The 2,6-Diaminopurine Riboside•5-Methylisocytidine Wobble Base Pair: An Isoenergetic Substitution for the Study of G•U Pairs in RNA†

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ABSTRACT: Phylogenetically invariant G•U wobble pairs are present in a wide variety of RNA's. As a means to study the contribution of individual chemical groups within a G•U pair, we have synthesized and thermodynamically characterized oligoribonucleotides containing the unnatural nucleosides 2,6-diaminopurine riboside (DAP) and 5-methylisocytidine (^{Me}iC). The DAP•^{Me}iC pair at the end of an RNA duplex is as stable as a G•U pair, consistent with formation of a wobble base pair with two hydrogen bonds. DAP•^{Me}iC is a valuable substitution for the study of G•U wobble pairs because it is conformationally similar to the G•U pair, but has a different array of functional groups in the major and minor grooves of the duplex and a reversed hydrogen bonding polarity between the bases. We also report the stability of several other terminal pairs proposed to be in a wobble configuration including inosine•U (I•U), A•^{Me}iC, DAP•C, A•C, G•5-methyl-U, 2′-deoxyguanosine•U, and 2′-deoxy-7-deazaguanosine•U. These pairs present a diversity of functional group substitutions in the context of a wobble conformation. Comparison of wobble pairs with and without the N2 exocyclic amine, i.e., G•U vs I•U, DAP•^{Me}iC vs A•^{Me}iC, and DAP•C vs A•C, demonstrates that the amine does not contribute to base pairing stability when the pair is located at the terminal position of the RNA duplex. However, at a position internal to the duplex, the exocyclic amine does improve helix stability. An internal I•U pair is less stable (≈1 kcal•mol^{-1}) than an internal G•U pair, and substantially less stable (≈2 kcal•mol^{-1}) than an internal A-U pair. These data provide quantitation for the reduced duplex stability observed upon conversion of A-U to I•U pairs by double-stranded RNA adenosine deaminase (dsRAD). This collection of wobble pairs will help identify the contribution made by individual functional groups in RNA/protein interactions and in the tertiary folding of RNA.

The G•U wobble pair originally proposed by Crick (1966) is a significant element of RNA secondary structure (Figure 1). Incidental G•U wobble pairs which are not phylogenetically conserved occur frequently within helices that otherwise contain Watson−Crick pairs (Gutell et al., 1994; Michel et al., 1989; Michel & Westhof, 1990). Mutation of such a G•U to a Watson−Crick pair has no negative effect on the structural integrity of the RNA. However, in other cases the G•U pair is nearly invariant. Mutation to a Watson−Crick pair is not observed phylogenetically, presumably because the wobble pair makes a special contribution to the structure and function of the RNA (Gutell et al., 1994; Michel et al., 1989; Michel & Westhof, 1990). In these cases, the G•U pair might be a critical element for higher order RNA folding or RNA/protein interactions. A wobble pair is a likely candidate for such a role because it has a unique local helical conformation (Holbrook et al., 1978, 1991), and it presents a distinctive array of functional groups in the major and minor grooves of the RNA duplex (Figure 1).

Examples of G•U pairs that are critical to RNA function include one that defines the 5′-exon/intron boundary in group I self-splicing introns (Doudna et al., 1989; Barfod & Cech, 1989; Green et al., 1991; Pyle et al., 1994), one in the acceptor stem of tRNA^{Ala} that is the major recognition element for binding by its cognate synthetase (Musier-Forsyth et al., 1991), and one at the cleavage site of the hepatitis delta virus ribozyme (Wu et al., 1993; Been, 1994). There is a conserved G•U pair within domain 5 of the group II intron (Michel et al., 1989) and a semiconserved G•U in the P6 helix of the group I intron (Michel & Westhof, 1990). A G•U pair might be involved in mRNA splicing when the G at the 5′-exon boundary of pre-mRNA (position −1) pairs with a phylogenetically invariant U in the loop of U5 snRNA (Newman & Norman, 1991, 1992; Wyatt et al., 1992; Moore et al., 1993). Two adjacent and invariant G•U pairs are present in an RNA identified by combinatorial selection that forms a hydrophobic pocket capable of binding valine (Majerfeld & Yarus, 1994).

A large number of phylogenetically conserved G•U pairs are present throughout the 16S and 23S rRNA's (Gutell et al., 1994). One helix in 16S rRNA (829−840:857−846) contains 5 or more G•U or U•G pairs among 10−12 total base pairs, though the exact position of the wobble pairs within the helix is variable. Many of the invariant G•U pairs throughout the rRNA's are near the ends of helices or adjacent to bulged regions (Gutell et al., 1994).

Considering the presumed importance of G•U pairs, we would like to examine the contribution of individual functional groups within the wobble pair to RNA structure and

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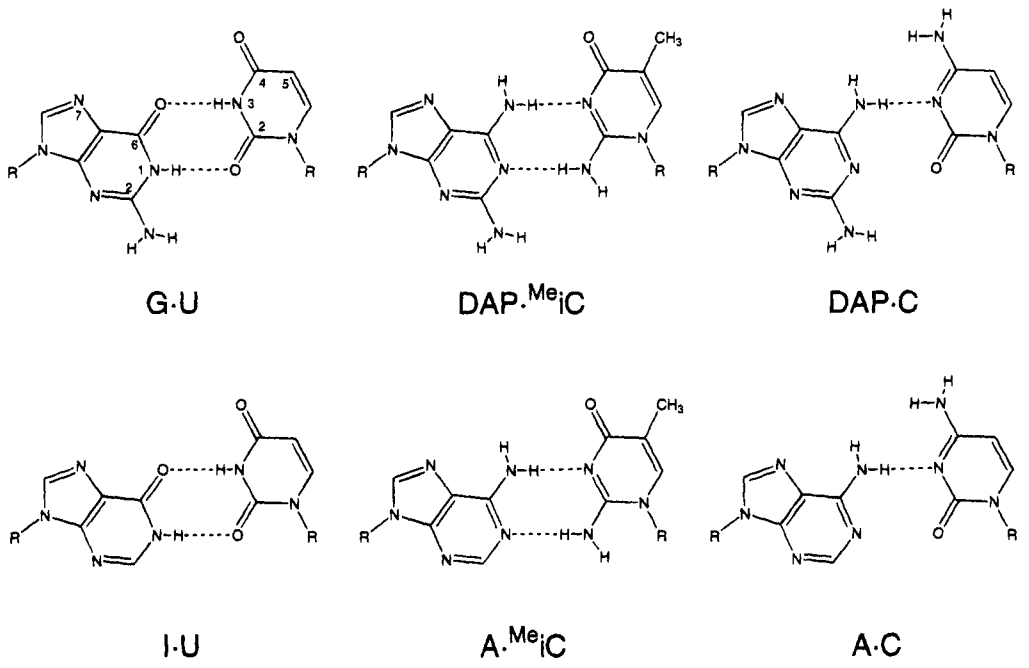

FIGURE 1: Hydrogen bonding and base configuration of proposed wobble pairs.

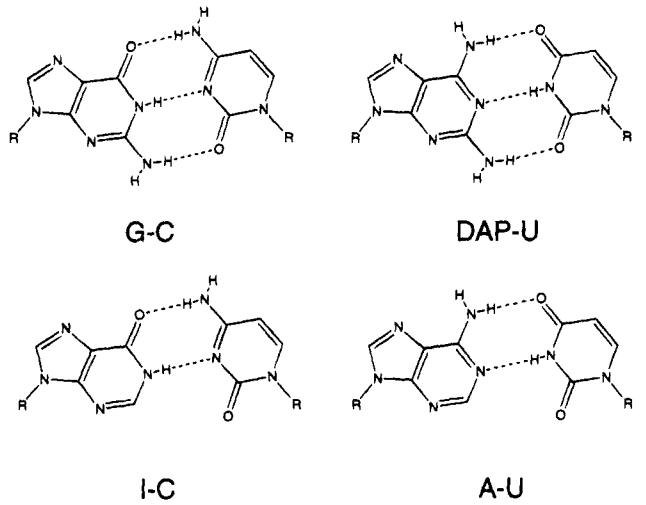

FIGURE 2: Hydrogen bonding and base configuration of base pairs with a Watson–Crick pairing scheme.

function. Such a characterization is hindered by the fact that other stable pairing combinations of the natural bases change the base pair to a Watson–Crick conformation (Figure 2) and thereby alter the structural context of the functional groups. One exception is the weak A•C pair which can be drawn in a wobble configuration, but with only one hydrogen bond in its unprotonated form (Figure 1). Although the A•C pair has weak base pairing properties (Hickey & Turner, 1985), mutation of the G•U to an A•C pair has been used to demonstrate the importance of the wobble conformation (Doudna et al., 1989). In addition, a G•U to A•C mutation is occasionally observed in phylogenetic comparisons (Gutell et al., 1994).

In an effort to extend the repertoire of analogs available for the study of G•U wobble pairs, we have synthesized and thermodynamically characterized a terminal 2,6-diaminopurine riboside•5-methylisocytidine (DAP•^{Me}iC)[1] base pair. The synthesis of ^{Me}iC solid support and DAP phosphoramidite facilitate incorporation of these novel bases into RNA oligonucleotides by automated synthetic methods. Duplex stability is measured for these bases with several pairing partners. This series includes the wobble pairs inosine•U, A•C, DAP•C, A•^{Me}iC, and DAP•^{Me}iC (Figure 1). These pairs provide a diversity of functional group substitutions within a wobble context and will help to define the role of specific G•U functional groups in RNA tertiary structure and RNA/protein recognition.

MATERIALS AND METHODS

Synthesis of Nucleoside Phosphoramidites and Solid Supports. Inosine (I) and 7-deaza-2′-deoxyguanosine (d7dG) phosphoramidites were purchased from ChemGenes and Glen Research, respectively. *N*-Acetyl protected cytidine phosphoramidite and *N*-acetyl protected cytidine polystyrene solid support were purchased from Pharmacia. The phosphoramidite of the purine riboside was prepared as previously described (Fu et al., 1993). Preparation of phosphoramidites and solid supports of 5-methylisocytidine, 5-methyluridine, and 2,6-diaminopurine riboside (Figure 3) followed the general procedures of Usman et al. (1987) and Scaringe et al. (1990).

