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A Packing Density Metric for Exploring the Interior of Folded RNA Molecules

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General. All reagents and anhydrous solvents were purchased from Aldrich; other solvents were from Fisher. All reactions using air-sensitive or moisture-sensitive reagents were carried out under an argon atmosphere. Reactions and workup procedures were conducted at room temperature unless otherwise noted. ^{1}H, ^{31}P, and ^{19}F NMR spectra were recorded on Bruker 500 or 400 MHz NMR spectrometers. ^{1}H chemical shifts are reported in ppm relative to tetramethylsilane. ^{31}P chemical shifts are reported relative to a standard of 85% aqueous H_3PO_4. ^{19}F chemical shifts are reported relative to a standard of trifluoroacetic acid. Mass spectra were obtained from the Department of Chemistry, University of California at Riverside, using a VG-ZAB instrument or from the Department of Chemistry, University of Chicago, using a HP ESI instrument. Merck silica gel (9385 grade, 230-400 mesh, 60 Å, Aldrich) was used for column chromatography. Silica gel on glass with fluorescent indicator (Aldrich) was used for TLC.

Nucleotide modifying enzymes were from Amersham Pharmacia or New England Biolabs. [γ-^{32}P]-Adenosine triphosphate was obtained from Perkin Elmer Life Sciences. Nucleoside triphosphates were from Amersham Pharmacia and 2'-ribo- and deoxy-α-triphosphates were from Glen Research. Denaturing polyacrylamide gel electorphoresis (DPAGE) was carried out using 6-9% polyacrylamide (Fisher; acrylamide:bis-acrylamide, 29:1)

with 7 M urea and TBE (89 mM Tris, 89 mM Boric Acid; 2 mM EDTA). DTT gels (10 mM DTT/7 M urea/TBE) were cast and allowed to polymerize overnight. Gel loading buffer contained 8 M urea (J.T. Baker), 50 mM EDTA (pH 8.0, Fisher), 0.02% bromophenol blue (EM Science), 0.02% xylene cyanol FF (Kodak), and 10 mM DTT.

Synthesis of nucleoside analogues and their α-thiotriphosphates.

The 2'-mercaptonucleoside α-thiotriphosphates were synthesized as previously described (Schwans et al., 2003). The synthesis of the 2'-deoxy-2'-methylnucleosides will be reported elsewhere.

2'-Chloro-2'-deoxynucleoside synthesis.

2'-Chloro-2'-deoxyuridine. 5'-*O*-Acetyl-2'-chloro-2'-deoxyuridine (Verheyden and Moffatt, 1972) (30 mg, 0.1 mmol) was dissolved in CH_2Cl_2 (10 mL). In a separate flask, sodium methoxide (40.5 mg, 0.75 mmol) was added to guanidine hydrochloride (313 mg, 3.75 mmol) in methanol (20 mL). The nucleoside solution was transferred to the guanidine/guanidinium hydrochloride (Ellervik and Magnusson, 1997; Dai and Piccirilli, 2003) solution via cannula. After being stirred for 15 min at room temperature, the reaction was quenched with DOWEX® 50WX8-200 ion-exchange resin. The resin was removed, and the solvent was evaporated. The resulting residue was purified by silica gel column chromatography (5-10% MeOH in CH_2Cl_2) to give 2'-chloro-2'-deoxyuridine (**27**) as a white foam in 80% yield. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.92 (d, 1H, J= 8.1 Hz), 6.0 (d, 1H, J = 5.7 Hz), 5.67 (d, 1H, J = 8.1 Hz), 4.54 (t, 1H, J = 5.4 Hz), 4.19 (m, 1H) 3.93 (m, 1H), 3.55-6.68 (m, 2H). MS (ESI): calculated for $C_9H_{10}ClN_2O_5$

(MH^-) 261.03; found 261.0.

