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The Internal Dynamics of Mini c TAR DNA Probed by EPR of Nitroxide Spin Labels at the Lower Stem, the Loop, and the Bulge †

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Abstract

Electron paramagnetic resonance (EPR) at 236.6 GHz and 9.5 GHz probed the tumbling of nitroxide spin probes in the lower stem, the upper loop, and near the bulge of mini c TAR DNA. High frequency 236.6 GHz EPR, not previously applied to spin labeled oligonucleotides, was notably sensitive to fast, anisotropic, hindered local rotational motion of the spin probe, occurring approximately about the NO nitroxide axis. Labels attached to the 2'-amino cytidine sugar in the mini c TAR DNA showed such anisotropic motion, which was faster in the lower stem, a region previously suggested to be partially melted. More flexible labels attached to phosphorothioates at the end of the lower stem tumbled isotropically in mini c TAR DNA, mini TAR RNA, and ψ_3 RNA, but at 5 °C the motion became more anisotropic for the labeled RNAs, implying more order within the RNA lower stems. As observed by 9.5 GHz EPR, the slowing of nanosecond motions of large segments of the oligonucleotide was enhanced by increasing the ratio of the nucleocapsid protein NCp7 to mini c TAR DNA from zero to two. The slowing was most significant at labels in the loop and near the bulge. At a 4:1 ratio of NCp7 to mini c TAR DNA all labels reported

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¹EPR (Electron Paramagnetic Resonance), SRLS (Slowly Relaxing Local Structure), NLSL (Nonlinear Least-Squares Limited), DEER (Double Electron Electron Resonance), PDS (Pulse Dipolar Spectroscopy)

Supporting Information Available

1) Protocols for labeling phosphorothioate linkages, including Figure 1S which shows HPLC purification trace for labeled mini c TAR DNA and Figure 2S which shows analytical gel traces for labeled mini c TAR DNA. 2) Protocols for preparation of spin-labeled mini c TAR DNA via the reaction of 2'-amino-2'-deoxycytidine and 4-Isocyanato-2, 2, 6, 6-tetramethylpiperidine 1-oxyl including Figure 3S to show analytical gel traces for mini c TAR DNA labeled by this method. 3) Non-denaturing gel shift assays showing the similarity of labeled and unlabeled mini c TAR DNA binding to NCp7, including Figure 4S which shows the gel scans. 4) Melting of labeled and unlabeled mini c TAR DNA as monitored by UV-Vis temperature melts at 260 nm, including Figure 5S to show the melting profiles ($\Delta A/\Delta T$ at 260 nm) for wild type and labeled mini c TAR DNA and Table 1S providing thermodynamic parameters ΔH , ΔS , ΔG , and T_m . 5) Figure 6S comparing simulated 9.5 GHz EPR spectra from SL1, SL2, SL3, SLA, and SLB to experimental spectra obtained in the presence of a two-fold excess of NCp7. 6) Figure 7S showing the effect of changing the ionic strength upon the breadth of the 9.5 GHz EPR spectrum from spin labeled mini c TAR DNA in the presence of 4:1 NCp7:mini c TAR DNA. 7) Simulations of the motion of spin labeled mini c TAR DNA as studied by high-field, high-frequency EPR, where axial symmetry is assumed for the local probe diffusion tensor, including Figure 8S showing SRLS simulations and Table 2S of the resultant EPR fitting parameters. This material is available free of charge via the Internet at <http://pubs.acs.org>.

tumbling times > 5 ns, indicating a condensation of NCp7 and TAR DNA. At the 4:1 ratio, pulse dipolar EPR spectroscopy of bi-labels attached near the 3' and 5' terminals showed evidence for an NCp7-induced increase in the 3' - 5' end-to-end distance distribution and a partially melted stem.