Nucleoside synthesis was carried out using reagent grade chemicals as received unless otherwise stated. Dry solvents were purchased in sure seal containers under N_2. Diaminopurine riboside (2-aminoadenosine) was purchased from RI Chemical Inc. (Orange, CA). Control pore glass long chain alkyl amine solid support was purchased from Sigma. Polystyrene solid support was obtained from Applied Bio-

[1] Abbreviations: iC, isocytidine; ^{Me}iC, 5-methylisocytidine; ^{Me}U, 5-methyluridine (synonymous with ribothymidine); DAP, 2,6-diaminopurine riboside (synonymous with 2-aminoadenosine); I, inosine; dG, 2′-deoxyguanosine; d7dG, 7-deaza-2′-deoxyguanosine; Pur, purine riboside; DMT, 4,4′-dimethoxytrityl; DMTCl, 4,4′-dimethoxytrityl-chloride; TBDMS, *tert*-butyldimethylsilyl; TBDMSCl, *tert*-butyldimethylsilyl chloride; DMAP, 4-dimethylaminopyridine; THF, tetrahydrofuran; DCC, dicyclohexylcarbodiimide; AMA, an equal mixture of 40% $MeNH_{2(aq)}$ and $NH_4OH_{(conc)}$; PAGE, polyacrylamide gel electrophoresis; IGS, internal guide sequence of the *Tetrahymena* ribozyme.

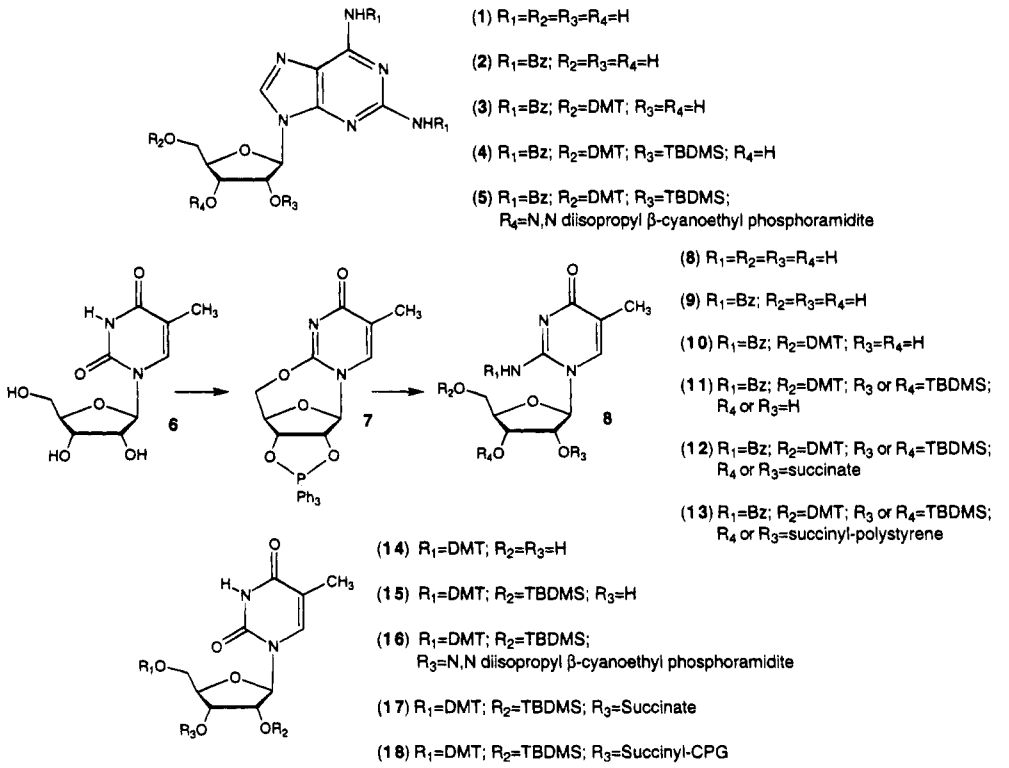

FIGURE 3: Scheme for the synthesis of nucleoside phosphoramidites and solid supports for 2,6-diaminopurine riboside (DAP), 5-methylisocytidine (^{Me}iC), and 5-methyluridine (^{Me}U).

systems. 5-Methyluridine was synthesized from tri-*O*-benzoyl-1-*O*-acetyl-D-ribofuranose and thymine as previously described (Mansuri et al., 1989). A 12 M solution of methanolic ammonia was prepared by bubbling NH_3 (g) through methanol at 0 °C. NMR spectra were recorded on a Varian XL-400 instrument operating at 400 MHz (^{1}H) and 121 MHz (^{31}P). Chemical shifts are reported in ppm relative to the solvent residual signal. FAB mass spectra were recorded on a VG 7070 EQ-HF mass spectrometer. Thin layer chromatography (TLC) was performed on silica gel 60 F-254 pre-coated plates. Flash column chromatography was carried out using silica gel grade 62 (60−200 mesh).

N^2,N^6-Dibenzoyl-2,6-diaminopurine Riboside (2). Diaminopurine riboside (**1**) (4.17 g, 14.8 mmol) was suspended as a slurry in dry pyridine (80 mL), chilled on ice, and reacted dropwise with benzoyl chloride (10.3 mL, 88.7 mmol, 6 equiv). The reaction was warmed to room temperature. The starting material dissolved completely within 30 min. The reaction was complete after 2 h as monitored by TLC. The reaction was quenched with methanol (5 mL) and concentrated *in vacuo*. The crude mixture was redissolved in methanol (100 mL) and pyridine (3 mL) and reacted for 10 min with sodium methoxide (7.2 g, 133.2 mmol, 9 equiv), quenched with Dowex resin (50 × 8−200 Py^+ form), filtered and washed with pyridine (2 × 100 mL) and methanol (2 × 50 mL), and concentrated. The resulting yellow paste was redissolved in a minimum volume of 10% MeOH/CH_2Cl_2, precipitated in stirring hexane, and collected by filtration to afford a slightly off white crystalline solid. TLC (5% MeOH/CH_2Cl_2) R_f = 0.24. Flash column chromatography (2−5% MeOH/CH_2Cl_2) of a small portion of the material afforded a white crystalline solid. ^{1}H NMR (DMSO-d_6) δ 11.21 (s, 1H, NHBz), 11.01 (s, 1H, NHBz), 8.63 (s, 1H, H8), 8.06 (d, 2H, Ph H2), 7.99 (d, 2H, Ph H2), 7.66−7.49 (m, 6H, Ph H3, Ph H4), 5.98 (d, 1H, H1′), 5.54 (d, 1H, OH2′, D_2O ex), 5.21 (d, 1H, OH3′, D_2O ex), 5.00 (t, 1H, OH5′, D_2O ex), 4.66 (dd, 1H, H2′), 4.18 (dd, 1H, H3′), 3.95 (m, 1H, H4′), 3.68−3.53 (m, 2H, H5′). MS (positive-ion FAB) calculated for $C_{24}N_6O_6H_{22}$ $[MH]^+$ 491; found 491.

5′-O-(4,4′-Dimethoxytrityl)-N^2,N^6-dibenzoyl-2,6-diaminopurine Riboside (3). N^2,N^6-Dibenzoyl-2,6-diaminopurine (**2**) (3.15 g, 6.43 mmol) was dried by coevaporation *in vacuo* with pyridine (3 × 50 mL). A suspension of **2** in dry pyridine (75 mL) was then treated with 4,4′-dimethoxytrityl chloride (DMTCl) (2.4 g, 7.07 mmol, 1.1 equiv) in three equal portions over 1 h. Compound **2** dissolved completely after the second DMTCl addition. On the basis of TLC, the reaction was half complete after 3 h. An additional portion of DMTCl (0.70 g, 2.07 mmol, 0.32 equiv) was added and the mixture left to react overnight. The reaction was quenched with MeOH (30 mL), concentrated to an oil, redissolved in CH_2Cl_2 (100 mL), and washed with saturated $NaHCO_3$ (2 × 50 mL), brine (2 × 50 mL), and water (2 × 50 mL). The organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. Flash column chromatography (1−3% MeOH/CH_2Cl_2) afforded 3.41g (67% yield) of a white crystalline solid. TLC (5% MeOH/CH_2Cl_2) R_f = 0.43. ^{1}H NMR (DMSO-d_6) δ 11.20 (s, 1H, NHBz), 10.91 (s, 1H, NHBz), 8.51 (s, 1H, H8), 8.06 (d, 2H, Ph H2), 7.92 (d, 2H, Ph H2), 7.66−7.49 (m, 6H, Ph H3, Ph H4), 7.32−6.70 (m, 13H, DMT), 6.03 (d, 1H, H1′), 5.63 (d, 1H, OH2′, D_2O ex), 5.18 (d, 1H, OH3′, D_2O ex), 4.76 (dd, 1H, H2′), 4.33 (m, 1H, H4′), 4.05 (m, 1H, H3′), 3.68 (s, 6H, OCH_3), 3.39−3.15 (m, 2H, H5′). MS (positive-ion FAB) calculated for $C_{45}N_6O_8H_{40}$ $[MH]^+$ 793; found 793.

5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-N^2,N^6-dibenzoyl-2,6-diaminopurine Riboside (4). Pyridine (1.05 mL, 13 mmol, 3.7 equiv) and $AgNO_3$ (0.71 g, 4.18 mmol, 1.2 equiv) were added to a mixture of nucleoside **3** (2.78 g, 3.5 mmol) dissolved in THF (40 mL). The mixture

was sonicated extensively to dissolve the $AgNO_3$. *tert*-Butyl dimethylsilyl chloride (TBDMSCl) (0.68 g, 4.52 mmol, 1.3 equiv) was added, and the solution became instantly turbid. After reacting overnight the reaction was about 70% complete, so an additional portion of pyridine (0.24 mL, 3.0 mmol, 0.8 equiv), $AgNO_3$ (0.16 g, 0.94 mmol, 0.28 equiv), and TBDMSCl (0.16 g, 1.06 mmol, 0.3 equiv) was added. After three additional hours the reaction was more than 90% complete with two major products. The 2′-regioisomer had the faster mobility and was produced in a 2:1 ratio over the 3′-regioisomer. The precipitate was removed by filtration through celite into a stirring solution of $NaHCO_3$ (sat), extracted into CH_2Cl_2, and the organic phase washed with $NaHCO_3$ (2 × 50 mL), brine (2 × 50 mL), water (2 × 50 mL), dried (Na_2SO_4), and concentrated *in vacuo*. The residue was purified by flash column chromatography (25−50% EtOAc/hexane) to afford 1.35 g (43% yield) of **4** as a white crystalline material. TLC (1:1 hexane/EtOAC) $R_f = 0.40$. ^{1}H NMR (DMSO-d_6) δ 11.18 (s, 1H, NHBz), 10.79 (s, 1H, NHBz), 8.52 (s, 1H, H8), 8.06 (d, 2H, Ph H2), 7.87 (d, 2H, Ph H2), 7.64−7.48 (m, 6H, Ph H3, Ph H4), 7.33−6.69 (m, 13H, DMT), 6.03 (d, 1H, H1′), 5.05 (d, 1H, OH3′, D_2O ex), 4.98 (t, 1H, H2′), 4.27 (dd, 1H, H3′), 4.07 (unresolved m, 1H, H4′) 3.67 (s, 6H, OCH_3), 3.45−3.17 (m, 2H, H5′), 0.76 (s, 9H, *tert*-butyl), −0.02 (s, 3H, $SiCH_3$), −0.14 (s 3H, $SiCH_3$). MS (negative-ion FAB) calculated for $C_{51}N_6O_8H_{54}Si$ $[M^{2-}H^+]^-$ 905; found 905.