N^4-Acetyl-1-[3, 5-*O*-(1, 1, 3, 3-tetraisopropyldisiloxane-1, 3-diyl)-β-D-arabinofuranosyl]cytosine. 1-[3, 5-*O*-(1, 1, 3, 3-Tetraisopropyldisiloxane-1, 3-diyl)-β-D-arabinofuranosyl]cytosine (Markiewicz et al., 1980, 760 mg, 1.56 mmol) was dissolved in DMF (10 mL). Acetic anhydride (0.18 ml, 1.56 mmol, 1 equiv) was added dropwise over 5 min. After stirring for 2 h, methanol (2 mL) was added to quench the reaction. The mixture was concentrated and purified by silica gel chromatography (0-3% MeOH in $CHCl_3$) to give the product as a white foam (quantitative conversion based on recovery of starting material). ^{1}H NMR ($CDCl_3$/TMS): δ 8.22 (d, 1H, *J*= 7.5 Hz), 7.47 (d, 1H, *J* = 7.5 Hz), 6.11 (d, 1H, *J* = 6.0 Hz), 4.59 (dd, 1H, *J* = 6.2 Hz), 3.80-4.14 (m, 4H) 2.25 (s, 3H), 0.99-1.08 (m, 28H). MS (ESI) Calcd for $C_{23}H_{42}N_3O_7Si_2$ [MH^+] 528.26; Found: 528.2.

N^4-Acetyl-1-[3, 5-*O*-(1, 1, 3, 3-tetraisopropyldisiloxane-1,3-diyl)-2-*O*-(trifluoromethanesulfonyl)-β-D-arabinofuranosyl]cytosine. N^4-Acetyl-1-[3, 5-*O*-(1, 1, 3, 3-tetraisopropyldisiloxane-1, 3-diyl)-β-D-arabinofuranosyl]cytosine (350 mg, 0.66 mmol) and 4-(dimethylamino)pyridine (122 mg, 1.0 mmol) were dissolved in CH_2Cl_2 (5 mL). The reaction mixture was cooled to 0 °C, and trifluoromethanesulfonyl chloride (0.1 ml, 0.8 mmol, 1.2 equiv) was added dropwise. The yellow solution was stirred for 30 min at 0 °C. The reaction mixture was partitioned between ice-cooled acetic acid/water (1:99, 25 mL) and extracted with CH_2Cl_2 (2x100 mL). The organic phases were combined and washed with ice-cooled saturated aqueous $NaHCO_3$, brine, and dried over $NaSO_4$. The solvent was evaporated, and column chromatography of the residue (0-5% MeOH in CH_2Cl_2) gave N^4-acetyl-1-[3, 5-*O*-(1, 1, 3, 3-tetraisopropyldisiloxane-1, 3-diyl)-2-*O*-(trifluoromethanesulfonyl)-β-D-arabinofuranosyl]cytosine (1.3 g, 76%) as a white foam. ^{1}H NMR ($CDCl_3$/TMS): δ 9.25 (bs, 1H), 7.88 (bs, 1H), 7.47 (d, 1H, *J* = 7.6 Hz), 6.27 (s, 1H) 5.47 (m, 1H), 4.62 (m, 1H), 4.14 (m, 1H),

3.96 (m, 2H), 2.27 (s, 3H), 1.00-1.09 (m, 28H). ^{19}F NMR ($CDCl_3$/TFA): δ –74.22 (s). MS (ESI) Calcd for $C_{24}H_{41}F_3N_3O_9SSi_2$ [MH^+] 660.20; Found: 660.1.

