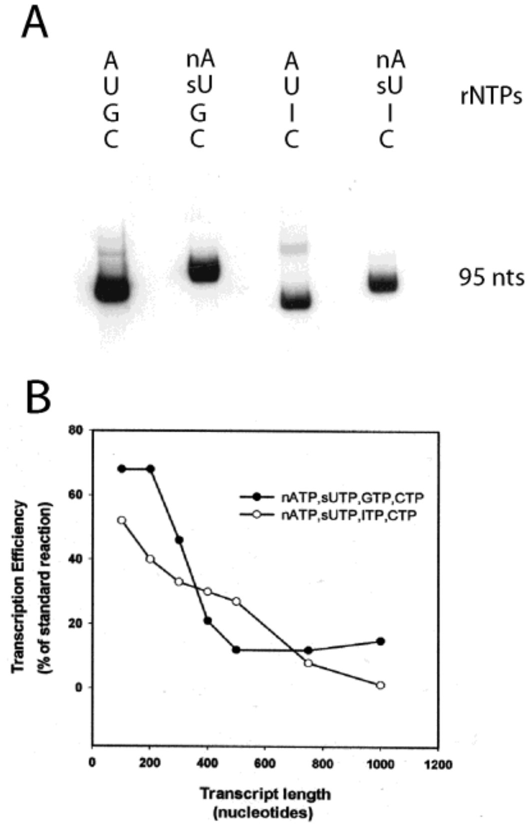


FIGURE 2: Uptake of nATP, sUTP, and ITP into RNA by T7 RNA polymerase. (A) Synthesis of a 95 nucleotide long runoff transcript from *Hin*dIII-restricted pTRIamp18 (Ambion) using different combinations of NTPs. (B) Synthesis of marker RNAs (100, 200, 300, 400, 500, 750, and 1000 nucleotides in length) from the RNA Century Marker Plus Template Set (Ambion) using different combinations of NTPs. The yield of modified RNA relative to unmodified RNA is plotted as a function of transcript length for nA/sU or nA/sU/I substitution.

stranded RNAs, which partly recapitulated half of the hairpin sequence, was also prepared using the same combinations of NTPs. These RNAs varied in their capacity to form a shorter hairpin structure, such as shown in Figure 3 for the SS32 RNA. DNA probes were hybridized to these transcripts, and the resulting DNA−RNA hybrids were tested for their hybridization properties. All of the transcripts were radiolabeled and were used directly after desalting on a spin column. The longest of these transcripts (SS32) when prepared with regular NTPs contained a stable secondary structure that was inaccessible to DNA probes.

Determination of Melting Temperatures of Short Hybrids by Gel Mobility Shift Assay. We developed a gel-based assay to monitor the melting of duplexes in solution that is 10−100-fold more sensitive than the standard optical melting technique and more versatile than the filter-based assay described by Wallace et al. (*29*). A schematic of the method as applied to a radiolabeled DNA−RNA hybrid or an RNA hairpin is shown in Figure 4A. Intermolecular hybrids were prepared by rapid heating and cooling of a labeled target strand (such as a transcript; usually 10−20 nM) with a complementary oligomeric probe (1 μM) in an appropriate buffer. Following annealing, the solution was placed in an ice bath and divided into aliquots, which were incubated in a thermocycler for 5 min at temperatures ranging from 10 to 90 °C. Before the aliquots were placed back in an ice bath, a "competitor" oligonucleotide was rapidly mixed into

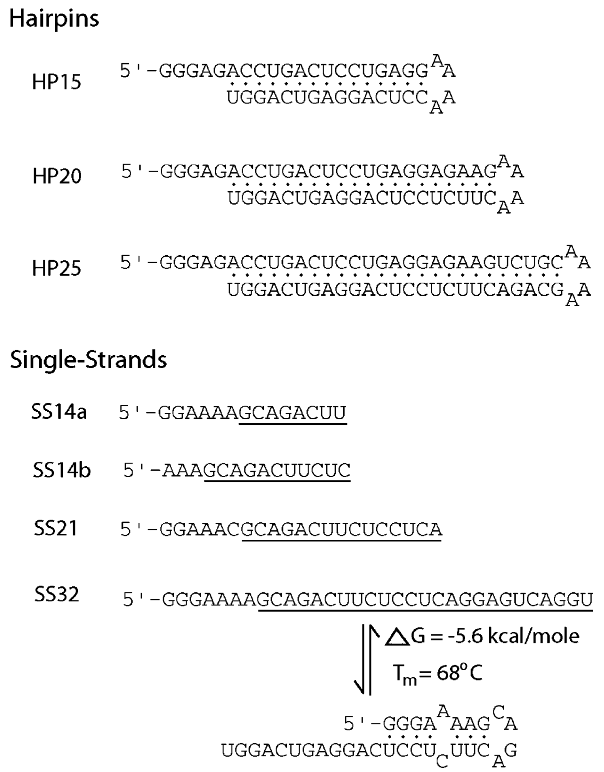


FIGURE 3: Hairpin and single-stranded RNAs used in this study. SS14a and SS14b were chemically synthesized and ^{32}P end-labeled with T4 polynucleotide kinase. The other RNAs were prepared by runoff transcription in the presence of [α-^{32}P]CTP with T7 RNA polymerase. Different versions of the same transcript were obtained by substituting NTP analogues for regular NTPs. The underlined sequences of the single-stranded RNAs are homologous to the 3′-arm of hairpin HP25.

the solution. The competitor was complementary in sequence to the probe and present in 10-fold molar excess. Upon cooling approximately 10% of any free target strand and competitor hybridized to the now limited amount of probe. Each reaction series was next electrophoresed through a native 12% polyacrylamide gel (containing 5 mM $MgCl_2$) in the cold room to resolve the hybridized and single-stranded forms of radiolabeled target (as shown for the melting of a 12 bp DNA−DNA hybrid in Figure 4B). Analysis of partitioning of the target between the free form and in complex with the probe allowed construction of a melting profile, in which the midpoint between the high- and low-temperature plateaus is the apparent melting temperature (T_m). Since the melting profiles were not determined under equilibrium conditions, they were not to be used for thermodynamic measurements.