Keywords

EPR; spin label; NCp7; mini c TAR DNA

Introduction

The purpose of this study is to understand the internal dynamics of a model stem-loop oligonucleotide from HIV-1 and the change in these dynamics upon its interaction with the HIV-1 nucleocapsid protein NCp7. The stem-loop structure is found in the TAR (Trans Activation Response) region of c TAR DNA and TAR RNA. As shown both *in vivo* (1, 2) and *in vitro* (3, 4), the binding of NCp7 inhibits self-priming within such a stem-loop and promotes annealing so as to form duplexes between complementary TAR RNA and TAR DNA. *In vitro* annealing has been carried out in quantitative kinetic detail (3, 4) using “mini c TAR DNA” (Figure 1A). As we have previously shown with the simpler ψ_3 RNA stem-loop (5), stem-loop oligonucleotide complexes with NCp7 undergo structural rearrangements, whose dynamics are amenable to EPR spin label methods. NCp7 (Figure 1B) is adapted for specific binding to a diversity of oligonucleotides in base-unpaired regions by hydrophobic and hydrogen bonding, and electrostatic interactions between cationic NCp7 and anionic oligonucleotides enhance both specific and non-specific binding.

Mini c TAR DNA (Figure 1A) has an apical loop and an internal bulge, both containing unpaired bases and potential centers for dynamic structural modulation and NCp7 binding. Imino hydrogen exchange has provided evidence for an intrinsically destabilized double strand region below the bulge (6). NMR techniques that resolve residual dipolar couplings have pointed to the internal bulge of TAR RNA as a locus for large bending motions and for exchange between conformations adapted to the recognition of small molecule inhibitors and TAR binding proteins like NCp7 and TAT (7–10). NMR structures of mini c TAR DNA are likewise consistent with several coexisting conformations (6, 11), and the TAR DNA structure, as opposed to the TAR RNA structure, is a less stable, more dynamic structure, and is more open to NCp7 perturbation.

In our previous EPR work only the 5' terminal of the ψ_3 RNA stem was labeled (5). In contrast, we now extend study to a comparison of dynamic EPR signatures in stem (SLA, SLB, SL2), loop (SL1), and bulge (SL3) of the more complex TAR, and we additionally use high sensitivity, high field EPR for this purpose (12). Previous spin label study on oligonucleotides used only low field 0.35 T, 9.5 GHz X-band EPR to monitor site-specific spin probes (13–20). High-field, high-frequency 8.4 T, 236.6 GHz EPR (12, 21–24) is now a mature technique with sufficient sensitivity to provide spectra from ~ 100 μ M solutions of oligonucleotides in aqueous solution. High-frequency, high-field EPR provides much better definition of the fast (sub-nanosecond) components of probe motion because the high field markedly increases the importance of the nitroxide g-tensor in determining spectral line shape. The Slowly Relaxing Local Structure (SRLS) model (12, 25–27) has become available for fitting spin label spectra that result from fast internal motion of a spin label restrained on a more slowly tumbling macromolecule, and it can be used to reduce spectral ambiguity by simultaneous analysis of spectra at, e.g., 236.6 and 9.5 GHz. Through a combination of 236.6 and 9.5 GHz EPR, supplemented by spectra from intermediate frequencies in the future, one will even more readily be able to spectroscopically separate

the global tumbling and large scale nanosecond bending motions from fast internal subnanosecond fluctuations at the probe (27).

The spin label attached at the 5' terminal of ψ_3 RNA through a thio-amido linkage (5) had its tumbling as reported in prior 9.5 GHz experiments progressively impeded by added NCp7 per Figure 1. In this work we have extended spin label-monitoring sites to the stem-loop-bulge structure of mini c TAR DNA using less mobile ureido-2' amino linkages at SL2, SL1, and SL3, respectively, and for the 3' and 5' ends, using the thio-amido linkages as previously with ψ_3 RNA. Loop, bulge, and destabilized stem regions have all been proposed and frequently found as specific targets for NCp7 in its interaction with TAR RNA and TAR DNA stem-loop oligonucleotides (7, 10, 28-30), and thus we monitor them. A recent NMR-monitored study of mini c TAR DNA bound to 11-55 NCp7 (which lacks the highly basic, cationic 1-10 tail of 1-55 NCp7) showed tryptophan intercalation and hydrogen bonding to unpaired bases in the lower stem below the bulge, but that study also reported gel retardation evidence for another weaker binding elsewhere (11). Although a previous 9.5 GHz EPR study by Sigurdsson and coworkers using a form of TAR RNA lacking the apical loop reported perturbation to the label EPR signal from binding of TAR inhibitors and peptides, there was no study of the interaction of TAR forms with NCp7 (14-16, 18).