***N*^{4}-Acetyl-3', 5'-*O*-(1, 1, 3, 3-tetraisopropyldisiloxane-1, 3-diyl)-2'-chloro-2'-deoxycytidine.** *N*^{4}-Acetyl-1-[3, 5-*O*-(1, 1, 3, 3-tetraisopropyldisiloxane-1, 3-diyl)-2-*O*-(trifluoromethanesulfonyl)-β-D-arabinofuranosyl]cytosine (1.3 g, 1.97 mmol) was dissolved in DMF (5 mL). Lithium chloride (827 mg, 19.7 mmol, 10 equiv) was added, and the reaction was heated to 80 °C. After 60 min, the reaction was allowed to cool to room temperature, and the solvent was evaporated. The residue was dissolved in CH_2Cl_2 and washed with 5% $NaHCO_3$, brine, and dried over $MgSO_4$. The solvent was evaporated, and the residue was purified by silica gel column chromatography (0-2% MeOH in $CHCl_3$) to give *N*^{4}-acetyl-3', 5'-*O*-(1, 1, 3, 3-tetraisopropyldisiloxane-1, 3-diyl)-2'-chloro-2'-deoxycytidine (450 mg, 40%) as a white foam. ^{1}H NMR ($CDCl_3$/TMS): δ 10.15 (bs, 1H), 8.66 (d, 1H, *J* = 7.5 Hz), 7.49 (d, 1H, *J* = 7.5 Hz), 5.97 (s, 1H) 4.67 (m, 1H), 4.60 (m, 1H) 3.88-4.18 (m, 3H) 2.22 (s, 3H), 0.99-1.08 (m, 28H). MS (ESI) Calcd for $C_{23}H_{41}ClN_3O_6Si_2$ [MH^+] 546.21; Found: 546.1.

2'-Chloro-2'-deoxycytidine. A solution of ammonium hydroxide/methylamine (2 mL; 1:1 vol/vol) was added to *N*^{4}-acetyl-3', 5'-*O*-(1, 1, 3, 3-tetraisopropyldisiloxane-1, 3-diyl)-2'-chloro-2'-deoxycytidine (150 mg, 0.27 mmol). The mixture was heated to 65 °C for 10 minutes and cooled in an ice bath (5 min) before opening. Methanol (5 mL) was added, and the solvent was evaporated. The residue was dissolved in CH_2Cl_2, and purified by short column silica gel chromatography (0-5% MeOH in CH_2Cl_2). Fractions containing the product were pooled and concentrated. The residue was dissolved in THF (5 mL), tetrabutylammonium fluoride hydrate (70 mg, 0.27 mmol) was added, and the solution was stirred at room temperature. After 5 min, TLC indicated that the reaction was complete. The solvent was removed under reduced pressure, and the residue was dissolved in 4% MeOH in CH_2Cl_2. Silica gel column chromatography (4-

15% MeOH in CH_2Cl_2) gave 2'-chloro-2'-deoxycytidine (60 mg, 60%) as a white foam. ^{1}H NMR (DMSO-d_6): δ 7.90 (d, 1H, J = 7.5 Hz), 7.28 (bd, 1H, J = 8.0 Hz), 6.03 (d, 1H, J = 5.1 Hz), 5.74 (d, 1H, J = 7.5 Hz), 4.46 (t, 1H, J = 5.1 Hz), 4.18 (m, 1H) 3.94 (m, 1H), 3.56-3.71 (m, 2H). MS (ESI) Calcd for $C_9H_{11}ClN_3O_4$ [MH^-] 260.05; Found: 260.0.

2'-Chloro-2'-deoxyadenosine. 3', 5'-*O*-(1, 1, 3, 3-tetraisopropyldisiloxane-1, 3-diyl)-2'-chloro-2'-deoxyadenosine (Robins et al., 1992; 1981; 50 mg, 0.09 mmol) was treated with TBAF/THF (1.0 M soln, 3.0 equiv) for 30 min. The reaction was quenched with 1.0 M triethylammonium acetate and concentrated under reduced pressure. Silica gel chromatography (4-20% MeOH in CH_2Cl_2) gave the product as a white solid (28%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.40 (s, 1H), 8.10 (s, 1H), 7.40 (bs, 1H), 6.14 (d, OH), 5.97 (d, 1H, J = 5 Hz), 5.40 (t, OH), 5.11 (dd, 1H, J = 4.5 Hz), 4.36 (m, 1H), 4.06 (m, 1H), 3.58-3.69 (m, 2H).