Using a 12 bp DNA hybrid (at 2 μM) and a competitor DNA of the same length as that of the target strand, the T_m determined by the gel shift was 40 °C, which was almost identical to the 41 °C determined by the spectrophotometric technique (Figure 4D). This verified the utility of the gel shift assay. To address if the assay maintained the same utility when using a competitor with a different backbone, which might skew the hybridization profile, we tested the same 12 bp hybrid but with a different competitor, in the form of RNA. The results showed that, despite differences in the two melting profiles, each yielded the same T_m for the test hybrid (Figure 4E). It should be noted that a higher percent of the test hybrid remained resistant to the RNA competitor relative to the DNA competitor. This is consistent with unpublished work showing that the RNA competitor formed a less stable hybrid with the DNA probe (T_m = 34 °C) than did the DNA target strand (T_m = 40 °C). In contrast, gel shift assays in which the competitor has a greater affinity for the probe than does the target strand risk underestimating the T_m of the test hybrid. While such competitors can reduce the amount of test hybrid obtained at low temperature, the T_m derived from the melting profile remains accurate as long as the stability difference between target-probe and competitor-probe hybrids is not too great (data not shown). To avoid time-dependent alteration of the ratio between the target-probe and competitor-probe hybrids, the competitor should be added at the conclusion of the 5 min incubation (instead of at the beginning), followed by rapid cooling of the solution in an ice bath.

A representative gel mobility shift series for the melting of a labeled 25 bp long RNA hairpin is presented in Figure 4C. Because of the hairpin structure, there was no requirement for a competitor DNA. Instead, a DNA probe (5 μM) that was complementary to one side of the hairpin stem was added to each aliquot of the hairpin after the 5 min incubation. As the temperature of the incubation increased, the hairpin began to melt, exposing the complementary RNA sequence to the probe. The hybrid migrated in two forms on the native PAGE, which may be attributable to alternative secondary structure features in the single-stranded RNA overhang.

Reduction of the Secondary Structure of RNA Containing 2-Aminoadenine, 2-Thiouracil, and/or Inosine. The RNA hairpins in Figure 3 were synthesized by T7 RNA polymerase with various combinations of regular and analogue NTPs to assess the extent to which substitution with nA, sU, and I reduced intramolecular base pairing. The melting profiles of selected hairpins were determined by the gel shift assay, using DNA probes that were complementary to one side of the entire stem region ("full-length" probes). For hybridization to occur, two conditions had to be satisfied. First, the hairpin had to be single stranded and largely free of secondary structure. Second, the probe had to form a stable hybrid with the complementary RNA sequence. Usually, these criteria are difficult to satisfy since the T_m of a hairpin is much higher than the T_m of the corresponding probe-target hybrid. By preparing RNA hairpins with pseudocomplementary bases, we hoped to overcome this situation by equalizing the T_m's.

The melting profile of a 25 bp RNA hairpin (HP25) that contained nA, sU, I, and C bases was monitored by its accessibility to a 25-mer DNA probe in two different buffers (Figure 5A). In buffer 1 (25 mM NaCl, 20 mM Hepes, pH 7.5), the 25-mer probe hybridized completely to the modified hairpin at temperatures as low as 45 °C. In buffer 2, which is the same as buffer 1 except with the presence of 5 mM $MgCl_2$, the probe also hybridized fully to the modified hairpin but at a higher temperature of 60 °C, consistent with the notion that Mg^{2+} ion stabilized the intramolecular RNA hairpin structure. At temperatures above 55 °C in buffer 1 the hybridization of probe to hairpin dropped off, possibly as a consequence of the T_m of the DNA−RNA hybrid being surpassed. According to this model, upon rapid cooling in an ice bath refolding of the RNA into a hairpin was favored over its intermolecular hybridization to the DNA probe.