At low ionic strength when the ratio of NCp7 to ψ_3 RNA bases was 6-7, a marked decrease in the rate of probe tumbling occurred (5). Such a decrease indicated large, slowly tumbling ψ_3 RNA-NCp7 complexes. The decreased rate of probe tumbling depended not just on ionic strength-dependent electrostatic attraction between cationic NCp7 and anionic RNA but also on the presence of intact Zn fingers of NCp7. Motivated by the *in vitro* annealing of mini c DNA and mini TAR RNA (3, 31), we follow now the change in dynamics of our mini c TAR DNA at a coverage of about 6-7 bases per NCp7. At a coverage of 6-7 bases per NCp7, NCp7 performs as a chaperoning agent that will recognize secondary structures within individual nucleotide strands, destabilize these secondary structures, and enhance subsequent annealing of complementary oligonucleotides into duplexes.

Previous FRET studies have provided evidence for the existence of destabilized secondary structure in the c TAR DNA when it is covered with NCp7 (32-34). Here pulse dipolar spectroscopy (PDS) is used to probe the distances and distance distributions between bi-labels connected to 3' (SLB) and 5' (SLA) ends of mini c TAR DNA at a coverage of about 6-7 bases per NCp7 as a monitor of the unwinding and destabilization of the stem structure. Because of the smaller nitroxide probes, their shorter tethers, and mathematically reliable method for extracting pair distributions (35, 36), the pulsed EPR technique provides not only precise distances (whereas FRET provides distance estimates), but also explicit quantitative information on the distribution of end-to-end distances in NCp7-destabilized mini c TAR DNA. Our present study is thus primarily a dynamic study, but pulse dipolar spectroscopy yields relevant structural information on the destabilization of the DNA double strand within the NCp7-destabilized c TAR DNA structure.

Methods and Materials

Preparation and characterization of spin labeled mini c TAR derivatives

Spin labels were attached at the positions SLA, SLB, SL1, SL2, SL3 shown in Figure 2.

The thio-amido phosphorothioate method of label attachment used for SLA and SLB is similar in its chemistry and its oligonucleotide end location to that used to label the ψ_3 RNA in our previous efforts (5) because study of end labeled mini c TAR DNA followed shortly upon the ψ_3 RNA work.. A 3-Iodomethyl compound with a shorter tether to the nitroxide has more recently been used for making thio-ether end labeled TAR RNA, and will be used

in the future for bi-label studies of both mini TAR RNA and mini c TAR DNA. Mini c TAR DNA with phosphorothioate modification was purchased from Trilink (TriLink Bio Technologies, San Diego, CA) or IDT (Integrated DNA Technologies, Inc., Skokie, IL). Spin labels SLA and SLB, respectively at the 5' and 3' terminals, were attached by reacting iodo-spin label precursors to phosphorothioate (37). 3-(2-iodoacetamide) Proxyl (IPSL) and 3-Iodomethyl-(1-oxy-2,2,5,5-tetramethylpyrroline) were purchased from Toronto Research Chemicals, North York, Ontario, Canada. Scheme 1 shows the thio-phosphorothioate methods of attaching spin labels.

Labels SL1, SL2, and SL3, respectively in the loop, stem, and near the bulge of mini c TAR DNA were attached by the reaction of a 4-Isocyanato TEMPO (Toronto Research Chemicals) spin label precursor with a 2'-amino group on a cytidine sugar (14) to form a 2'-ureido-2'-amino linkage as shown in Scheme 2. Mini c TAR DNAs with specific 2'-amino groups in loop (SL1), stem (SL2) or bulge (SL3) were obtained from TriLink Bio Technologies, San Diego, CA. The ureido linkage to 2'-amino was chosen rather than a phosphorothioate linkage for our initial study of stem, loop, and bulge regions, because it was expected that the 2'-amino linkage would have less intrinsic mobility and would better report motion of the oligonucleotide to which it was tethered. For both the phosphorothioate and the 2'-amino methods of attachment, the detailed protocols for label attachment, for gel and HPLC purification, and for analysis of labeled product are provided in the Supporting Information, **Figures 1S, 2S, 3S**. (Possible Rp and Sp diastereomers of phosphorothioate linkages (38) were not separated.).