*5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-N^2,N^6-dibenzoyl-2,6-diaminopurine Ribosyl 3′-O-β-Cyanoethyldiisopropylaminophosphoramidite (**5**).* A solution of nucleoside **4** (0.40 g, 0.44 mmol) was dissolved in dry CH_2Cl_2 (10 mL) and cooled to 0 °C. In a separate flask, 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.15 mL, 0.66 mmol, 1.5 equiv), diisopropylethylamine (0.15 mL, 0.88 mmol, 2.0 equiv), and N-methyl imidazole (18 μL, 0.22 mmol, 0.5 equiv) were added to an additional portion of dry CH_2Cl_2 (10 mL), cooled to 0 °C, and added dropwise to the solution of nucleoside **4**. The reaction was warmed slowly to room temperature. NMR of the crude reaction mixture in the region of the H1′ proton showed nearly complete conversion to phosphoramidite **5** after 1 h. The reaction mixture was concentrated and the residue purified by flash column chromatography (1% triethylamine in 1:1 hexane/EtOAc) to afford 353 mg (72% yield) of compound **5** as a white foam. The diastereomer of faster R_f was purified in greater yield due to difficulty separating the slower migrating isomer from the starting material. TLC (1% triethylamine in 1:1 hexane/EtOAc) $R_f = 0.45$ and 0.55. ^{31}P NMR ($CDCl_3$) δ 153.27 and 150.57 (two phosphoramidite diastereomers).

*2′,3′-O-(Triphenylphosphanediyl)-O^2,5′-cyclo(5-methyl)-uridine (**7**).* 5-Methyluridine (**6**) (3.87 g, 15 mmol) and triphenylphosphine (11.8 g, 45.0 mmol, 3.0 equiv) were dissolved in dry THF (30 mL) under argon. A solution of diethylazodicarboxylate (7.0 mL, 45 mmol, 3.0 equiv) dissolved in dry THF (7 mL) was added dropwise. The solution warmed slightly and within 5 min became a gelatin like solid that was quantitatively converted to nucleoside **7**. The solid was crushed and washed extensively with ether followed with 10% THF in ether to afford 7.95 g (53% yield) of nucleoside **7** as an off white powder. TLC (20% MeOH/CH_2Cl_2) $R_f = 0.36$. ^{1}H NMR (DMSO-d_6) δ 7.96 (s, 1H, H6), 7.63−7.28 (m, 15H, Ph), 4.79 (m, 1H, H4′), 4.71 (s, 1H, H1′), 5.54−4.48 (m, 2H, H2′ and H3′), 4.10 (d, 1H, H5′), 1.77 (s, 2H, CH_3). MS (positive-ion FAB) calculated for $C_{28}N_2O_5P_1H_{25}$ $[MH]^+$ 501; found 501.

*5-Methylisocytidine (**8**).* Nucleoside **7** (6.0 g, 12.0 mmol) was combined with 12 M methanolic ammonia (200 mL) in a sealed chamber and heated to 100 °C overnight. After reaction, the solution was filtered to remove a small amount of UV nonabsorbent insoluble residue and concentrated to a paste. The product was redissolved in a minimum volume of MeOH and recrystallized in acetonitrile. Filtration and washing with cold acetonitrile afforded 2.1 g (68% yield) of nucleoside **8**. TLC (20% MeOH/CH_2Cl_2) $R_f = 0.10$. ^{1}H NMR (DMSO-d_6) δ 7.48 (s, 1H, H6), 6.76 (s, 2H, NH2), 5.41 (d, 1H, OH, D_2O ex), 5.37 (d, 1H, H1′), 5.29 (t, 1H, OH5′, D_2Oex), 5.21 (d, 1H, OH, D_2Oex), 4.12 (dd, 1H, H2′), 3.97 (dd, 1H, H3′), 3.89 (unresolved m, 1H, H4′), 3.60 (m, 2H, H5′), 1.70 (s, 3H, CH_3). MS (positive-ion FAB) calculated for $C_{10}N_3O_5H_{15}$ $[MH]^+$ 258; found 258.

*N^2-Benzoyl-5-methylisocytidine (**9**).* A cold suspension of 5-methylisocytidine (**8**) (1.0 g, 3.9 mmol) in dry pyridine (40 mL) was treated dropwise with benzoyl chloride (5.8 mL, 46.7 mmol, 12 equiv). The reaction mixture was allowed to warm to room temperature and stirred for 2.5 h. The reaction was then chilled, quenched by the addition of MeOH (20 mL), and concentrated to an oil. The oil was redissolved in CH_2Cl_2 (100 mL), washed with $NaHCO_3$ (2 × 50 mL), water (2 × 50 mL), dried (Na_2SO_4), and concentrated *in vacuo*. The residue was redissolved in MeOH (40 mL) and reacted with NaOMe (1.97 g, 35.1 mmol, 9 equiv) at room temperature for 5 min. The reaction was quenched with Dowex resin (50 × 8−200 Py^+ form), filtered, washed with pyridine (100 mL) and methanol (50 mL), and concentrated to an oil. The residue was purified by flash column chromatography (5−15% MeOH/CH_2Cl_2) to afford 0.83 g (65% yield) of nucleoside **9** as a white foam. TLC (5% MeOH/CH_2Cl_2) $R_f = 0.16$. ^{1}H NMR (DMSO-d_6) δ 8.29 (s, 1H, H6), 8.19 (d, 2H, Ph H2), 7.57 (t, 1H, Ph H4), 7.47 (t, 2H, Ph H3), 6.37 (d, 1H, H1′), 5.48 (d, 1H, OH2′, D_2O ex), 5.33 (t, 1H, OH5′, D_2O ex), 5.19 (d, 1H, OH3′, D_2O ex), 4.16 (dd, 1H, H2′), 4.05 (dd, 1H, H3′), 3.95 (unresolved m, 1H, H4′), 3.82−3.62 (m, 2H, H5′), 1.87 (s, 3H, CH_3). MS (positive-ion FAB) calculated for $C_{17}N_3O_6H_{19}$ $[MH]^+$ 362; found 362.

*5′-O-(4,4′-Dimethoxytrityl)-N^2-benzoyl-5-methylisocytidine (**10**).* Nucleoside **9** (0.83 g, 2.3 mmol) was dissolved in dry pyridine (40 mL) and DMTCl (0.83 g, 2.5 mmol, 1.1 equiv) was added to the reaction in three equal portions over the period of 90 min. The reaction was 80% complete after stirring overnight so an additional portion of DMTCl (0.16 g, 0.5 mmol, 0.2 equiv) was added. After 3 additional hours, the reaction was more than 90% complete. The unreacted DMTCl was quenched by the addition of MeOH (10 mL), and the solution was concentrated to an oil. The residue was dissolved in CH_2Cl_2, extracted twice with $NaHCO_3$ (sat), once with water, dried (Na_2SO_4) and reconcentrated. Purification of the oil by flash column chromatography (0−3% MeOH/CH_2Cl_2) resulted in 1.16 g (76% yield) of nucleoside **10** as a light yellow foam. TLC (5% MeOH/CH_2Cl_2) $R_f =$ 0.45. ^{1}H NMR (DMSO-d_6) δ 8.22 (d, 2H, Ph H2), 7.84 (s, 1H, H6), 7.59−7.22 (m, 12H, DMT Ph), 6.91 (d, 4H, DMT), 6.36 (s, 1H, H1′), 5.59 (d, 1H, OH2′, D_2O ex), 5.30 (d, 1H, OH3′, D_2O ex), 4.30 (unresolved m, 1H, H2′), 4.22 (dd, 1H, H3′), 4.11 (unresolved m, 1H, H4′), 3.73 (s, 6H, OCH_3), 3.34−3.27 (m, 2H, H5′), 1.41 (s, 3H, CH_3). MS (positive-

ion FAB) calculated for $C_{38}N_3O_8H_{37}$ $[MH]^+$ 664; found 664.

*5′-O-(4,4′-Dimethoxytrityl)-2′,3′-O-(tert-butyldimethylsilyl)-N^2-benzoyl-5-methylisocytidine (**11**).* Compound **10** (1.16 g, 1.75 mmol) was dissolved in dry THF and combined with pyridine (0.52 mL, 6.5 mmol, 3.7 equiv) and $AgNO_3$ (0.36 g, 2.1 mmol, 1.2 equiv). The mixture was sonicated extensively to dissolve the $AgNO_3$. TBDMSCl (0.34 g, 2.3 mmol, 1.3 equiv) was added, whereupon the solution became instantly turbid. Reaction overnight was about 70% complete, so an additional portion of pyridine (0.11 mL, 1.4 mmol, 0.80 equiv), $AgNO_3$ (0.083 g, 0.50 mmol, 0.28 equiv), and TBDMSCl (0.080 g, 0.53 mmol, 0.3 equiv) was added. After three additional hours the reaction was more than 90% complete. The precipitate was removed by filtration through Celite into a stirring solution of $NaHCO_3$ (sat), extracted into CH_2Cl_2, and the organic phase washed with $NaHCO_3$ (2 × 50 mL), brine (2 × 50 mL), and water (2 × 50 mL), dried (Na_2SO_4), and concentrated *in vacuo*. NMR of the H1′ proton region indicated the presence of both isomers in a ratio of approximately 2:1. Because the 2′ and 3′ regioisomers had identical retention in all solvents tested they could not be separated by conventional column chromatography. However, because the oligonucleotides synthesized in this study contain the ^{Me}iC modification exclusively at the 3′-end, and oligonucleotide deprotection and desilylation relieves the regioisometry of the 3′-terminal base, the regioisomeric mixture of nucleosides **11** was used for preparation of the ^{Me}iC solid support. Flash column chromatography afforded 1.16 g (85% yield) of a mixture of regioisomers of nucleoside **11** as a white foam. TLC (3:1 hexane/EtOAc) $R_f = 0.27$.

Preparation of 5-Methylisocytidine Polystyrene Solid Support. Nucleosides **11** (0.71g, 0.92 mmol) were dissolved in pyridine (10 mL), reacted with succinic anhydride (0.92 g, 9.2 mmol, 10 equiv) and 4-dimethylaminopyridine (DMAP) (0.17g, 1.38 mmol, 1.5 equiv), and stirred overnight. The mixture was evaporated to an oil, dissolved in CH_2Cl_2 (50 mL), and washed with 10% citric acid (2 × 30 mL), brine (2 × 30 mL), and water (2 × 30 mL), dried (Na_2SO_4) and concentrated *in vacuo*. Flash column chromatography (0−5% MeOH/CH_2Cl_2) afforded 0.55 g (68.3% yield) of nucleosides **12** as a white foam. TLC (5% MeOH/CH_2Cl_2) $R_f = 0.37$. MS (positive-ion FAB) calculated for $C_{48}N_3O_{11}H_{55}Si$ $[MH]^+$ 878; found 878.