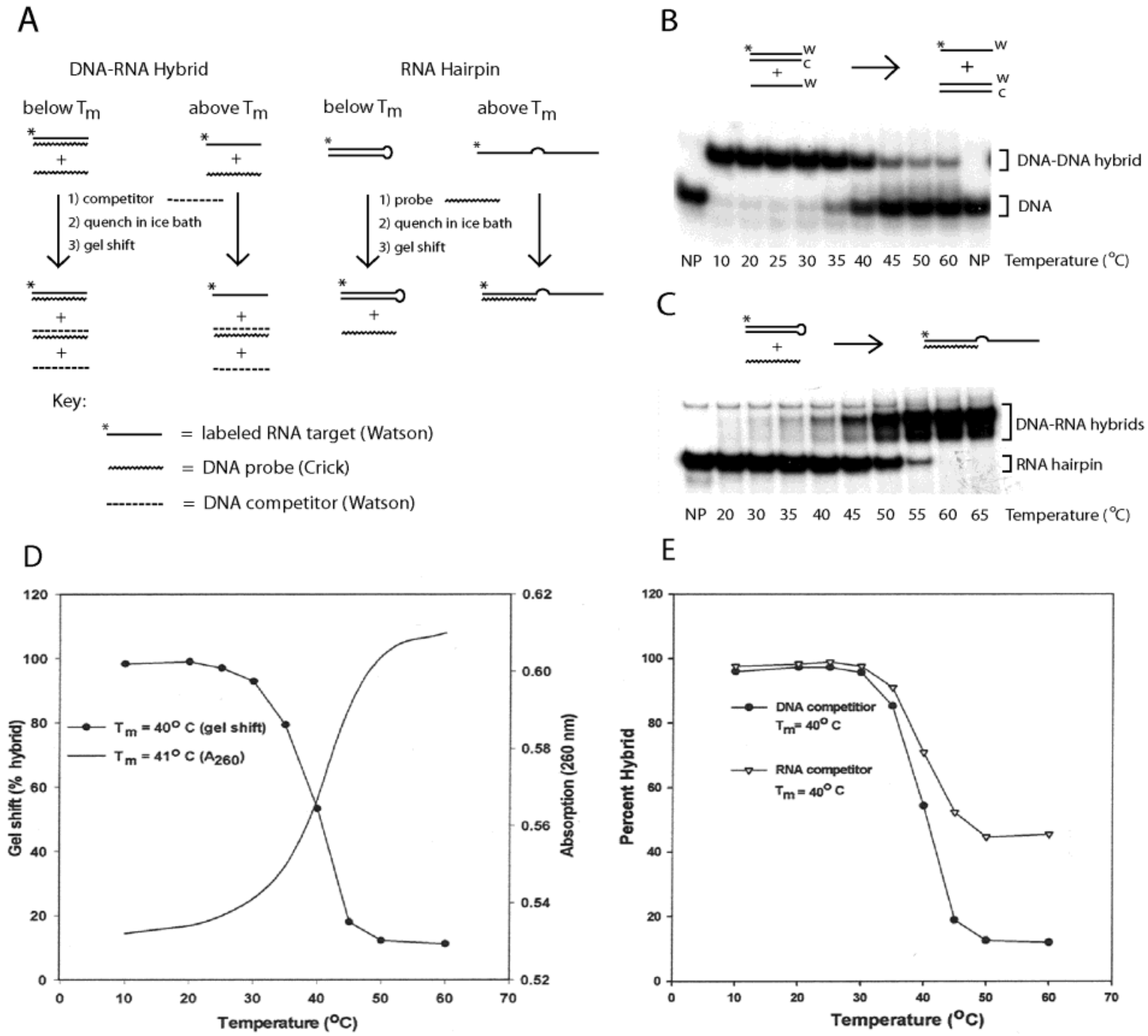


FIGURE 4: Validation of the gel mobility shift assay for determining melting temperature. (A) Protocol for determining the melting temperature of a short hybrid or hairpin by gel mobility shift assay. (B) Gel mobility shift pattern for melting of a 12 bp DNA−DNA hybrid present at 10 nM concentration in buffer 1 (25 mM NaCl, 20 mM Hepes, pH 7.5). Electrophoretic analysis was carried out in a 12% polyacrylamide gel prepared in 90 mM Tris−borate buffer and 5 mM $MgCl_2$ and set up in a cold room. The model hybrid had a target strand sequence of 5′-GCAGACTTCTCC-3′. NP = no probe added. (C) Melting profiles of the model DNA−DNA hybrid (2 μM concentration in buffer 1) determined by spectrophotometric and gel shift methods. The competitor concentration in the gel shift method was 20 μM. (D) Melting profiles of the model DNA−DNA hybrid (10 nM concentration in buffer 1) when using a DNA or RNA competitor (10 μM concentration) in the gel shift method. (E) Gel mobility shift pattern for melting of HP25 in buffer 2 (5 mM $MgCl_2$, 25 mM NaCl, 20 mM Hepes, pH 7.5). The hairpin contained nA, sU, I, and C and was present at 20 nM concentration. A 25-mer DNA probe (5 μM concentration) was used to trap any hairpin in the single-stranded state. NP = no probe added.

Modified versions of hairpins HP20 and HP15 were designed and tested. Both were completely accessible to full-length probes at permissive temperature (not shown). Using the gel shift assay, the T_m of HP25, HP20, and HP15, substituted with nA, sU, and I, was determined to be 48, 45, and 36 °C, respectively, in the presence of 5 mM $MgCl_2$ and to be 38, 33, and 26 °C in the absence of $MgCl_2$. As expected, the unmodified hairpins were not accessible to full-length probes at any temperature up to 90 °C. When analyzed by the program Mfold (*30*, *31*), all three unmodified hairpins were predicted to have T_m's greater than 100 °C. The accessibility of the modified RNA hairpins demonstrates that substitution with nA, sU, and I significantly reduced the strength of RNA secondary structure.

RNA hairpins that were only substituted with nA and sU, or with I alone, were inaccessible to full-length probes. In the special case of nA/sU-substituted RNA hairpins, however, efficient hybridization was observed when the length of the probe was increased. The longer probes extended past the core stem of the hairpin and could hybridize to bases in the single-stranded leader sequence or to bases in the hairpin loop. The accessibility of longer probes to the 25 bp RNA hairpin containing the nA/sU pair was monitored as a function of temperature (Figure 5B). While no hybridization was observed with 25-mer and 26-mer probes, longer probes of 27−30 nucleotides in length gave V-shaped profiles of different magnitude. Conceivably, the longer probes hybridized to the hairpin by a two-step mechanism, where nucleation of base pairing occurred in the first step and strand invasion in the second. In this two-step mechanism, the "melting" should reflect the frequency with which productive base pairing was initiated. While increasing the temperature should initially promote fraying of the hairpin and nucleation of the probe, further increases in temperature would inhibit

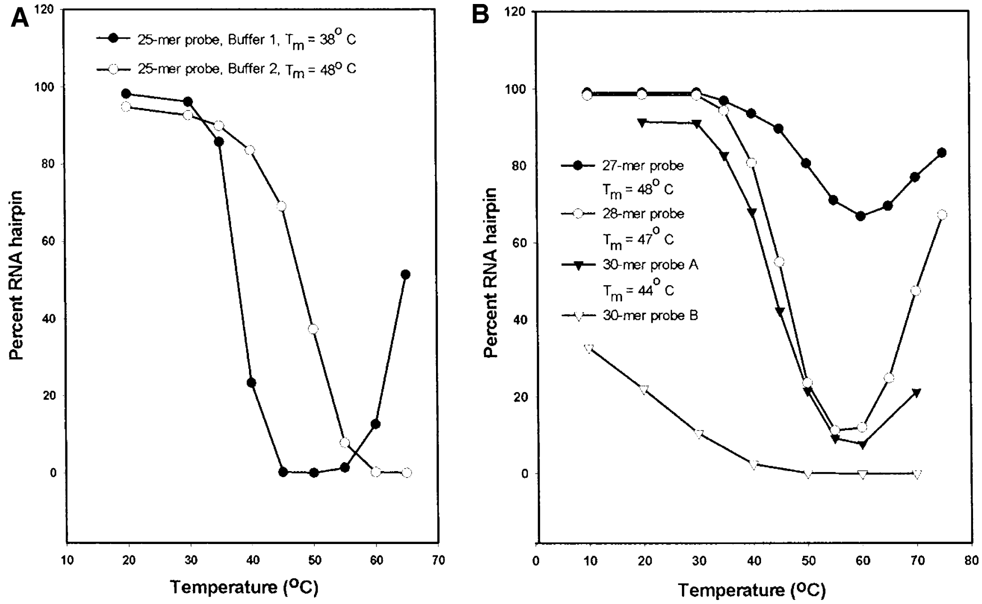
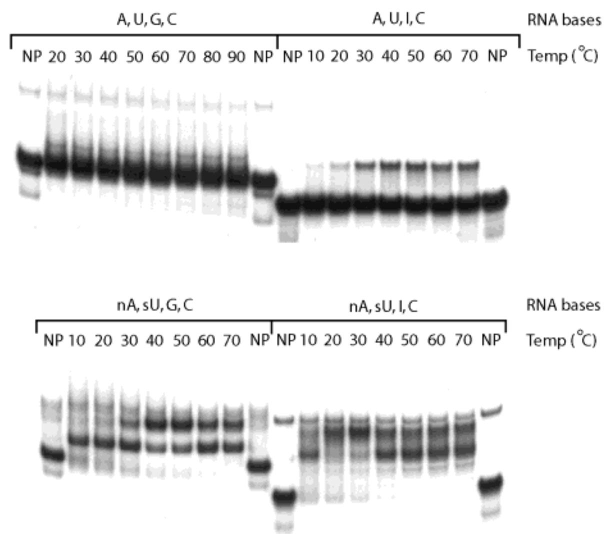


FIGURE 5: Apparent melting profiles of HP25 transcripts with (A) nA, sU, I, and C or (B) nA, sU, G, and C bases. Analyses in (B) were conducted in buffer 1 (25 mM NaCl, 20 mM Hepes, pH 7.5) while those in (A) were conducted in buffers 1 and 2 (5 mM $MgCl_2$, 25 mM NaCl, 20 mM Hepes, pH 7.5).

nucleation by reducing hybrid stability. Notably, probe B in Figure 5B was unique in that it hybridized to the hairpin at all temperatures. This 30-mer probe was complementary to five bases of the leader sequence, a length sufficient to enable productive nucleation even at low temperatures. Probe A, also a 30-mer, was complementary to three bases of the leader sequence and two bases of the hairpin loop, neither of which supported efficient nucleation at low temperature. Strand invasion of the hairpin by longer probes implies that the DNA−RNA hybrid was more stable than the RNA hairpin.