Preparation of nucleocapsid protein NCp7

NCp7 was prepared by solid phase peptide synthesis and with analysis methods similar to those described previously (5, 39-41). The final NCp7 concentration was determined by using an extinction coefficient of $\epsilon_{280} = 6050 \text{ M}^{-1} \text{ cm}^{-1}$ (42).

Non denaturing gel shift assays

The interaction of NCp7 and mini c TAR DNA was monitored by non-denaturing gel assays as done previously for ψ_3 RNA (5), and these are shown in Figure 4S to provide at various ratios of NCp7 to c TAR DNA a comparison of NCp7 binding to spin-labeled mini c DNA and NCp7 binding to unlabeled mini c TAR DNA. The spin labeled and non-labeled forms of mini c TAR DNA showed extremely similar NCp7 binding. The mini c TAR DNA by itself, either labeled or not, traveled fastest with unlabeled traveling slightly faster. At a 1:1 or higher ratio of NCp7 to mini c TAR DNA there appeared a low molecular weight complex, traveling slightly slower than the 30 bp marker which, in analogy with the findings on ψ_3 RNA, indicated a 1:1 complex. At higher ratios more diffuse, more slowly moving, higher molecular weight complexes appeared.

Melting of labeled and unlabeled mini c TAR DNA

UV-260 nm absorbance profiles, reflecting the hyperchromic increase brought on by duplex melting, were obtained as a function of temperature in the 20-95 °C range for both labeled and unlabeled mini c TAR DNA at a 2 μM concentration. The melting profile was obtained by taking the first derivative of the absorbance with respect to temperature, and from these profiles estimates were obtained by non-linear least-squares parameter estimation of T_m (melting temperature), ΔH (van't Hoff enthalpy), ΔS (van't Hoff entropy) using a model that assumes a two-state sequential unfolding (43). Melting temperatures of all mini c TAR DNA's, both labeled and unlabeled, were similar to within 1 °C under the same solution conditions (Figure 5S), but the enthalpy of melting was diminished by labels in the bulge (SL3) and at the 5' end (SL2) of mini c TAR DNA (Table 1S).

EPR Spectroscopy

Apparatus—At Albany X-band (9.5 GHz) EPR spectra of spin labeled mini c TAR DNA were taken at room temperature using a high sensitivity 9.5 GHz dielectric resonator that holds samples of approximately 1 μL volume (5, 44-46). Microwave power of 0.64 mW (milliwatt) and modulation of about 1 G (gauss) were chosen so as not to broaden the EPR spectrum. For 9.5 GHz study at ACERT a Bruker ElexSys E500 spectrometer was used at room temperature (20 °C) and at 5 °C with a cavity resonator (SHQE4122). For 9.5 GHz study at ACERT, samples of 1-1.5 μL were placed in a 0.50 i.d. X 0.70 o.d. quartz capillary with end sealed by Dow Corning Silicon vacuum grease, and the spectrometer parameters were: 1 Gauss modulation amplitude, 100 kHz modulation frequency, 2 mW microwave power, 100 Gauss magnetic field sweep.

High-field EPR spectra were taken on a state-of-the-art home built-spectrometer operating at 236.6 GHz (12) to study more explicitly the rapid probe motion. The field modulation was 9 Gauss p.t.p. Twelve mm diameter, ~ 0.17 mm thickness quartz cover slips (ESCO) were used as the basis for double stacked “sandwich” sample holders (27, 47). About 2 μL of sample were placed between a flat quartz cover slip and an etched quartz cover slip containing a circular well in the middle. The diameter of the well was about 0.8 cm. A thin layer of vacuum grease was applied on the edges to seal the two cover slips. Two such samples were fixed together by vacuum grease to provide the sandwich samples containing ~ 4.0 VL. The vertical distance between the centers of the two samples is one half of a wavelength (= 0.63 mm). At 236.6 GHz very low concentrations of Mn^{++} contaminant can provide a background signal because the Mn^{++} line width is vastly sharpened at high frequency, since its width is inversely proportional to the square of the microwave frequency. This Mn^{++} signal is well understood, can be removed from spectra by computer methods, and in fact, provides a field marker.