2'-Chloro-2'-deoxyguanosine. 3', 5'-*O*-(1, 1, 3, 3-tetraisopropyldisiloxane-1, 3-diyl)-2'-chloro-2'-deoxyguanosine (Ikehara and Imura, 1981; 60 mg, 0.11 mmol) was treated with TBAF/THF (1.0 M soln, 330 mL, 0.33 mmol, 3.0 equiv) for 30 min. The reaction mixture was quenched with 1.0 M triethylammonium acetate and concentrated under reduced pressure. Silica gel chromatography (4-20% MeOH in CH_2Cl_2) gave the product as a white solid (28 mg, 85%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.80 (s, 1H), 7.98 (s, 1H), 6.55 (bs, 1H), 6.0 (d, 1H), 5.19 (t, OH), 4.95 (dd, 1H, J = 4.8 Hz), 4.38 (m, 1H), 3.98 (m, 1H), 3.50-3.15 (m, 2H).

Nucleoside α-thiotriphosphate synthesis. The 2'-modified nucleosides were converted to the corresponding α-thiotriphosphates as described by Schwans et al. for the synthesis of 2'-mercaptonucleoside α-thiotriphosphates (Schwans et al., 2003).

2'-Chloro-2'-deoxyadenosine-α-thiotriphosphate. ^{31}P NMR (D_2O, 400 MHz): δ 43.5 m, -10.7 d, -23.5 m. MS (ESI): calculated for $C_{10}H_{14}ClN_5O_{11}P_3S^-$ (M-): 539.93; found 539.9.

2'-Chloro-2'-deoxycytidine-α-thiotriphosphate. ^{31}P NMR (D_2O, 400 MHz): δ 41.1 m, -13 dd, -26.2 m. MS (ESI): calculated for $C_9H_{14}ClN_3O_{12}P_3S^-$ (M^-): 515.92; found 515.8.

2'-Chloro-2'-deoxyguanosine-α-thiotriphosphate. ^{31}P NMR (D_2O, 500 MHz): δ 43.8 m, -10.7 d, -23.7 m. MS (ESI): calculated for $C_{10}H_{14}ClN_5O_{12}P_3S_1^-$ (M^-): 555.93; found 555.9.

2'-Chloro-2'-deoxyuridine-α-thiotriphosphate. ^{31}P NMR (D_2O, 400 MHz): δ 41.2 m, -9.3 d, 23.4 m. MS (ESI): calculated for $C_9H_{13}ClN_2O_{13}P_3S^-$ (M^-): 516.90; found 516.8.

2'-Deoxy-2'-methyladenosine-α-thiotriphosphate. ^{31}P NMR (D_2O, 400 MHz): δ 42.0 m, -10.6 d, -23.9 m. MS (ESI): calculated for $C_{11}H_{17}N_5O_{11}P_3S^-$ (M^-): 519.99; found 520.0.

2'-Deoxy-2'-methylcytidine-α-thiotriphosphate. ^{31}P NMR (D_2O, 400 MHz): δ 44 m, -10 dd, -23 m. MS (ESI): calculated for $C_{10}H_{17}N_3O_{12}P_3S^-$ (M^-): 495.97; found 495.8.

2'-Deoxy-2'-methylguanosine-α-thiotriphosphate. ^{31}P NMR (D_2O, 400 MHz): δ 43.6 m, -10.4 d, -23.0 m. MS (ESI): calculated for $C_{11}H_{17}N_5O_{12}P_3S^-$ (M^-): 535.98; found 535.8.