To address the importance of the modified bases in reducing the secondary structure of RNA hairpins, the ability of probe B to invade the HP25 was examined as a function of temperature for transcripts that contained different combinations of bases (Figure 6). Four RNA transcripts were generated, containing normal bases with no substitution (A, U, G, C), substitution of G with I, substitution of A and U with nA and sU, or substitution of A, U, and G with nA, sU, and I, respectively. The two hairpins that contained nA and sU were able to hybridize to the probe at all temperatures, resulting in a shift of the labeled hairpin. Interestingly, hybridization gave rise to two distinct products, which remain to be identified. In the case of the I-substituted hairpin, hybridization occurred at elevated temperatures, although there appeared to be equilibrium established between the hybrid and hairpin. The low ratio of hybrid to hairpin indicates that the 30 bp hybrid was less stable than the 25 bp hairpin. In contrast, the unmodified hairpin failed to hybridize to the probe at all of the temperatures tested. This clearly emphasizes that the presence of any of these substitutions can reduce the secondary structure of RNA hairpins but that the combination of nA and sU is much more effective than the action of I alone.

Reduction of the secondary structure of the RNA hairpin should confer single-stranded character to the RNA. To test this hypothesis, we asked whether a set of tiled 15-mer DNA probes could hybridize to an HP25 hairpin that contained all three modifications, nA, sU, and I, at 55 °C in the



Target: 5′-GGGAGACCUGACUCCUGAGGAGAAGUCUGC A A
UGGACUGAGGACUCCUCUUCAGACG A A

Probe: 3′-CCCTCTGGACTGAGGACTCCTCTTCAGACG

FIGURE 6: Hybridization of a 30-mer probe to HP25 transcripts with different combinations of bases. Reactions were conducted for 5 min at temperatures ranging from 10 to 90 °C in buffer 2 with 5 μM probe and 20 nM transcript. Following quenching in an ice bath, an aliquot of each reaction was analyzed electrophoretically. NP = no probe added.

presence of 5 mM $MgCl_2$. The melting profile of this hairpin (see Figure 5A) indicated that it should be primarily single stranded under these conditions. The DNA probes had overlapping sequences so that they collectively encompassed the entire region of the RNA that had the potential to form a hairpin. The same set of probes formed stable hybrids under identical conditions with a similarly modified single-stranded RNA (data not shown). Following hybridization, the reactions were placed in an ice bath and analyzed by electrophoresis in a nondenaturing polyacrylamide gel. The resulting gel shift

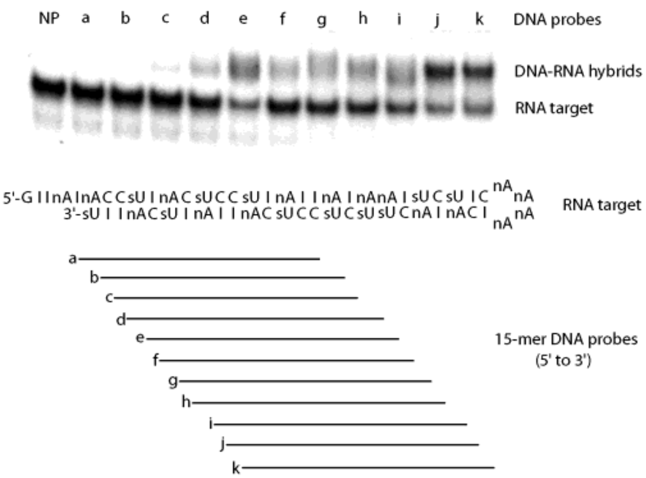


FIGURE 7: Hybridization of tiled 15-mer probes to an HP25 transcript with nA, sU, I, and C bases. Reactions were conducted for 10 min at 55 °C in buffer 2 with 5 μM probe and 20 nM transcript. Following quenching in an ice bath, an aliquot of each reaction was analyzed by electrophoresis. NP = no probe added.

Table 1: Melting Temperatures of a 12 bp DNA−RNA Hybrid with nA, sU, and/or I Substitutions in the RNA Strand[a]

base composition of RNA	T_m (°C) 0 mM $MgCl_2$	T_m (°C) 5 mM $MgCl_2$
A, U, G, C	37	52
nA, sU, I, C	52	68
nA, sU, G, C	57	72
A, U, I, C	21	39
A, sU, G, C	52	71
nA, U, G, C	32	48

[a] The sequence of the probe was 5′-GGAGAAGTCTGC-3′. SS21 RNAs with the indicated bases were prepared by in vitro transcription.

pattern (Figure 7) showed that the hybridization signal increased as the target sequence shifted toward the hairpin loop. Presumably, all of the probes hybridized to the single-stranded RNA target at 55 °C. Upon cooling, however, intramolecular I-C pairing that initiated from the hairpin loop refolded the RNA and disrupted downstream probe-target hybrids. In contrast, those hybrids that were located adjacent to the stem loop blocked initiation of this process and were not displaced by branch migration.

Stability and Specificity of Base Pairing to the RNA Target Containing Base Modifications. The SS21 single-stranded RNA target (Figure 3) was synthesized with different base analogues (nA, sU, and/or I) and used to form a 12 bp hybrid with a complementary DNA probe. The stability of this RNA−DNA hybrid was determined by the gel shift assay. If the presence of the base analogues enhanced intermolecular base pairing, this would increase the melting temperature of the hybrid. Analysis of the melting temperatures, summarized in Table 1, showed that the unmodified hybrid had a T_m of 37 °C in buffer 1. However, introduction of nA, sU, and I into the same RNA increased the T_m to 52 °C. Introduction of only nA and sU increased the T_m further to 57 °C, indicating that the presence of I had a destabilizing effect. This can be explained by the ability of I to base pair with C in only two hydrogen bonds, rather than the three hydrogen bonds between G and C. The destabilizing effect of I is also evident when I was introduced alone, which decreased the T_m to 21 °C. In contrast, sU had a stabilizing effect, which is supported by the increase of the T_m to 52 °C upon introduction of sU alone. The stabilizing effect of sU has been attributed to several factors including increased stacking interactions, increased rigidity of the backbone favoring hybridization, and increased acidity of the N3 proton that strengthens hydrogen bonding (*15*). Interestingly, although nA was expected to stabilize the RNA−DNA hybrid by forming the more stable nA•T base pair (three hydrogen bonds, Figure 1C), introduction of this base analogue had mixed results. Whereas the T_m of the nA-containing hybrid (32 °C) was somewhat lower than the unmodified hybrid (37 °C), the T_m of the nA/sU-containing hybrid (57 °C) was higher than the sU-containing hybrid (52 °C). Each of the individual substitutions was also tested in buffer 2. These yielded parallel effects, confirming the properties of these substitutions in general.