EPR line shape analysis for the estimate of tumbling correlation times. 9.5

GHz NLSL analysis—Random tumbling, as it modulates the anisotropic hyperfine and Zeeman interactions, causes the three hyperfine lines of the nitrogen $I = 1$ ^{14}N nucleus of the nitroxide spin label to vary differently in amplitude and line width. As the spin label becomes less mobile, the outlying ($M = \pm 1$) peak heights diminish with respect to the central ($M = 0$) peak. This line width variation at 9.5 GHz is primarily due to the motional averaging of the nitrogen hyperfine interaction. 9.5 GHz EPR line shape simulations and correlation time determinations were based on the **Nonlinear Least-Squares Limited stochastic Liouville (NLSL)** fitting program developed by Freed and co-workers (48). These simulations model the motion of the nitroxide by a rotational diffusion tensor, **R**. For our 9.5 GHz simulations, an isotropic tumbling diffusion rate, $R_{\text{iso}} = 1/(6\tau_{\text{iso}})$, generally sufficed, where τ_{iso} is the isotropic tumbling time (although an anisotropic rotation tensor was tried in a number of cases). For samples having slower tumbling brought by a 4:1 ratio of NCp7 to mini c TAR DNA, NLSL was used to estimate the percentage of slower and faster tumbling sites.

Multi-frequency SRLS analysis—Rapid subnanosecond dynamic modes, i.e., internal fluctuations, which may differ between stem, loop, and bulge, were best resolved at higher frequencies. In contrast, nanosecond or longer global tumbling, large bending motions, and nanosecond conformational distortions, would appear frozen at such high frequencies as 236.6 GHz but could affect the low frequency (9.5 GHz) spectra. The high-frequency high-field sensitivity to rapid tumbling motion is a consequence of the increased importance of the g-tensor as the magnetic field is increased. The increased sensitivity to the g-tensor enables high frequency EPR to distinguish rapid rotations about the g_x , the g_y , or the g_z tensor directions so that at high frequencies one can “read off” from the spectrum the nature

of anisotropic motions (12, 49). An important aspect of the SRLS model is that the local reorientation of the spin label may be restricted by a local potential and that order parameters, S_{20} and S_{22} , reflecting axial and non-axial contributions to the ordering, may be derived from this potential.

Pulse dipolar spectroscopy - double electron-electron resonance (DEER)—

Using a 17.35 GHz home-built Ku-band pulse spectrometer (50, 51), DEER measurements were performed at 60 K. A 4-pulse DEER sequence was applied with respective $\pi/2$ - π - π pulse widths of 16 ns, 32 ns and 32 ns, and a 32 ns π pump pulse was used. The detection pulses were positioned at the low-field edge, and the pump pulse was positioned at the center of nitroxide spin-label spectrum so that the frequency separation between detected and pumped pulses was 70 MHz. Distances measured were in the range of 2 to 5 nm, and the DEER evolution time period (τ_2) was 1.6 μ s, covering at least 2.5 periods of dipolar oscillations. The exponentially decaying background was removed from the raw time-domain DEER signals. By application of the Tikhonov regularization (L-curve) method (36) and refinement by the Maximum Entropy Method (MEM) (35), distances were reconstructed from the base-line corrected and normalized signals.

Results

Motion of spin labeled mini c TAR DNA studied by high-field, high-frequency EPR

Because of recent sensitivity advances (27) in high-frequency, high-field EPR, faster internal probe motion can be better understood. The spectra in Figure 3 provide a comparison of 9.5 GHz and 236.6 GHz EPR spectra from mini c TAR DNA labeled at SL1, SL2, SL3, SLB, mini TAR RNA 3' end labeled at SLB, and from ψ_3 RNA labeled at its 5' end.² The 9.5 GHz spectra, all having three fairly narrow lines, indicated an ostensibly isotropic tumbling with correlation time of the order one nanosecond, like that of previously reported spin labeled ψ_3 RNA (5). However, there was a spectroscopic contrast at 236.6 GHz between the samples of mini c TAR DNA, mini TAR RNA, and ψ_3 RNA which were phosphorothioate end-labeled and the samples which were labeled at by ureido-2'-amino linkages at SL1, SL2, and SL3.² The contrast is due to differences in fast subnanosecond tumbling that is well sensed at 236.6 GHz, but not at 9.5 GHz. SL1, SL2, and SL3 performed rapid, sub-nanosecond anisotropic motion about a preferential axis, which was at or near their nitroxide g_x magnetic axis (See Scheme 3 above for definition of axes.). The reorientation of the g_x axis itself, with respect to the overall macromolecule, was considerably slower. The details of such anisotropic motions, in terms of a local rotation tensor and an ordering potential, are provided in the Discussion. The end-labeled species of SLB mini c TAR DNA, SLB mini TAR RNA, and ψ_3 RNA all showed a rapid isotropic tumbling which interchanged x, y, and z axes in less than a nanosecond.