Nucleosides **12** were coupled to a polystyrene solid support (1.2 g) by dissolving **12** (0.175 g, 0.2 mmol) in CH_2Cl_2 (10 mL) with dicyclohexylcarbodiimide (DCC) (0.41 g, 2.0 mmol, 10 equiv). The slurry was placed on a shaker and allowed to react overnight. The polystyrene support was filtered and washed with acetonitrile (3 × 30 mL) and ether (3 × 30 mL) and air-dried. The nucleoside loading was determined by reacting a small portion of the support (3.1 mg) with 3% trichloroacetic acid in CH_2Cl_2 (1.0 mL), followed by 0.1 M toluene sulfonic acid in acetonitrile (3.0 mL) and measuring the optical absorbance at 498 nm. This gave an estimated nucleoside loading of 25 μmol/g of support. Unreacted sites on the support were capped by suspending the polystyrene in pyridine (5 mL), adding acetic anhydride (0.3 mL) and DMAP (0.01 g), and shaking for 3 h. The support was then filtered and washed as described above. The polystyrene support was dispensed into 1 μmol scale portions (40 mg of support) and used for solid-phase oligoribonucleotide synthesis.

*5′-O-(4,4′-Dimethoxytrityl)-5-methyluridine (**14**).* 5-Methyluridine (**6**) (1.66 g, 6.43 mmol) was dissolved in pyridine (25 mL). DMTCl (2.6 g, 7.7 mmol, 1.2 equiv) was added in three equal portions over 2 h and allowed to react overnight. The reaction mixture was concentrated to an oil, dissolved in CH_2Cl_2 (100 mL), washed with $NaHCO_3$ (2 × 50 mL), brine (2 × 50 mL), and water (1 × 50 mL), dried (Na_2SO_4) and reconcentrated *in vacuo*. Flash column chromatography (5−10% MeOH/CH_2Cl_2) of the residue afforded 2.35 g (65.2% yield) of nucleoside **14** as a white foam. TLC (5% MeOH/CH_2Cl_2) $R_f = 0.19$. ^{1}H NMR ($CDCl_3$) δ 10.11 (s, 1H, NH), 7.76 (s, 1H, H6), 7.43−7.24 (m, 9H, DMT), 6.85 (d, 4H, DMT), 5.96 (d, 1H, H1′), 5.40 (unresolved d, 1H, OH2′, D_2O ex), 4.44 (unresolved m, 2H, H2′ and H3′), 4.24 (unresolved m, 1H, H4′), 3.80 (s, 6H, OCH3), 3.56−3.43 (m, 2H, H5′), 3.30 (unresolved d, 1H, OH3′, D_2O ex), 1.44 (s, 3H, CH3). MS (negative-ion FAB) calculated for $C_{31}N_2O_8H_{32}$ $[M^{2+}H]^-$ 559; found 559.

*5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-5-methyluridine (**15**).* Nucleoside **14** (2.15 g, 3.84 mmol) was dissolved in THF (30 mL) and combined with pyridine (1.15 mL, 14.2 mmol, 3.7 equiv) and $AgNO_3$ (0.78 g, 4.6 mmol, 1.2 equiv). After sonicating extensively to dissolve the $AgNO_3$, TBDMSCl (0.75 g, 5.0 mmol, 1.3 equiv) was added to the reaction which became instantly cloudy. After reacting overnight the reaction was examined by TLC. The starting material was more than 90% reacted with a 5:1 ratio of the regioisomers. The precipitate was removed by filtration through Celite into a stirring $NaHCO_3$ (sat) solution, extracted into CH_2Cl_2, and the organic phase washed with $NaHCO_3$ (2 × 50 mL), brine (2 × 50 mL), and water (2 × 50 mL), dried (Na_2SO_4), and concentrated *in vacuo*. Flash column chromatography of the residue (25−50% EtOAc/hexane) afforded 2.08 g (80% yield) of nucleoside **15** as a white foam. TLC (1:1 hexane/EtOAc) $R_f = 0.36$. ^{1}H NMR ($CDCl_3$) δ 8.15 (s, 1H, NH), 7.69 (s, 1H, H6), 7.43−7.27 (m, 9H, DMT), 6.86 (d, 4H, DMT), 6.06 (d, 1H, H1′), 4.51 (dd, 1H, H2′), 4.31 (unresolved m, 1H, H3′), 4.19 (m, 1H, H4′), 3.82 (s, 6H, OCH3), 3.56−3.38 (m, 2H, H5′), 2.76 (unresolved d, 1H, OH3′, D_2O ex), 1.37 (s, 3H, CH3), 0.95 (s, 9H, *tert*-butyl), 0.16 (s, 3H, $SiCH_3$), 0.15 (s, 3H, $SiCH_3$). MS (negative-ion FAB) calculated for $C_{37}N_2O_8H_{46}Si$ $[M^{2+}H]^-$ 673; found 673.

*5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-5-methyluridine-3′-O-β-cyanoethyldiisopropylaminophosphoramidite (**16**).* A solution of nucleoside **15** (0.31 g, 0.46 mmol) was dissolved in dry CH_2Cl_2 (10 mL) and cooled to 0 °C. In a separate flask, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.15 mL, 0.66 mmol, 1.5 equiv), diisopropylethylamine (0.15 mL, 0.88 mmol, 2.0 equiv), and N-methyl imidazole (18 μL, 0.22 mmol, 0.5 equiv) were added to an additional portion of dry CH_2Cl_2 (10 mL), cooled to 0 °C, and added dropwise to the solution of nucleoside **15**. The reaction was warmed slowly to room temperature. NMR of the crude reaction mixture in the region of the H1′ proton showed near complete conversion to phosphoramidite **16** after 1 h. The reaction mixture was concentrated and the residue purified by flash column chromatography (1% triethylamine in 1:1 hexane/EtOAc) to afford 270 mg (67% yield) of compound **5** as a white foam. TLC (1:1 hexane/EtOAc) $R_f = 0.43$ and 0.40. ^{31}P NMR ($CDCl_3$) δ 152.70 and 150.79 (two phosphoramidite diastereomers). MS (posi-

tive-ion FAB) calculated for $C_{46}N_4O_9H_{63}SiP$ $[MH]^+$ 875; found 875.

Preparation of 5-Methyluridine CPG Solid Support. Nucleoside **15** (0.400 g, 0.59 mmol) was dissolved in pyridine (10 mL) and reacted overnight with succinic anhydride (0.59 g, 5.9 mmol, 10 equiv) and DMAP (0.11 g, 0.89 mmol, 1.5 equiv). The mixture was evaporated to an oil *in vacuo*, dissolved in CH_2Cl_2 (50 mL), washed with 10% citric acid (2 × 30 mL), brine (1 × 30 mL), and water (2 × 30 mL), dried (Na_2SO_4), and concentrated. Flash column chromatography (0−5% MeOH/CH_2Cl_2) afforded 0.38 g (84% yield) of nucleoside **17** as a white foam. TLC (5% MeOH/CH_2Cl_2) $R_f = 0.10$. Nucleoside **17** was coupled to a control pore glass solid support (0.5 g) by dissolving **17** (0.155 g, 0.2 mmol) in CH_2Cl_2 (10 mL) with dicyclohexylcarbodiimide (0.41 g, 2.0 mmol, 10 equiv). The slurry was placed on a shaker and allowed to react overnight. The polystyrene support was filtered and washed with acetonitrile (3 × 30 mL), ether (3 × 30 mL), and air-dried. The nucleoside loading was determined to be 27 μmol/g as described above. Unreacted sites were capped by suspending the support in pyridine (5 mL), adding acetic anhydride (0.3 mL) and DMAP (0.01 g), and shaking for 3 h. The support was filtered and washed. The support was dispensed into 1 μmol scale portions (≈40 mg of support) and used for solid-phase oligonucleotide synthesis.

Oligonucleotide Synthesis, Deprotection, and Characterization. Oligoribonucleotide synthesis was carried out as described by Usman et al. (1987) on an Applied Biosystems 394 synthesizer. The coupling efficiency of the unnatural base phosphoramidites was comparable to that obtained with the commercially available phosphoramidites (98−100%). The oligonucleotide was cleaved from the support and the bases deprotected in 1.5 mL of an equal mixture of 40% $MeNH_{2(aq)}$ and $NH_4OH_{(conc)}$ (AMA) at 70 °C for 30 min. *N*-Acetylcytidine phosphoramidite was used in place of *N*-benzoyl-protected phosphoramidite to prevent transamination to *N*-methyl-C upon treatment with AMA (Reddy et al., 1994). No noticeable oligonucleotide degradation was observed under these conditions. The oligonucleotides were dried and desilylated in 1.0 M tetrabutylammoniumfluoride in THF (Aldrich) (0.5 mL). The oligonucleotides were ethanol precipitated, purified to single base resolution by 20% PAGE, visualized by UV shadowing, and eluted from the polyacrylamide in 10 mM Tris, 1 mM EDTA, pH 7.5. The oligonucleotides were concentrated and the residual salts removed by reverse phase column chromatography using a disposable cartridge (Waters). The oligonucleotides were dried and stored at −20 °C until use.

Base Composition Analysis. Oligonucleotide base composition was confirmed by digesting each oligonucleotide (5 nmol) with nuclease P1 (0.4 unit) for 12 h at 50 °C and dephosphorylating with bacterial alkaline phosphatase (Boehringer Mannheim) (4.0 units) at 37 °C for 6 h. The nucleoside products were resolved by reverse-phase HPLC using a Rainin Microsorb ODS 4 × 250 mm column with 50 mM potassium phosphate (pH 7.0) and methanol as buffers (Figure 4).