Table 2: Melting Temperatures of Perfect-Match and Single-Mismatch 12 bp DNA−RNA Hybrids with Regular or nA/sU-Substituted RNA[a]

RNA target with A, U, G, C bases: mismatch	T_m (°C)	ΔT_m (°C)	RNA target with nA, sU, G, C bases: mismatch	T_m (°C)	ΔT_m (°C)
none	52		none	72	
T-U	41	11	T-sU	60	12
G-U	45	7	G-sU	43	29
C-U	35	17	C-sU	44	28
A-C	36	16	A-C	37	35
T-C	38	14	T-C	35	37
C-C	34	18	C-C	NH	
A-A	37	15	A-nA	41	31
G-A	41	11	G-nA	46	26
C-A	37	15	C-nA	51	21
A-G	39	13	A-G	43	29
T-G	42	10	T-G	47	25
G-G	42	10	G-G	43	29
av mismatch	39	13	av mismatch	45	27

[a] Hybrids were formed in buffer 2 with unmodified SS21 and nA/sU-substituted SS32 RNAs. The perfect-match probe was 5′-GGAGAAGTCTGC-3′. Mismatch probes contained a single base substitution in the underlined region. NH = no hybrid detected.

The specificity of the DNA−RNA hybridization was addressed by determining the effect of mismatches on melting temperature. While mismatches would reduce the T_m of a hybrid, we expected that the reduction would be more dramatic with higher specificity. The SS21 RNA was synthesized with normal bases, or with the nA/sU substitutions, and was hybridized to 12-mer DNA probes containing single mismatches. Analysis of the T_m of the hybrids by the gel shift assay (Table 2) showed that the presence of mismatches had a greater effect when the RNA was substituted with nA and sU than when it was unmodified. For example, the hybrid of the RNA containing the nA/sU modifications had a T_m of 72 °C, but the presence of a G-sU mismatch lowered the T_m by 29 °C to 43 °C. In contrast, the hybrid of the RNA without modifications had a T_m of 52 °C, but the presence of a G-U mismatch lowered the T_m by only 7 °C to 45 °C. Similarly, the C-sU mismatch lowered the T_m of the hybrid containing the modified RNA by 28 °C, but the corresponding C-U mismatch lowered the T_m of the hybrid without modification by 17 °C. While different mismatches had different effects, the average decrease in T_m attributable to a mismatch was 27 °C in hybrids that contained modified RNA and 13 °C in hybrids that contained unmodified RNA. Thus, the specificity of hybridization was

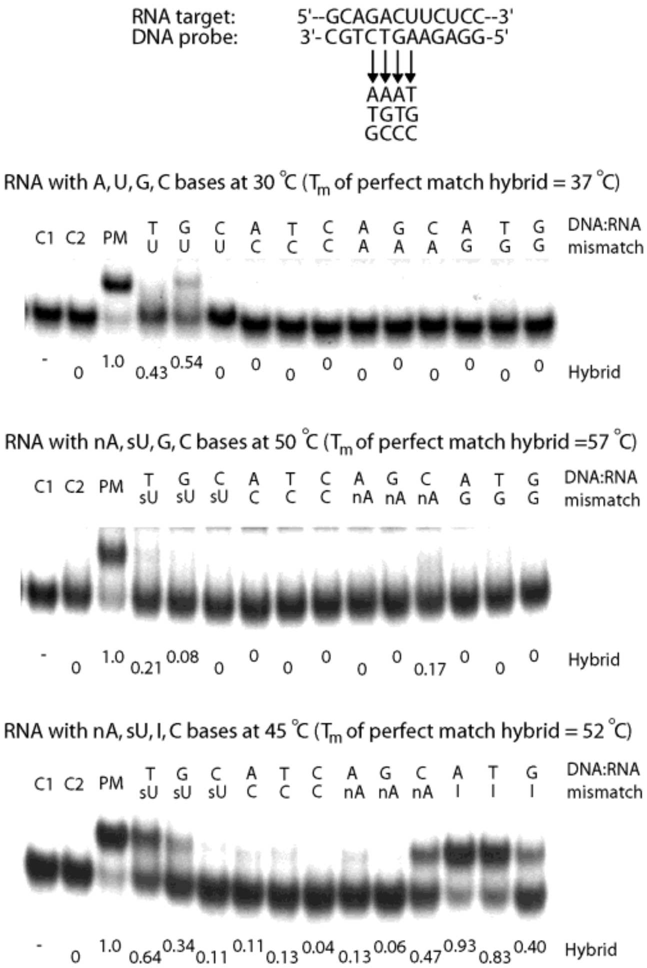


FIGURE 8: Hybridization of perfect-match and single-mismatch 12-mer probes to the same sequence in single-stranded control and nA-, sU-, and I-substituted RNAs. Each of 13 probes (1 μM) was incubated for 5 min in 10 μL of buffer 1 with three separate radiolabeled transcripts (10−20 nM). The control RNA was unmodified SS21. The two modified RNAs were base-substituted versions of SS32. Hybridization reactions were terminated by adding a competitor oligonucleotide (5 μM) and analyzed as described for the melting point determinations. PM = perfect-match hybrid; C1 = no probe added; C2 = competitor oligonucleotide added prior to 5 min incubation with the perfect-match probe.

much greater for the hybrid that contained modified RNA.