2'-Deoxy-2'-methyluridine-α-thiotriphosphate. ^{31}P NMR (D_2O, 400 MHz): δ 41.1 m, -13.0 dd, -25.8 m. MS (ESI): calculated for $C_{10}H_{16}N_2O_{13}P_3S^-$ (M^-): 496.96; found 497.00.

Transcription Reactions. ΔC209 P4-P6 was transcribed from *Ear I* digested ΔC209 plasmid (gift from K. Juneau and T. Cech). Transcription reactions contained 40 mM tris(hydroxylmethyl)aminomethane hydrochloride (Tris-HCl, pH 7.5), 4 mM spermidine, 10 mM dithiothreitol (DTT), 15 mM $MgCl_2$, 0.05% Triton X-100, 1.0 mM NTPs, 0.05 μg/μL DNA

template, and 0.08 μg/μL Y639F T7 RNA polymerase. NTPs were added to the concentrations listed in the table below. All transcription reactions were incubated for 2 hours at 37 °C. A level of approximately 5% analogue incorporation was achieved by comparing I_2 induced cleavage intensities to a NTP□S standard (Ryder et al., 2000). Transcripts were purified by denaturing PAGE (10 mM DTT in gel and buffer). Samples were eluted, ethanol precipitated, washed with 70% ethanol and resuspended in 1X TE (10 mM Tris·HCl pH 7.5; 0.1 mM EDTA) with 10 mM DTT.

2'-analogue	αS NTP conc. (mM)	Parental NTP conc. (mM)
2'-Cl: ACl	0.05	0.5
CCl	0.5	0.5
GCl	0.1	0.5
UCl	0.5	0.5
2'-CH_3: ACH_3	1.0	1.0
CCH_3	0.6	1.0
GCH_3	0.5	1.0
UCH_3	0.5	1.0
2'-SH: ASH	0.4	1.0
CSH	0.4	1.0
GSH	0.5	1.0
USH	0.4	1.0

ΔC209 P4-P6 interference mapping experiments.

ΔC209 P4-P6 interference mapping experiments were conducted as described in Schwans et al. (Schwans et al., 2003 and references therein).

Transcription of 5'-GAU$_x$GGC-3'. Transcription reactions contained 40 mM Tris-HCl pH 7.5, 4 mM spermidine, 10 mM DTT, 15 mM $MgCl_2$, 0.05 % Triton X-100, 2 Units inorganic pyrophosphatase, 0.5 μg DNA template, and 0.05 μg/μl Y639F T7 RNA polymerase. ATP, CTP, and GTP at 1.0 mM. In separate reactions, uridine-α-thiotriphosphate and 2'-chlorouridine-α-thiotriphosphate were added to a concentration of 0.5 mM. The transcription reactions were incubated at 37 °C for 30 min. The reactions were extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol (10 μg glycogen was added to assist precipitation of the short transcript). The reactions were resuspended in calf alkaline phosphatase buffer and treated with 1 Unit calf alkaline phosphatase at 37 °C for 30 min. The reactions were extracted with phenol/chloroform/isoamyl alcohol and precipaited with ethanol (10 μg glycogen). The transcripts were 5'-radiolabeled using T4 PNK and γ^{32}P ATP according to the manufacture's instructions. The radiolabeling reactions were quenched by the addition of one volume "stop" solution (8 M urea; 50 mM EDTA) and purified by 20 % DPAGE. The full-length transcript was excised from the gel and eluted into 1X TE at 4 °C overnight. The eluted RNAs were used directly in base-catalyzed cleavage and ribonuclease A assays.

Characterization of a transcript containing a single 2'-chloro-2'-deoxyuridine residue.