Oligonucleotide Melting Studies and Thermodynamic Calculations. Oligonucleotide concentrations were calculated from absorbance at 260 nm. For oligonucleotides containing only A, G, C, and U, molar extinction coefficients were calculated from the nearest neighbor approximation

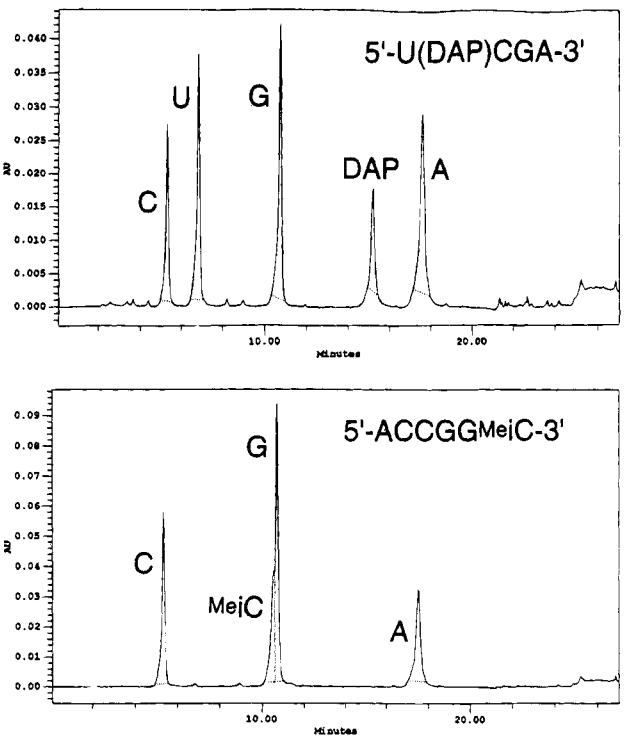

FIGURE 4: Base composition analysis of oligonucleotides containing DAP and ^{Me}iC. (Upper) Chromatogram of oligonucleotide 5′-U(DAP)CGA-3′ containing DAP and all four natural bases. DAP is fully deprotected and transamination of the other bases is not detected. (Lower) Chromatogram of oligonucleotide 5′-ACCGG-^{Me}iC-3′ after gel purification to remove the fraction of the material that was postsynthetically modified during deprotection. Although ^{Me}iC partially elutes with G, the assignment of the shoulder peak was confirmed by coinjection with authentic sample and matching the spectrum of the peak to that of a ^{Me}iC standard. Neither the deamination product 5-methyluridine (elutes just after G at ≈12 min) nor the putative transamination product *N*-methyl-5-methylisocytidine (elutes at ≈23 min) was detected in this oligonucleotide preparation.

(Puglisi & Tinoco, 1989). For oligonucleotides containing unnatural purine base substitutions, molar extinction coefficients were estimated by replacing adenosine ($\epsilon_{260} = 15300$) with purine riboside ($\epsilon_{260} = 6000$), inosine ($\epsilon_{260} = 7100$), 2′-deoxy-7-deazaguanosine (assume equal to G; $\epsilon_{260} = 12200$), or 2,6-diaminopurine riboside ($\epsilon_{260} = 7370$). For oligonucleotides containing pyrimidine substitutions, extinction coefficients were estimated by replacing uridine ($\epsilon_{260} = 10200$) with 5-methylisocytidine ($\epsilon_{260} = 6300$) and 5-methyluridine ($\epsilon_{260} = 9000$).

Absorbance vs temperature curves were measured at 260 nm in 1.0 M NaCl, 1 mM EDTA, and 10 mM sodium cacodylate (pH 7.0) with a computer interfaced Cary 1 spectrophotometer at a heating rate of 1 °C min^{-1}. Van't Hoff enthalpies ($\Delta H°$) and melting temperatures (T_m) were determined according to the derivative method of Gralla and Crothers (Gralla & Crothers, 1973; Marky & Breslauer, 1987). The entropy of formation ($\Delta S°$) was calculated from

$$\frac{1}{T_m} = \frac{R}{\Delta H°} \ln Ct + \frac{\Delta S°}{\Delta H°} \quad (1)$$

for a self-complementary duplex and

$$\frac{1}{T_m} = \frac{R}{\Delta H°} \ln Ct + \frac{(\Delta S° - R \ln 4)}{\Delta H°} \quad (2)$$

for a nonself-complementary duplex (Puglisi & Tinoco, 1989). The reported thermodynamic values are an average of at least three trials at different total strand concentrations (Ct) between 2 and 40 μM. For a subset of the duplexes $\Delta H°$ and $\Delta S°$ were calculated from plots of $1/T_m$ vs ln Ct using eq 1 or 2. Oligonucleotides of the sequence XGAGGG were heated to 85 °C for 5 min prior to the addition of oligonucleotide GGCCCUCY to prevent multimer formation of the G-rich oligonucleotide (Pyle et al., 1994).

RESULTS

Phosphoramidite and Solid Support Synthesis. Phosphoramidites and solid supports of 5-methyluridine and 2,6-diaminopurine riboside and the solid support of 5-methylisocytidine were synthesized and used to incorporate the unnatural nucleosides into RNA oligonucleotides. The 2,6-diaminopurine phosphoramidite was prepared using 2,6-diaminopurine riboside as starting material. Both amino groups were protected by perbenzoylation with benzoyl chloride, and the hydroxyl groups of the ribose were selectively debenzoylated with NaOMe in MeOH. The 5′-hydroxyl was protected by dimethoxytritylation. The 2′-hydroxyl was protected by silylation with *tert*-butyldimethylsilyl chloride (TBDMSCl) in the presence of pyridine and $AgNO_3$ (Hakimelahi et al., 1982). The 3′-hydroxyl was activated with β-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite.

5-Methylisocytidine was synthesized from 5-methyluridine. The *N*-benzoyl-protected solid support of ^{Me}iC (Kimura et al., 1980) was prepared as described above, except instead of synthesizing the phosphoramidite, the mixture of 2′ and 3′ TBDMS regioisomers was used to prepare the succinate derivative. This was coupled to the polystyrene solid support with a loading of 25 μmol/g using dicyclohexylcarbodiimide. The 5-methyluridine phosphoramidite and solid support were prepared using the same substitutions on the ribose hydroxyls as described above. The nucleoside loading on the solid support was 27 μmol/g.

Oligonucleotide Synthesis and Base Composition Analysis. In previous syntheses of DNA oligonucleotides containing 2,6-diaminopurine (DAP) substitutions, difficulty was reported in removal of the protecting group on the N2 amino group (Gaffney et al., 1984; Chollet et al., 1986; Fathi et al., 1990). In these studies, DNA oligonucleotides were incubated at high temperature in concentrated aqueous ammonia for several days to achieve complete deprotection. Such conditions are incompatible with RNA synthesis in good yield.

An equal mixture of 40% $MeNH_{2(aq)}$ and $NH_4OH_{(conc)}$ (AMA) has been reported to give complete deprotection of DNA oligonucleotides in less than 15 min (Reddy et al., 1994). A similar study using AMA for the deprotection of RNA indicates that this method can be applied to oligoribonucleotides (F. Wincott, A. DiRenzo, D. Tracz, C. Shaffer, S. Grimm, C. Workman, D. Sweedler, and N. Usman, manuscript in preparation). To achieve complete deprotection of the DAP, the silylated RNA oligonucleotide was incubated in AMA for 30 min at 70 °C. Base composition analysis demonstrated that the DAP was completely deprotected (Figure 4).

The use of AMA for the deprotection of DNA oligonucleotides has been reported to give 5−10% transamination of C when the base was *N*-benzoyl or *N*-isobutyryl protected, but *N*-acetyl protected 2′-deoxy-C did not undergo this side reaction (Reddy et al., 1994). For this reason *N*-acetyl protected ribo-C was used exclusively in the synthesis of these RNA oligonucleotides. As expected, base composition analysis detected no significant modification of A, G, C or U after treatment with AMA, including transamination of C to *N*-methyl-C (Figure 4). Base composition analysis also demonstrated the integrity of oligonucleotides containing 5-methyluridine, inosine, 2′-deoxyguanosine, and 7-deaza-2′-deoxyguanosine.

Purification of oligonucleotides containing ^{Me}iC was more difficult because ^{Me}iC undergoes post-synthetic modification in strong base. Previous work on 2′-deoxyisocytidine reported a significant level of hydrolytic deamination to 2′-deoxyuridine upon base deprotection with concentrated ammonia (Switzer et al., 1993). Deamination was significantly reduced by introducing a methyl group at the 5 position of the base (Tor & Dervan, 1993). Based upon that observation, we synthesized 5-methylisocytidine (^{Me}iC). However, a low level (4−8%) of deamination to 5-methyluridine (^{Me}U) was still observed upon deprotection of the oligoribonucleotide with 3:1 NH_3(aq):EtOH. Deprotection with AMA also postsynthetically modified the ^{Me}iC. While base composition analysis detected no ^{Me}U, approximately 50% of the ^{Me}iC had been converted to another product. Based on its UV absorbance spectrum (λ_{max} = 302 nm) and retention time, this derivative might be the transamination product, *N*-methyl-5-methylisocytidine. Although this side reaction is substantial, oligonucleotides containing unmodified ^{Me}iC (Figure 4) could be isolated because the oligonucleotide containing the modified base migrated significantly slower in 20% denaturing PAGE than the unmodified oligomer. Based on the observations with *N*-acetyl-C, the extent of the putative transamination reaction might be reduced using *N*-acetyl protected ^{Me}iC.

Measurement of Base Pair Stability. The stabilities of a terminal DAP•^{Me}iC pair and other terminal pairing combinations were measured for the self-complementary sequence XCCGGY, where X represents purine and Y pyrimidine nucleotides (Table 1). This sequence was selected because a complete set of natural base pair combinations for X and Y have been measured in this sequence context (Petersheim & Turner, 1983; Hickey & Turner, 1985; Freier et al., 1985a, 1986a,b; Turner et al., 1987). Although many of the oligonucleotides in the previous studies contained a 3′-terminal phosphate, it contributed very little to duplex stability (Petersheim & Turner, 1983). We repeated a subset of these measurements to confirm the reproducibility of the reported results in our system.

Terminal base pair stabilities were also measured for the non-self complementary sequences GGCCCUCY and XGAGGG which mimic the 5′-exon and internal guide sequence (IGS) of the 5′ splice site duplex of the *Tetrahymena* group I intron, respectively (Table 2). Although our conclusions are based primarily upon the data from the XCCGGY duplex, because it contains two pairs and provides a more accurate measure of base pair stability, all of the pairing stabilities are qualitatively similar for both duplexes. Subtle differences between the results with the two helices are likely due to sequence context effects.