The effect of the modified bases on the ability of the SS21 RNA to hybridize to DNA probes containing single mismatches was evaluated under stringent conditions. The SS21 RNA was synthesized with normal bases or with nA/sU or nA/sU/I substitutions. The hybridization between the RNA and 12-mer DNA probes was conducted at a temperature 7 °C below the T_m of the perfect-match hybrid. This temperature was 30 °C for the unmodified RNA, 50 °C for the nA/sU-substituted RNA, and 45 °C for the nA/sU/I-substituted RNA. At the conclusion of the hybridization, the ratio of the annealed to free RNA was fixed by adding a competitor DNA identical in sequence to the radiolabeled target and placing the solution in an ice bath. The fraction of RNA that hybridized to the probe was analyzed by the gel shift assay, and the results were normalized to the perfect-match hybrid (Figure 8). The unmodified RNA showed no hybrid formation in the presence of several mismatches, including C•U, A•C, T•C, C•C, A•A, G•A, C•A, A•G, T•G, and G•G, confirming the capacity for discrimination against

Table 3: Variation in Melting Temperatures of a Set of 12 bp DNA−RNA Hybrids with Regular or nA/sU/I-Substituted RNA[a]

tiled 12-mer DNA probes	G/I-C base pairs in hybrid (%)	T_m (°C) bases in RNA target: A, U, G, C	nA, sU, G, C	nA, sU, I, C
5′-GTCTGCTTTTCC	50	37	62	40
5′-AGTCTGCTTTTC	42	33	58	40
5′-AAGTCTGCTTTT	33	31	55	42
5′-GAAGTCTGCTTT	42	44	58	45
5′-AGAAGTCTGCTT	42	51	55	44
5′-GAGAAGTCTGCT	50	46	55	45
melting range (°C)		20	7	5
% RNA in hybrid[b]		1.5	88	88

[a] Hybrids were formed in buffer 2 with unmodified SS14a and SS14b RNAs and in buffer 1 with nA/sU- and nA/sU/I-substituted SS32 RNAs. [b] This is a measure of the efficiency of hybrid formation following the heat−cool cycle.

these mismatches. However, this RNA did not discriminate against T•U or G•U, forming hybrids at 43% or 54% of the level observed with the perfect-match probe (Figure 8, top panel). The RNA substituted with nA and sU, however, showed improved discrimination, as the frequency of hybrid formation at T•sU or G•sU decreased to 21% and 8%, respectively (middle panel). Thus, the presence of sU in RNA improved specificity over U. With the exception of a low level of hybrid formation with C to form the C•nA mismatch, the presence of nA in RNA maintained the same specificity as A. Interestingly, the nA/sU-substituted RNA containing I had a much reduced specificity. This RNA displayed hybrid formation with mismatches that were not tolerated by the unmodified RNA, including C•nA, A•I, T•I, and G•I (bottom panel). As reported by others (*32*, *33*), inosine mispaired to A, T, and G. Its presence also decreased the specificity of both sU and nA.

Partial Equalization of Base Pairing Strength in RNA Substituted with nA and sU. Introduction of nA and sU into RNA stabilizes DNA−RNA duplexes by replacing A-T and A-U base pairs with the more stable nA-T and A-sU base pairs. Given that the modified base pairs are closer in stability to G-C base pairs, thermal stability of modified DNA−RNA hybrids should be less dependent on G/C content. To test this expectation, we chose the SS32 RNA as the target (Figure 3) and formed hybrids with several 12-mer DNA probes to determine the stability of these hybrids in which G-C base pairs accounted for 33−50% of base composition (Table 3). The SS32 RNA in these hybrids contained regular bases or was substituted with nA/sU or nA/sU/I. When the SS32 RNA target contained modified bases, the selected sequences readily hybridized to complementary probes. However, when the RNA contained no modification, the selected sequences were inaccessible to probes due to participation in secondary structure (Figure 3). To circumvent this problem, two 14-mer RNAs (SS14a and SS14b) were chemically synthesized as targets. Low levels of hybridization sufficient for T_m determination were observed with these two targets.

The melting temperatures of the various DNA−RNA hybrids are listed in Table 3. The hybrids with nA/sU substitution showed much less variation in T_m than did the unmodified hybrids. For example, the unmodified hybrids ranged in T_m from 51 °C (with the AGAAGTCTGCTT

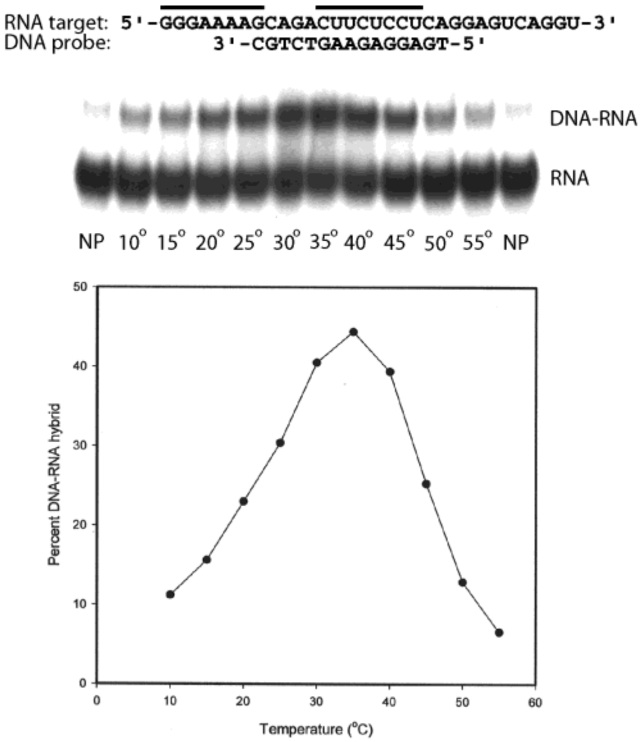


FIGURE 9: Hybridization of a 15-mer probe to a hairpin element in unmodified SS32 RNA. Reactions were conducted over different temperatures with 1 μM probe and 10 nM transcript in 10 μL of buffer 1. After 5 min incubation the reactions were quenched in an ice bath and analyzed by electrophoresis. NP = no probe added.

probe) to 31 °C (with the AAGTCTGCTTTT probe), displaying a difference of 20 °C. In contrast, the nA/sU-substituted hybrids ranged in T_m from 62 °C (with the GTCTGCTTTTCC probe) to 55 °C (with the AAGTCTGCTTTT probe, for example), displaying a difference of 7 °C. This confirmed our expectation that base pairing strength in the modified hybrids was less influenced by base pair composition. The same effect was observed with the nA/sU/I-substituted hybrids, which ranged in T_m from 45 to 40 °C. The remarkably high variation in T_m of the regular DNA−RNA hybrids might be attributable to base pair composition, as well as to secondary structure in the target RNA. It has been shown that self-structure in a probe or target can depress the melting temperature of an intermolecular hybrid (*34*). With hybridization efficiencies of less than 2%, the unmodified RNA targets could suffer from secondary structure that depressed the T_m of each hybrid to a differing extent. This source of variation in T_m was not a factor in hybrids substituted with nA and sU, because these base analogues destabilized the secondary structure element.