There was a difference in the 236.6 GHz EPR line shape between the mini c TAR DNA and the two RNA derivatives, all labeled at or near the end of their stem. This difference, although marginally evident in the 20 °C spectra of Figure 3, was noteworthy at 5 °C as shown in Figure 4. The motion of the label on the mini TAR RNA slowed more than the corresponding label on the mini c TAR DNA, and by better resolution of its low field g_x shoulder, gave evidence for an increased anisotropy to its label motion at 5 °C. The label at the 5' end of the ψ_3 RNA similarly slowed and showed evidence for anisotropic motion at

²Labeling of a phosphorothioate group by an iodo spin label precursor was done on SLB mini TAR DNA, SLB mini TAR RNA, and ψ_3 RNA. For the SLB mini c TAR DNA, a phosphorothioate between the 3' G and its neighboring G was labeled with an iodoacetamide spin label precursor. For the SLB mini TAR RNA, a phosphorothioate between the 3' G and its neighboring G was labeled with an iodo methyl spin label precursor. For the ψ_3 RNA, a phosphorothioate at the terminal 5' G was labeled with iodoacetamide spin label precursor.

lower temperature. The thio-amido label was used for labeling the mini c TAR DNA and the ψ_3 RNA, and this label has potentially more flexible bonds between its phosphorothioate point of attachment and the nitroxide than does the thio-ether bond used for labeling the mini TAR RNA. However, see Footnote 4.⁴ Nevertheless the label on the ψ_3 RNA, even though attached to the very end of the ψ_3 RNA oligonucleotide rather than between the final and the penultimate nucleotides as in SLB mini c TAR DNA, showed less mobility and more anisotropy at 5 °C than did the same label on the mini c TAR DNA.

Motion of spin labeled mini c TAR DNA in the presence of NCp7

This section focuses on the dynamic changes due to added NCp7 as reported by spin labels on mini c TAR DNA. Because 9.5 GHz EPR is sensitive to slower nanosecond tumbling times, these studies with NCp7 binding at a number of mini c TAR DNA/NCp7 ratios were undertaken with 9.5 GHz EPR.³ The changes in the spin label spectra upon addition of the first NCp7 and then the second NCp7 are shown in Figure 5A. In all cases the tumbling of the spin labels was slowed by the NCp7, and different spin label sites reported different sensitivity to NCp7. The probes at the loop (SL1) and the bulge (SL3) were the slowest to begin with and were most slowed by binding of the NCp7. SL2 in the stem was faster and was less slowed by binding of NCp7. The simplified line shape analysis by a single isotropic correlation time, τ_{iso} , (48, 52) provided a semiempirical parameter for comparing differences in probe motion. Thus, a bar graph of τ_{iso} values is provided in Figure 5B, to show which probes were most impeded by addition of NCp7. Representative simulations used to obtain τ_{iso} from 9.5 GHz data are provided in the Supporting Information, **Figure 5S**.

When the ratio of NCp7 to mini c TAR DNA was increased to 4:1, there was a considerable slowing (from ~1.0 ns to > 5 ns tumbling time) in the motion of the spin label, where the broadening of the signals due to slowing of the tumbling is shown best by the integrated absorption EPR presentation in Figure 6. This phenomenon occurred for all the spin labels studied and at a coverage of ~7 nucleotides/NCp7, corresponding to 4 NCp7 molecules per mini c TAR DNA (noting that mini c TAR DNA is a 27-mer). The line shape broadening occurred at about the same NCp7 coverage where the previous study of ψ_3 RNA stem-loops (5) also showed considerable slowing. Interestingly, Figure 7 in the first derivative mode indicates two differently mobile species for the spin labeling site SLB under the condition where the molecular ratio of NCp7 to mini c TAR DNA is 4:1. As in the case of ψ_3 RNA, the immobilization in the presence of NCp7 coverage could be largely eliminated by increasing the ionic strength. We show representative 9.5 GHz EPR spectra in the presence of a 4:1 NCp7 to mini c TAR DNA molecular ratio in the Supporting Information, **Figure 7S**, where there is progressive line shape narrowing as the NaCl concentration was raised from 20 to 150 to 400 mM.