As a preliminary study of the relative stability of A-U, G•U, and I•U pairs at internal positions within an RNA

Table 1: Thermodynamic Parameters for Duplex Formation by the Self-Complementary Oligonucleotide XCCGGY[a]

pair (X, Y)	$-\Delta H^\circ$ (kcal•mol^{-1})[b]	$-\Delta S^\circ$ (cal•mol^{-1}•K^{-1})[b]	$-\Delta G^\circ_{37}$ (kcal•mol^{-1})	T_m (°C)	stability relative to G•U pair (kcal•mol^{-1})[c]
wobble pairs					
G•U	61.50 ± 0.6	168.1 ± 1.7	9.4 ± 0.10	56.7 ± 0.4	(0)
G•U	*58.2*	*158.1*	*9.2*	*57.0*	*(0)*
I•U	55.66 ± 1.7	152.1 ± 5.2	8.5 ± 0.09	53.5 ± 0.2	−0.4
G•^{Me}U	62.51 ± 3.0	169.2 ± 9.0	10.0 ± 0.25	60.2 ± 0.4	0.3
DAP•^{Me}iC	54.10 ± 1.9	145.3 ± 6.2	9.0 ± 0.08	57.4 ± 1.1	−0.2
A•^{Me}iC	57.77 ± 3.0	156.8 ± 9.3	9.1 ± 0.16	56.8 ± 1.0	−0.1
	(55.39)	(149.5)	(9.0)	(56.9)	(−0.2)
DAP•C	47.86 ± 2.4	130.8 ± 7.4	7.3 ± 0.12	47.8 ± 0.7	−1.0
	(50.67)	(139.9)	(7.3)	(47.1)	(−1.0)
A•C	*48.5*	*134.7*	*6.7*	*44.2*	*−1.2*
Watson−Crick pairs					
G-C	67.48 ± 2.4	179.5 ± 7.5	11.8 ± 0.11	68.0 ± 0.7	1.2
G-C	*62.7*	*166.0*	*11.2*	*67.2*	*1.0*
I-C	*53.6*	*146.9*	*8.0*	*51.3*	*−0.7*
DAP-U	57.19 ± 3.3	154.1 ± 9.8	9.4 ± 0.25	58.5 ± 0.6	0.0
	(51.59)	(136.8)	(9.2)	(59.4)	(−0.1)
DAP-^{Me}U	57.93 ± 2.3	153.3 ± 6.8	10.4 ± 0.17	64.3 ± 0.5	0.5
	(58.71)	(155.7)	(10.4)	(64.1)	(0.5)
A-U	54.85 ± 2.2	149.6 ± 6.9	8.5 ± 0.54	53.5 ± 0.4	−0.5
A-U	*59.5*	*164.5*	*8.5*	*52.4*	*−0.4*
A-^{Me}U	58.04 ± 3.4	156.2 ± 9.9	9.6 ± 0.30	59.4 ± 0.5	0.1
other pairs					
G-^{Me}iC	54.89 ± 2.3	148.5 ± 6.9	8.8 ± 0.16	55.9 ± 0.3	−0.3
	(48.84)	(129.7)	(8.6)	(56.8)	(−0.4)
DAP-A	44.63 ± 1.8	121.0 ± 5.6	7.1 ± 0.11	47.1 ± 0.5	−1.1
DAP-G	47.30 ± 0.3	128.4 ± 0.5	7.5 ± 0.14	49.1 ± 0.9	−0.9
A-A	*44.6*	*122.1*	*6.7*	*44.4*	*−1.3*
A-G	*48.4*	*131.2*	*7.7*	*50.8*	*−0.8*
unpaired 5′ extensions					
DAP-( )	35.53 ± 4.5	95.0 ± 14.9	6.1 ± 0.15	40.5 ± 1.6	−1.6
A-( )	*38.6*	*106.4*	*5.6*	*36.4*	*−1.8*
G-( )	*32.5*	*88.9*	*5.0*	*30.6*	*−2.1*
I-( )	*36.1*	*101.2*	*4.7*	*29.0*	*−2.4*
no pair					
( )-( )	*34.2*	*95.6*	*4.6*	*27.1*	*−2.4*

[a] Values in this table represent the average of at least three melting experiments performed in 1.0 M NaCl, 10 mM sodium cacodylate, and 1 mM EDTA at total strand concentrations between 2 and 35 μM. The T_m is calculated for 100 μM oligonucleotide. Values in parentheses are calculated from $1/T_m$ vs ln Ct plots. Agreement of ΔH° values within 15% confirms that the oligonucleotide obeys a two-state binding model. Values in italics are from previous work using the same experimental conditions, calculated from plots of $1/T_m$ vs ln Ct (Petersheim & Turner, 1983; Hickey & Turner, 1985; Freier et al., 1985a, 1986a,b; Turner et al., 1987). Many of the oligonucleotides in the prior studies contained a terminal 3′-phosphate. Even though the phosphate contributed very little to duplex stability (Petersheim & Turner, 1983), relative stabilities for the italicized values are calculated relative to the oligonucleotide GCCGGUp which also contains the terminal 3′-phosphate. [b] Four significant figures are reported for ΔH° and ΔS° to prevent the compounding of rounding errors when using the values to calculate ΔG° at other temperatures. [c] Relative stabilities are calculated by subtracting the $-\Delta G^\circ$ of the GCCGGU duplex from the $-\Delta G^\circ$ of the XCCGGY duplex and dividing the result by two. A positive value in this column indicates that the pair is more stable than G•U.

duplex, we studied the self-complementary oligonucleotides GUCUAGXC and GUCUXGAC, where X represents nucleotides A, G, or I (Table 3). All three duplexes obeyed two state melting behavior with sloping upper and lower baselines.

DISCUSSION

We prepared oligoribonucleotides containing the unnatural nucleosides DAP and ^{Me}iC. The goal was to generate a base pair in a wobble conformation that forms two hydrogen bonds and is thermodynamically equivalent to G•U but has a different hydrogen bonding polarity between the bases and different functional groups in the major and minor grooves of the duplex. The thermodynamic characterization indicates that a DAP•^{Me}iC pair achieves these objectives, at least for substitutions at the terminal position of an RNA duplex.

Terminal DAP•^{Me}iC and G•U Pairs Are Isoenergetic. Based on the melting analysis of the self-complementary duplexes, a terminal DAP•^{Me}iC pair is of similar stability to a G•U pair, with a calculated difference of only 0.2 kcal•mol^{-1}. The difference is even less in the context of the 5′-exon•IGS duplex (0.1 kcal•mol^{-1}). Furthermore, DAP•^{Me}iC is significantly more stable than DAP•C which at neutral pH could be stabilized by at most a single hydrogen bond. The DAP•^{Me}iC is 0.8 and 0.4 kcal•mol^{-1} more stable than the DAP•C pair in the self-complementary duplex and the 5′-exon•IGS duplex, respectively. This suggests that the DAP•^{Me}iC pair is stabilized by more than a single hydrogen bond. These two comparisons show that DAP and ^{Me}iC form a stable base pair. Because it is almost energetically equivalent to a G•U pair, we postulate it is in a wobble configuration stabilized by two hydrogen bonds.

Terminal DAP•^{Me}iC and G•U pairs are equivalent despite a significantly different hydrogen bonding pattern between the two base pairs (Figure 1). In a G•U wobble pair the C6 carbonyl of G accepts a hydrogen bond from the N3 of U and the C2 carbonyl of U accepts a hydrogen bond from the N1 of G. For a DAP•^{Me}iC pair drawn in a wobble configuration, the polarity of both the hydrogen bonds is reversed. The N6 amine of DAP donates a proton to the N3 of ^{Me}iC and the N2 amine of ^{Me}iC donates a proton to the N1 of DAP. Thus, hydrogen bond polarity and functional

Table 2: Thermodynamic Parameters for Duplex Formation by XGAGGG with GGCCCUCY, Oligonucleotide Mimics of the *Tetrahymena* Ribozyme 5′-Exon·IGS Duplex[a]

pair (X, Y)	$-\Delta H°$ (kcal·mol^{-1})[b]	$-\Delta S°$ (cal·mol^{-1}·K^{-1})[b]	$-\Delta G°_{37}$ (kcal·mol^{-1})	T_m (°C)	stability relative to G·U pair (kcal·mol^{-1})[c]
wobble pairs					
G·U	54.87 ± 2.7	146.3 ± 8.7	9.5 ± 0.04	54.7 ± 0.8	(0)
G·^{Me}U	56.98 ± 1.8	152.1 ± 5.9	9.8 ± 0.12	55.9 ± 1.0	0.3
I·U	54.80 ± 1.1	146.2 ± 3.6	9.5 ± 0.02	54.5 ± 0.3	0.0
I·^{Me}U	55.77 ± 1.4	149.0 ± 4.3	9.5 ± 0.08	54.7 ± 0.1	0.0
DAP·^{Me}iC	51.78 ± 3.1	136.8 ± 9.8	9.4 ± 0.13	54.8 ± 1.3	−0.1
A·^{Me}iC	51.58 ± 2.2	136.4 ± 6.9	9.3 ± 0.09	54.5 ± 0.8	−0.2
DAP·C	48.85 ± 2.8	128.4 ± 8.9	9.0 ± 0.18	53.7 ± 1.2	−0.5
A·C	47.47 ± 4.8	124.1 ± 15	9.0 ± 0.17	54.0 ± 0.9	−0.5
dG·U	55.57 ± 5.1	149.5 ± 16	9.2 ± 0.04	52.7 ± 1.3	−0.3
d7dG·U	48.53 ± 3.5	127.5 ± 11	9.0 ± 0.03	53.6 ± 1.4	−0.5
Watson−Crick pairs					
G-C	60.43 ± 1.7	160.6 ± 5.4	10.6 ± 0.07	59.6 ± 0.6	1.1
I-C	53.90 ± 2.8	143.2 ± 8.7	9.5 ± 0.08	55.0 ± 0.5	0.0
DAP-U	57.80 ± 1.7	154.6 ± 5.3	9.9 ± 0.08	55.9 ± 0.3	0.4
DAP-^{Me}U	58.30 ± 0.6	155.3 ± 2.2	10.1 ± 0.05	57.4 ± 0.5	0.6
A-U	53.00 ± 0.6	140.2 ± 2.2	9.5 ± 0.11	55.4 ± 1.0	0.0
A-^{Me}U	53.10 ± 2.4	140.2 ± 7.3	9.6 ± 0.12	56.1 ± 0.1	0.1
other pairs					
G-^{Me}iC	60.98 ± 4.8	165.2 ± 15	9.7 ± 0.18	54.2 ± 1.1	0.2
Pur-U	52.07 ± 5.0	138.4 ± 16	9.1 ± 0.22	53.4 ± 0.3	−0.4
Pur-^{Me}U	51.33 ± 0.6	135.2 ± 1.8	9.4 ± 0.06	55.3 ± 0.2	−0.1
Pur-C	45.90 ± 3.5	119.2 ± 11	8.9 ± 0.01	54.0 ± 1.3	−0.6
Pur·^{Me}iC	51.98 ± 3.9	138.4 ± 12	9.1 ± 0.15	52.9 ± 1.1	−0.4

[a] Values in this table represent the average of at least three melting experiments performed in 1.0 M NaCl, 10 mM sodium cacodylate, and 1 mM EDTA at total strand concentrations between 2 and 35 μM. The T_m is calculated for 100 μM oligonucleotide. [b] Four significant figures are reported for $\Delta H°$ and $\Delta S°$ to prevent the compounding of rounding errors when using the values to calculate $\Delta G°$ at other temperatures. [c] Relative stabilities are calculated by subtracting the $-\Delta G°$ of the GGAGGG·GGCCCUCU duplex from the $-\Delta G°$ of the XGAGGG·GGCCCUCY duplex. A positive value in this column indicates that the pair is more stable than G·U.