Substitution of Structured RNA with nA and sU Facilitates Hybridization to DNA Probes. A strong indication of secondary structure in the unmodified SS32 RNA (Figure 3) was obtained when a 15-mer DNA probe was hybridized to this radiolabeled target as a function of temperature. Following hybridization, the reactions were placed in an ice bath until the entire set could be analyzed by the gel shift assay in the cold room. Figure 9 demonstrates that hybridization peaked at 35 °C, whereas at lower temperatures the probe had increasing difficulty in gaining access to the complementary sequence that was part of a stable hairpin element in the RNA target. At temperatures higher than 35 °C, hybridization of any sort became increasingly unlikely. When such reactions were quenched in ice, rapid cooling favored the intramolecular hairpin formation over the intermolecular probe-target hybrid formation. Only one hairpin structure out of the several predicted by MFold could account for the secondary structure in the target sequence. The putative base pairing elements of this hairpin in the SS32 sequence are indicated by the bars.

The SS32 hairpin provided an opportunity to test whether substitution of a structured RNA with nA and sU could facilitate hybridization to short DNA probes by reducing intramolecular base pairing. The radiolabeled SS32 RNA, either unmodified or substituted, was hybridized to a set of 12-mer probes (Figure 10). Hybridizations were conducted at 10 °C in the presence of 5 mM $MgCl_2$, and hybrids were detected by electrophoresis in a native 12% sequencing gel in a cold room. While the unmodified RNA was inaccessible to most of the complementary probes, due to secondary structure, the modified RNAs that contained nA/sU were accessible to most probes. The gel shift patterns show that every probe hybridized to the RNA with nA/sU/G/C bases, producing hybrids with unique gel mobilities. In RNA substituted with I alone, or together with nA/sU, the gel shift pattern was less consistent, suggesting interference by residual secondary structure and dissociation of hybrids during electrophoresis. Increasing the stringency of hybridization by raising the temperature or removing $MgCl_2$ from the buffer did not alter the results for any of the RNA targets.

DISCUSSION

The modified bases 2-aminoadenine and 2-thiouracil meet the definition of a pseudocomplementary base pair. Whereas the nA-sU couple acts as a nonpairing substitute for A-U in RNA, the nA-T/U and A-sU couples are stable base pairs in both RNA duplexes and DNA−RNA hybrids. In this respect nA and sU function similarly in RNA as nA and sT in oligodeoxynucleotides and peptide nucleic acids (*12*, *13*). Previously we proposed that steric clash between the 2-amino group of nA and the 2-thiocarbonyl group of sT interferes with hydrogen bonding between these bases. The same interaction probably obstructs pairing between nA and sU. In the DNA−RNA hybrid studied here the presence of A-sU and nA-T base pairs afforded significant stabilization, consistent with the induction of an A-form duplex. Single substitution experiments demonstrated that the A-sU base pair accounted for most of the stabilization. Introduction of each sU residue raised the T_m by 5−6 °C whereas introduction of each nA or I residue lowered the T_m by 2 or 6−8 °C, respectively. When these RNA substitutions were introduced together into the same hybrid, the stabilization attributable to A-sU base pairs predominated.

We have shown that nATP and sUTP can be incorporated into short transcripts by T7 RNA polymerase and that the resulting RNA has reduced secondary structure. Hybridization of short DNA probes to this RNA was promoted by both enhanced accessibility to target sequences and increased stability of the newly formed DNA−RNA hybrids. Under conditions where probes can form stable hybrids, long single-stranded DNA and RNA targets possess substantial secondary structure that can reduce accessibility. This is particularly true for RNA targets, since RNA duplexes are usually more stable than DNA−RNA hybrids (*35*, *36*). Substitution of RNA with nA and sU thus provides a partial solution to the

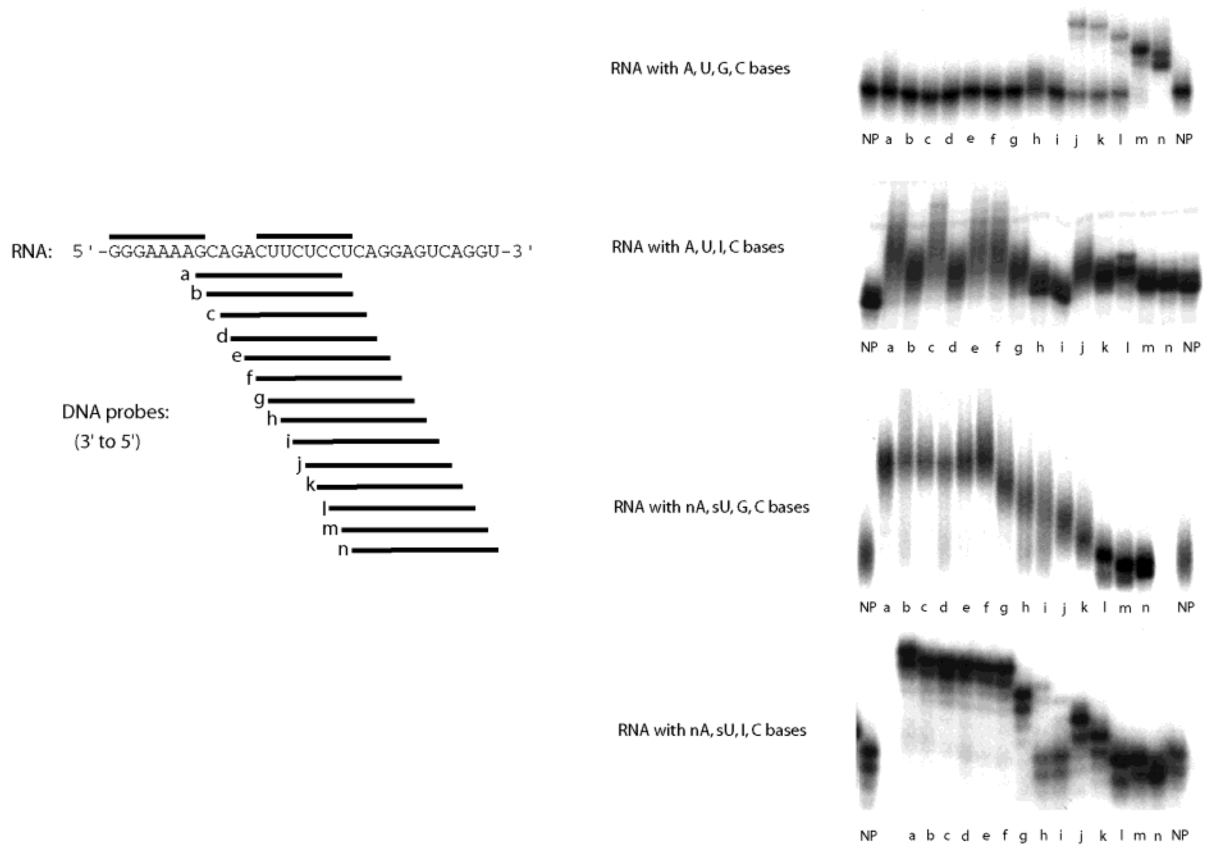


FIGURE 10: Hybridization of tiled 12-mer probes to SS32 transcripts with different combinations of bases. Probe (1 μM) and transcript (10 nM) were incubated at 10 °C in 5 μL of buffer 2. After 5 min competitor oligonucleotide (7.5 μM final concentration) was added, and reaction aliquots were analyzed in a 40 cm long, 1 mm thick 12% polyacrylamide gel prepared in the presence of 1 mM $MgCl_2$. Electrophoresis was conducted in a cold room. NP = no probe added.