Hobbs et al. demonstrated that poly(2'-chlorouridylic acid) and poly(2'-chlorocytidylic acid) generated by *Micrococcus lysodeikticus* polynucleotide phosphorylase were stable against base-catalyzed cleavage and ribonuclease A catalyzed cleavage (Hobbs et al., 1972). To

examine whether transcripts containing a 2'-chlorouridine exhibited the same behavior, we generated a transcript, 5'-GAU$_x$GGC-3', (Piccirilli et al., 1991; Moroney and Piccirilli, 1991) in which the uridine residue was substituted with 2'-chloro-2'-deoxyuridine-5'-phosphorothioate (5'-GAU$_{2'\text{-Cl}}$GGC-3'). We also generated a transcript containing uridine-5'-phosphorothioate to control for the effect of the phosphorothioate substitution alone (5'-GAU$_{2'\text{-OH}}$GGC-3'). We subjected the 5'-radiolabeled transcripts to base-catalyzed cleavage. While 5'-GAU$_{2'\text{-OH}}$GGC-3' was cleaved at every residue, 5'-GAU$_{2'\text{-Cl}}$GGC-3' resisted base-catalyzed cleavage at position 3, indicating the absence of the 2'-hydroxyl group at the site of 2'-chlorouridine incorporation. We also exposed the transcripts to ribonuclease A, which cleaves RNA at pyrimidine residues. Whereas 5'-GAU$_{2'\text{-OH}}$GGC-3' reacted quantitatively, 5'-GAU$_{2'\text{-Cl}}$GGC-3' resisted reaction. These results show that transcripts containing a 2'-chlorouridine nucleoside analogue exhibit the same behavior as poly(2'-Cl)-oligonucleotides.

Base-catalyzed cleavage of 5'-GAU$_x$GGC-3'. Each 5'-radiolabeled transcript was treated with 33 mM $NaHCO_3$ (pH 9) for 8 min at 90 °C and subsequently analyzed by 20% DPAGE.

Ribonuclease A Cleavage of 5'-GAU$_x$GGC-3'. Each radiolabeled transcript was treated with 1 ng ribonuclease A at 37 °C for 2 min. The reactions were quenched by the addition of stop solution, placed at 0 °C, and analyzed by 20% DPAGE.

Calculation of van der Waals Repulsion Energy. Models containing a series of 2'-nucleotide analogues (-Cl, -S-, -Br) at specific locations in the ΔC209 P4-P6 domain were generated using Sybyl 6.9 (Tripos Inc.). Bond lengths were set to the average C–X bond length for gas-phase molecules (Lide, 1993). We used the Tripos force field (Clark et al., 1989) without energy

minimization to calculate the total energy for each modified structure. Each calculated energy value was normalized against the energy of the unmodified structure. Although all force potentials (bond motions and van der Waals interactions) were included in the calculation (Eq. 1), changes in bond stretching, bending, and twisting are not observed, as energy minimization was not conducted. Therefore, the energy difference (ΔE) represents solely changes in van der Waals overlap between the different structures (Eq. 2)

$$E_{Total} = \Sigma E_{str} + \Sigma E_{bend} + \Sigma E_{oop} + \Sigma E_{tors} + \Sigma E_{vdW} \quad (1)$$

$$\Delta E = E^{2'\text{-}X}_{Total} - E^{2'\text{-}O}_{Total} = E^{2'\text{-}X}_{vdW} - E^{2'\text{-}O}_{vdW} \quad (2)$$

E_{str} = bond stretching energy term
E_{bend} = angle bending energy term
E_{oop} = out of plane bending energy term
E_{tors} = torsional energy term
E_{vdW} = van der Waals energy term