Table 3: Relative Stability of Internal A-U, G·U, and I·U Pairs in the Context of the Self-Complementary Oligonucleotides GUCUAGXC and GUCUXGAC[a]

sequence	$-\Delta H°$ (kcal·mol^{-1})	$-\Delta S°$ (cal·mol^{-1}·K^{-1})	$-\Delta G°_{37}$ (kcal·mol^{-1})	T_m (°C)	stability relative to A-U pair (kcal·mol^{-1})[b]
A-U pairs					
GUCUAGAC	73.79 ± 2.0	205.9 ± 5.9	9.9 ± 0.15	56.2 ± 0.4	(0)
	76.0	*212.5*	*10.1*	*56.2*	*(0)*
G·U pairs					
GUCUAGGC	72.10 ± 1.9	206.6 ± 5.8	8.0 ± 0.15	47.4 ± 0.5	−1.0
GUCUGGAC	75.75 ± 1.2	216.5 ± 3.6	8.6 ± 0.10	49.4 ± 0.3	−0.7
I·U pairs					
GUCUAGIC	61.04 ± 1.9	176.6 ± 6.1	6.3 ± 0.06	40.1 ± 0.3	−1.8
GUCUIGAC	65.11 ± 0.6	193.0 ± 1.6	5.3 ± 0.18	34.9 ± 0.8	−2.4

[a] Values in this table represent the average of at least four melting experiments performed in 1.0 M NaCl, 10 mM sodium cacodylate, and 1 mM EDTA at total strand concentrations between 2 and 35 μM. The T_m is calculated for 100 μM oligonucleotide. Values in italics are the result of previous work using the same experimental conditions and calculated from plots of $1/T_m$ vs ln Ct (Freier et al., 1986b). [b] Four significant figures are reported for $\Delta H°$ and $\Delta S°$ to prevent the compounding of rounding errors when using the values to calculate $\Delta G°$ at other temperatures. [c] Relative stabilities are calculated by subtracting the $-\Delta G°$ of the GUCUAGAC duplex from the $-\Delta G°$ of the GUCUAGXC or GUCUXGAC duplex and dividing the difference by two. A negative value in this column indicates that the pair is less stable than A-U.

groups in the major and minor grooves have been changed without significantly affecting the stability of the terminal base pair. This makes DAP·^{Me}iC an ideal compensatory base pair substitution for the study of G·U pairs in RNA, such as the invariant G·U found in the group I ribozymes. For example, if DAP·^{Me}iC is functionally equivalent to the G·U pair, it supports the proposed wobble configuration of the bases and suggests the hydrogen bonding polarity between the bases is unimportant.

Stabilization from the 5-Methyl Group. Previous work has shown that a methyl group at the 5 position of pyrimidines provides a modest stabilization of DNA triplexes (Povsic & Dervan, 1989). This is postulated to arise from a stabilizing hydrophobic effect. We observe a similar enhancement in the duplex stability of terminal wobble and Watson−Crick pairs resulting from a 5-methyl substitution in RNA. For both duplexes, a terminal G·^{Me}U pair is 0.3 kcal·mol^{-1} more stable than a terminal G·U pair. Similar stabilization is observed for terminal pairs in a Watson−Crick configuration. In the self-complementary duplex, A-^{Me}U and DAP-^{Me}U are 0.5 kcal·mol^{-1} more stable than A-U and DAP-U, respectively. The effect is smaller in the context of the 5′-exon·IGS duplex.

The observation that the 5-methyl group has some stabilizing effect on a terminal RNA wobble pair suggests that the DAP·^{Me}iC pair is also stabilized by the 5-methyl group of ^{Me}iC. If DAP·^{Me}iC and G·^{Me}U are compared, the DAP·^{Me}iC pair is about 0.5 kcal·mol^{-1} less stable. The small energetic difference could reflect weaker hydrogen bonding in the DAP·^{Me}iC pair, possibly resulting from differences in secondary electrostatic interactions between the bases (Pranata et al., 1991). One possibility is a destabilizing dipole−dipole interaction between the N6 exocyclic amine of DAP and the N2 exocyclic amine of ^{Me}iC. It could also result from less

favorable stacking interaction with the neighboring pair.

The Exocyclic Amine of G Does Not Stabilize a Terminal Wobble Pair. The N2 exocyclic amine of G does not form a direct hydrogen bond with the opposing base in a G•U wobble pair. However, a crystal structure of RNA containing an internal G•U pair showed a highly ordered water molecule bridging between the N2 exocyclic amine of G and the 2′-hydroxyl of U (Holbrook et al., 1991). It is not clear if such an interaction makes a significant contribution to base pair stability. Substitution of G with inosine (I) replaces the exocyclic amine of G with a hydrogen (Figure 1). We measured the stability of an I•U wobble pair to assess the contribution of the exocyclic amine to the stability of a terminal G•U pair. Deletion of the exocyclic amine resulted in little (0.4 kcal•mol^{-1} for the self-complementary duplex) or no (0.0 kcal•mol^{-1} for the 5′-exon•IGS duplex) thermodynamic destabilization of the terminal G•U pair (Tables 1 and 2).

A more significant difference is observed between G-C and I-C pairs (Figure 2) (Turner et al., 1987) (Tables 1 and 2). A terminal G-C Watson−Crick pair is worth 1.1−1.9 kcal•mol^{-1} more than a G•U or an A-U pair. An I-C pair, which lacks the N2 exocyclic amine and is therefore only able to form a base pair with two hydrogen bonds, is 1.1 kcal•mol^{-1} less stable than G-C and energetically equivalent to the G•U and A-U pairs, which also form only two hydrogen bonds. Therefore, deletion of the N2 exocyclic amine significantly destabilizes a Watson−Crick base pair, but has little effect on the stability of a terminal wobble pair.

If the DAP•^{Me}iC and DAP•C pairs are also in a wobble configuration, then the simplistic prediction is that substitution of the N2 exocyclic amine with a hydrogen will have no energetic consequences to the stability of the duplex. DAP•^{Me}iC and A•^{Me}iC pairs are energetically equivalent within 0.1 kcal•mol^{-1} (Tables 1 and 2). This is also true for pairs DAP•C and A•C. The absence of an effect upon deleting the exocyclic amine provides an additional line of thermodynamic evidence that the bases are paired as depicted in Figure 1. The observation that deletion of the exocyclic amine of a terminal wobble pair has little or no effect on duplex stability suggests that the water molecule, if present in this case, makes a negligible contribution to duplex stability.

The Exocyclic Amine of G Stabilizes an Internal Wobble Pair. Although A-U, G•U and I•U base pairs are equally stable at the terminal base pair of a helix, a significant difference in A-U and I•U base pair stability has been implied from studies of the double-stranded RNA adenosine deaminase activity (Bass & Weintraub, 1987, 1988; Rebagliati & Melton, 1987). This enzyme selectively converts A's to I's within double-stranded RNA, which results in conversion of A-U pairs to I•U pairs. The activity of this enzyme was originally characterized by the observation that RNA duplexes became sensitive to single-strand specific nucleases after modification (Bass & Weintraub, 1988). If the A to I conversion destabilizes the duplex, as implied by the nuclease sensitivity, then I•U pairs must be substantially less stable than A-U pairs. While we do not observe this destabilization at the terminal base pair of the helix, greater instability might result from an I•U pair at internal positions within the RNA duplex, such as was observed for internal I•T pairs in a DNA duplex (Kawase et al., 1986). To test this possibility we measured the relative stability of A-U, G•U and I•U pairs at two internal positions using the self-complementary oligoribonucleotides GUCUAGXC and GUCUXGAC, where X represents A, G, or I (Table 3).

As expected from the adenosine deaminase modification experiments, an internal I•U pair is significantly less stable than an A-U pair. While an internal G•U wobble destabilizes the helix by an average of 0.8 kcal•mol^{-1} at these two positions, the I•U pair is more than 2.0 kcal•mol^{-1} less stable than A-U. A complete characterization of the destabilization of an I•U pair requires the measurement of a large number of sequences to generate a complete set of nearest neighbor parameters (He et al., 1991). However, from these few sequences we conclude that an internal I•U pair is significantly more destabilizing than a terminal pair, and therefore the exocyclic amine of G is stabilizing at internal positions. Similar context affects are likely to be important when considering the stability of the DAP•^{Me}iC pair.

Other Stable Base Pair Combinations. While this study primarily focuses on base pairs in a wobble configuration, other pairing partners for DAP and ^{Me}iC were also tested at the terminal position of the helix. Two of these combinations were more stable than an A-U pair: DAP-U and G•^{Me}iC.

DAP-U can be drawn with three hydrogen bonds in a Watson−Crick configuration (Figure 2). It has been previously shown to form a stable base pair in DNA and RNA duplexes (Howard et al., 1966; Howard & Miles, 1984; Muraoka et al., 1980). As the terminal pair in an RNA duplex, DAP-U is 0.5 kcal•mol^{-1} more stable than A-U, but 1.2 kcal•mol^{-1} less stable than G-C which also forms three hydrogen bonds. The lower stability of a DAP-U pair has been attributed to repulsive electrostatic interactions between the juxtaposed functional groups of the base pair (Pranata et al., 1991).

Surprisingly, G-^{Me}iC also forms a terminal base pair that is at least as stable as A-U and almost as stable as DAP-U. This is surprising since ^{Me}iC has no obvious hydrogen bonding pattern with G in either the Watson−Crick or wobble configuration that should produce this level of base pairing stability (Figure 5). Furthermore, G is not incorporated opposite iC when iC or ^{Me}iC is used as a template for transcription or replication (Switzer et al., 1993; Tor & Dervan, 1993). Three models to explain this data are shown in Figure 3. In the first model (Figure 5A), G adopts a syn torsion angle and pairs with ^{Me}iC pair in a reverse Watson−Crick configuration to form three hydrogen bonds. It seems unlikely a reverse Watson−Crick pair would yield a base pair of the observed stability; however, the G•^{Me}iC forms the terminal pair of the helix and might have sufficient flexibility to form a stable pair in this way. Another potential pairing alignment (Figure 5B) displaces the G into the major groove with the formation of two hydrogen bonds to ^{Me}iC. A third possibility (Figure 5C) shows ^{Me}iC drawn in its other tautomeric form, resulting in a Watson−Crick pair with three hydrogen bonds. The equivalent iminol tautomer of protonated C has been proposed to stabilize the A•C wobble pair (Purrello et al., 1993). The thermodynamic data alone do not allow us to distinguish between these three models.