problem of secondary structure. However, previous studies in which RNA was substituted with 2-aminoadenine, 5-methyluracil, 5-propynyluracil, or 5-methylcytidine did not provide compelling evidence that the modified RNA was a better target for DNA probes (*2*, *37*, *38*). It should be noted that substitution of an RNA target with a single base pair stabilizing analogue would be expected to enhance both intramolecular pairing in the RNA and intermolecular pairing in the DNA−RNA hybrid. In this case, no advantage would be gained unless the hybrid was stabilized to a greater extent than the RNA self-structure.

Prior to hybridization many nucleic acid targets are amplified and labeled by enzymatic reactions. This presents an opportunity for preparing modified DNA or RNA targets with reduced secondary structure. In unpublished work we found that dnATP and dsTTP were efficiently incorporated into long primer extension products by T7 DNA polymerase. However, the current study showed that the synthesis of long transcripts by T7 RNA polymerase was significantly reduced by nATP and sUTP. If such synthesis were more efficient, enzymatic incorporation of the pseudocomplementary nA-sU couple into RNA could be seamlessly incorporated into amplification protocols that rely upon transcription as the final step in generating labeled RNA from a DNA or RNA sample (*39*−*41*). In vitro engineering of T7 RNA polymerase to improve the uptake of modified NTPs such as nATP and sUTP might provide a solution to this issue.

Preparation of truly structure-free DNA or RNA would require the development of a pseudocomplementary G-C base pair. This task is nontrivial since three hydrogen bonds already exist in a G-C pair. Elimination of G-C base pairing by substitution of G or C with a nonpairing base analogue would require the presence of a second base analogue capable of pairing to the first in templates for synthesis of the modified target and in probes that hybridize to it. One possibility would be to substitute 6-thioguanine (sG) for G. Even though the sG-C couple is unstable (*42*−*45*), sG is taken up into DNA (*46*−*48*) and RNA (*49*) opposite C residues in the template. Besides not pairing with C, sG does not support G-tetrad formation (*43*, *50*, *51*). DNA probes targeted to sG-modified DNA or RNA would necessarily have to contain an analogue of C that hydrogen bonded to sG. Several years ago Rappaport (*42*, *46*) described a stable base pair between 6-thioguanine and 2-pyrimidinone (P). The sG-P base pair was equal in stability to an A-T base pair, and neither base analogue formed a stable pair with any of the four standard bases. Protocols have been described for the synthesis of P-substituted oligonucleotides (*42*, *52*, *53*).

Our results indicate that most RNA secondary structure can be eliminated or substantially reduced when A, U, and G are replaced by nA, sU, and I. Unfortunately, mispairing attributable to I precludes the use of such RNA as a target in hybridization assays. In this context 7-deazaguanine (cG) might be an acceptable substitute for G. The NTPs of cG are readily incorporated into DNA (*54*, *55*) and RNA (*23*). Stability of the cG-C base pair is greater than I-C but less than that of G-C (*56*, *57*). Unlike G, cG does not participate in Hoogsteen pairing, thus eliminating another source of potential secondary structure (*58*). Importantly, there are no reports that this base exhibits degeneracy in pairing. Short of developing a new pseudocomplementary G-C pair, the use of cG in combination with nA and sT/sU warrants further investigation.

Development of a robust enzymatic protocol for preparing structure-free pseudocomplementary DNA and RNA would provide major benefits in the use of DNA probes. While short probes are uniquely suited to the detection of single nucleotide polymorphisms and point mutations, removal of secondary structure in the target would permit the use of shorter probes that possess the minimal length required for specificity. Hybridization efficiency as well as reproducibility would be expected to increase. Equalization of base pairing strength, as shown here with nA and sU substitution, would reduce or eliminate the variation in hybrid stability attributable to base pair composition. Pseudocomplementary targets would facilitate the development of generic oligonucleotide microarrays consisting of every possible permutation of a given probe. In the future, such arrays could find application in the resequencing of specific genes or transcripts for SNP identification or in the detection of specific organisms or cell types based on unique hybridization profiles or "signatures". Other potential applications of pseudocomplementary nucleic acid include nanopore sequencing, DNA computing, and nanodevice fabrication.

The enhanced stability of DNA−RNA hybrids that contain nA and sU in the RNA strand permits the use of shorter DNA probes than would otherwise be possible. When we determined the shortest DNA probe that could stably hybridize to a single-stranded sequence in regular RNA and nA/sU-substituted RNA, gel mobility shift analysis (at low temperature in the presence of $MgCl_2$) indicated that this limit was an 11-mer for regular RNA and a 10-mer for modified RNA (data not shown). For applications requiring even shorter probes, such as universal microarrays, the use of peptide nucleic acid or locked nucleic acid probes would have merit. Both types of oligomers form unusually stable hybrids with DNA and RNA (*10*, *11*).

In the course of studying base-modified RNA, we developed a new gel mobility shift assay for determining the melting temperatures of hybrids that contained a radiolabeled transcript or synthetic DNA/RNA oligonucleotide. While one cannot extract thermodynamic parameters from the melting profile, the technique has certain advantages over the traditional optical melting protocol. Approximately 150−200 μL of 1−10 nM hybrid is sufficient for determining a melting profile with the gel shift technique versus 1 mL of 0.2−2.0 μM hybrid required for optical melting. Since the ratio of free to annealed target is determined by detecting each species in a gel, the radiolabeled target need not be purified as long as the relevant bands can be detected in the gel. For the same reason, one can determine the melting temperatures of hybrids that are formed with low efficiency or hybrids in which the target is several times longer than the probe. The primary drawback of this technique is the processing time and the lack of real-time data acquisition. On occasion, we have been unable to resolve target from hybrid in the gel, thus preventing acquisition of a melting profile. Finally, care must be exercised when the competitor-probe hybrid differs greatly in stability from the target-probe hybrid, leading to the determination of an incorrect T_m. This can be addressed by using competitor oligonucleotides that mimic the target strand as much as possible.

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