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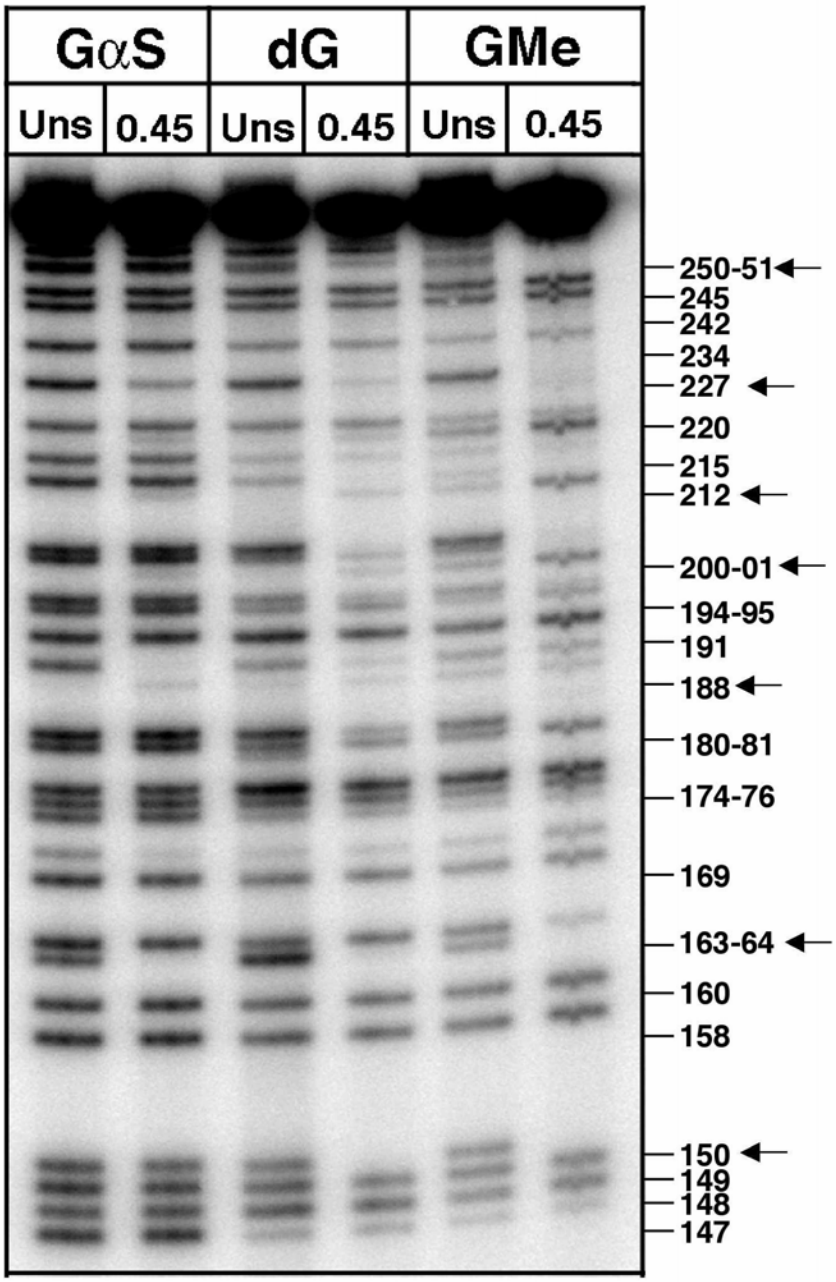
Figure SI-A. Nucleotide analogue interference mapping of ΔC209 P4-P6 folding using 2'-deoxy-2'-methylguanosine. Transcription with Y639F T7 RNA polymerase in the presence of α-thiotriphosphates of guanosine (GαS), 2'-deoxyguanosine (dG), and 2'-deoxy-2'-methylguanosine (GMe) generated populations of ΔC209 P4-P6. The I_2-induced sequencing ladders demonstrate that the polymerase incorporated 2'-deoxy-2'-methylguanosine evenly throughout the ΔC209 P4-P6 domain. Arrows indicate gaps in the sequencing ladder compared to the unselected control representing sites at which the nucleotide analogue interferes with the folding of ΔC209 P4-P6.


GαS
dG
GMe
Uns
0.45
Uns
0.45
Uns
0.45
250-51
245
242
234
227
220
215
212
200-01
194-95
191
188
180-81
174-76
169
163-64
160
158
150
149
148
147