Substitutions at Non-Hydrogen-Bonding Positions. Substitutions at non-hydrogen-bonding positions within the G•U pair were also studied, but only in the sequence context of the 5′-exon•IGS duplex. 2′-deoxy-G substitution resulted in a base pair that is 0.3 kcal•mol^{-1} weaker than G•U. This likely results from destabilizing conformational effects within

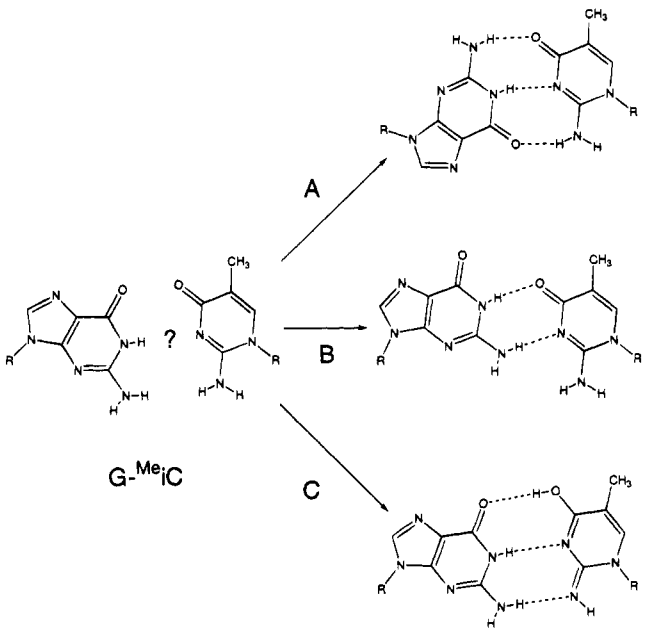

FIGURE 5: Potential base pairing schemes between G and ^{Me}iC that might explain the observed thermodynamic stability. (A) Reverse Watson–Crick pairing with the G adopting a *syn* torsion angle. (B) Wobble pairing with the G shifted toward the major groove and the ^{Me}iC shifted toward the minor groove. (C) Alternative tautomeric pairing with the ^{Me}iC adopting the other tautomeric form.

the furanose ring (Hall & McLaughlin, 1991; Chou et al., 1991; Salazar et al., 1993). A slight additional destabilization (0.2 kcal·mol^{-1}) is observed for replacement of the N7 with a carbon (7-deaza-2′-deoxyguanosine). This might result from weakened hydrogen bonding due to altered aromatic conjugation within the purine ring or reduced stacking potential of the base.

Conclusions. All Watson–Crick and wobble base pairs that form two hydrogen bonds are of approximately equal stability at terminal positions of a duplex. These include the pairs G·U, I·U, DAP·^{Me}iC, A·^{Me}iC, A-U, and I-C. There is energetic equivalence despite a significant diversity of functional groups in the major and minor grooves of the duplex. Furthermore, for a terminal base pair, non-hydrogen-bonding groups in the major and minor grooves of the helix have only a modest effect on stability. This includes the minor groove N2 exocyclic amino and major groove N7 imino groups of the purine base, and the major groove 5-methyl group of the pyrimidine base. Because none of these substitutions affects base pairing stability more than 0.5 kcal·mol^{-1} at 37 °C, this is a useful series of base analogs for identifying the role played by individual functional groups of a G·U pair in tertiary RNA structure and RNA/protein interactions. Significant thermodynamic differences observed in the context of a folded RNA structure can then be attributed to tertiary interactions involving the functional group that differs between the two base pairs.

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REFERENCES

Barfod, E. T., & Cech, T. R. (1989) *Mol. Cell. Biol. 9*, 3657–3666.
Bass, B. L., & Weintraub, H. (1988) *Cell 55*, 1089–1098.
Bass, B. L., & Weintraub, H. (1987) *Cell 48*, 607–613.
Been, M. D. (1994) *Trends Biochem. Sci. 19*, 251–256.
Chollet, A., Chollet-Damerius, A., & Kawashima, E. H. (1986) *Chem. Scr. 26*, 37–40.
Chou, S.-H., Flynn, P., Wang, A., & Reid, B. (1991) *Biochemistry 30*, 5248–5257.
Crick, F. H. C. (1966) *J. Mol. Biol. 19*, 548–555.
Doudna, J. A., Cormack, B. P., & Szostak, J. W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7402–7406.
Fathi, R., Goswami, B., Kung, P., Gaffney, B. L., & Jones, R. A. (1990) *Tetrahedron Lett. 31*, 319–322.
Freier, S. M., Sinclair, A., Neilson, T., & Turner, D. H. (1985) *J. Mol. Biol. 185*, 645–647.
Freier, S. M., Alkema, D., Sinclair, A., Neilson, T., & Turner, D. H. (1985) *Biochemistry 24*, 4533–4539.
Freier, S. M., Kierzek, R., Jaeger, J. A., Sugimoto, N., Caruthers, M. H., Neilson, T., & Turner, D. H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9373–9377.
Freier, S. M., Kierzek, R., Caruthers, M. H., Neilson, T., & Turner, D. H. (1986) *Biochemistry 25*, 3209–3213.
Fu, D., Rajur, S. B., & McLaughlin, L. W. (1993) *Biochemistry 32*, 10629–10637.
Gaffney, B. L., Markey, L. A., & Jones, R. A. (1984) *Tetrahedron 40*, 3–13.
Gralla, J., & Crothers, D. M. (1973) *J. Mol. Biol. 78*, 301–319.
Green, R., Szostak, J. W., Benner, S. A., Rich, A., & Usman, N. (1991) *Nucleic Acids Res. 19*, 4161–4166.
Gutell, R. R., Larsen, N., & Woese, C. R. (1994) *Microbiol. Rev. 58*, 10–26.
Hakimelahi, G. H., Proba, Z. A., & Ogilvie, K. K. (1982) *Can. J. Chem. 60*, 1106–1113.
Hall, K. B., & McLaughlin, L. W. (1991) *Biochemistry 30*, 10606–10613.
He, L., Kierzek, R., SantaLucia, J., Walter, A. E., & Turner, D. H. (1991) *Biochemistry 30*, 11124–11132.
Hickey, D. R., & Turner, D. H. (1985) *Biochemistry 24*, 3987–3991.
Holbrook, S. R., Sussman, J. L., Warrant, R. W., & Kim, S.-H. (1978) *J. Mol. Biol. 123*, 631–660.
Holbrook, S. R., Cheong, C., Tinoco, I., & Kim, S.-H. (1991) *Nature 353*, 579–581.
Howard, F. B., Frazier, J., & Miles, H. T. (1966) *J. Biol. Chem. 241*, 4293–4295.
Howard, F. B., & Miles, H. T. (1984) *Biochemistry 23*, 6723–6732.
Kimura, J., Yagi, K., Suzuki, H., & Mitsunobu, O. (1980) *Bull. Chem. Soc. Jpn. 53*, 3670–3677.
Kawase, Y., Iwai, S, Inoue, H., Miura, K., & Ohtsuka, E. (1986) *Nucleic Acids Res. 14*, 7727–7736.
Majerfeld, I., & Yarus, M. (1994) *Nature Struct. Biol. 1*, 287–292.
Marky, L. A., & Breslauer, K. J. (1987) *Biopolymers 26*, 1601–1620.
Martin, F. H., Castro, M. M., Aboul-ela, F., & Tinoco, I. (1985) *Nucleic Acids Res. 13*, 8927–8938.
Mansuri, M. M., et al. (1989) *J. Org. Chem. 54*, 4780.
Michel, F., Umesono, K., & Ozeki, H. (1989) *Gene 82*, 5–30.
Michel, F., & Westhof, E. (1990) *J. Mol. Biol. 216*, 585–610.
Moore, M. J., Query, C. C., & Sharp, P. A. (1993) *The RNA World*, pp 303–358, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Muraoka, M., Miles, H. T., & Howard, F. B. (1980) *Biochemistry 19*, 2429–2439.

Musier-Forsyth, K., Usman, N., Scaringe, S., Doudna, J. A., Green, R., & Schimmel, P. (1991) *Science 253*, 784−786.
Newman, A., & Norman, C. (1991) *Cell 65*, 115−123.
Newman, A. J., & Norman, C. (1992) *Cell 68*, 743−754.
Petersheim, M., & Turner, D. H. (1983) *Biochemistry 22*, 256−263.
Povsic, T. J., & Dervan, P. B. (1989) *J. Am. Chem. Soc. 111*, 3059−3061.
Pranata, J., Wierschke, S. G., & Jorgensen, W. L. (1991) *J. Am. Chem. Soc. 113*, 2810−2819.
Puglisi, J. D., & Tinoco, I. (1989) *Methods Enzymol. 180*, 304−325.
Purrello, R., Molina, M., Wang, Y., Smulevich, G., Fossella, J., Fresco, J. R., & Spiro, T. G. (1993) *J. Am. Chem. Soc. 115*, 760−767.
Pyle, A. M., Moran, S., Strobel, S. A., Chapman, T., Turner, D. H., & Cech, T. R. (1994) *Biochemistry* (in press).
Rebagliati, M. R., & Melton, D. A. (1987) *Cell 48*, 599−605.
Reddy, M. P., Hanna, N. B., & Forooqui, F. (1994) *Tetrahedron Lett. 35*, 4311−4314.
Salazar, M., Fedoroff, O. Y., Miller, J. M., Ribeiro, N. S., & Reid, B. R. (1993) *Biochemistry 32*, 4207−4215.
Scaringe, S. A., Francklyn, C., & Usman, N. (1990) *Nucleic Acids Res. 18*, 5433−5441.
Switzer, C., Moroney, S. E., & Benner, S. A. (1989) *J. Am. Chem. Soc. 111*, 8322−8323.
Switzer, C. Y., Moroney, S. E., & Benner, S. A. (1993) *Biochemistry 32*, 10489−10496.
Tor, Y., & Dervan, P. B. (1993) *J. Am. Chem. Soc. 115*, 4461−4467.
Turner, D. H., Sugimoto, N., Kierzek, R., & Dreiker, S. D. (1987) *J. Am. Chem. Soc. 109*, 3783−3785.
Usman, N., Ogilvie, K. K., Jiang, M., & Cedergren, R. J. (1987) *J. Am. Chem. Soc. 109*, 7845−7854.
Walker, G. T. (1988) *Nucleic Acids Res. 16*, 3091−3099.
Wu, H.-N., Lee, J.-Y., Huang, H.-W., Huang, Y.-S., & Hsueh, T.-G. (1993) *Nucleic Acids Res. 21*, 4193−4199.
Wyatt, J. R., Sontheimer, E. J., & Steitz, J. A. (1992) *Genes Dev. 6*, 2542−2553.