Changes to mini c TAR DNA at 4:1 NCp7: mini c TAR DNA

The structure of the mini c TAR DNA within the slowly moving multi NCp7 complexes provided a useful complement to the dynamic studies addressed above, especially since such

⁴Work in progress at X-band with thio-ether label linkages in the bulge, stem, and loop near the positions of previous ureido-2'-amino labeling sites SL1, SL2, and SL3 has shown the same order of mobility as SL1, SL2, and SL3 did although the motion of the thio-ether labels is overall faster than the ureido labels. A related study with thio-ether linkages at corresponding stem, loop, and bulge positions is underway to compare motions of mini c TAR DNA and mini TAR RNA. For our future experimental design we are grateful to a reviewer for suggesting looking for Rp Sp diastereomeric differences of the phosphorothioate labeling site between mini c TAR DNA and mini TAR RNA.

³In order to assess the utility of high field EPR to studies of oligonucleotides, our initial high field study was limited to spin labeled mini c TAR DNA and mini TAR RNA oligonucleotides by themselves in the absence of NCp7. In synchrony with technical improvements to high field EPR for aqueous samples, additional high frequency EPR directed at NCp7 complexes of mini TAR is underway.

complexes are not amenable to standard structural NMR and X-ray methods. For this reason, pulse dipolar spectroscopy (DEER) was performed on mini c TAR DNA which had been bi-labeled at the SLA, SLB end positions by thio-amido labels. DEER spectra were taken both in the absence of NCp7 and in the presence of a 4:1 ratio of NCp7 to mini c TAR DNA. Figure 8 indicates the increased interprobe distance and the broadening of the distance distribution between bi-labels SLA and SLB in the presence of a 4:1 ratio of NCp7 to mini c TAR DNA. There was still a large fraction of the mini c TAR DNA which approximately maintained the original SLA-SLB distance, while the remainder adopted a longer interprobe distance with greater breadth overall to its inter-probe distribution.

Discussion

Detailed motion inferred from simultaneous SRLS simulations of 9.5 and 236.6 GHz EPR spectra

In Figure 3 the effects of the motion of the probe, notably for SL1, SL2, and SL3 which have ureido-2' amino spin label linkages, are better resolved at high frequency. The spin labels on mini c TAR DNA, mini TAR RNA, and ψ_3 RNA coupled by the more flexible phosphorothioate linkages at the end of the stem-loop, showed isotropic tumbling at 236.6 GHz at 20 °C, but at 5 °C the tumbling reported by the RNA samples showed incipient anisotropic motion about the g_x axis, as pointed out in Figure 4.

Because of the anisotropy of motion observed from their 236.6 GHz spectra, SL1, SL2, and SL3 were chosen for a comprehensive SRLS fit that included the local rapid diffusion tensor of the spin probe, a potential that in coupling the local rapid diffusion tensor to the global macromolecular tumbling led to an order tensor, and the global diffusion tensor of the entire mini c TAR DNA molecule. Such multi-parameter, multi-frequency simulations have been used to good effect to elucidate label motion for site-directed labels on proteins, notably in the study on T4 Lysozyme (27). The present work is the first application of high-sensitivity, high-frequency EPR to spin labeled oligonucleotides.

The combined 9.5 and 236.6 GHz spectra of SL1, SL2, and SL3 were simulated using the SRLS model program, where $\mathbf{R}_x$, $\mathbf{R}_y$, $\mathbf{R}_z$, c_{20} , and $\mathbf{R}_c$ were varied as fitting parameters. $\mathbf{R}_x$, $\mathbf{R}_y$, $\mathbf{R}_z$ are the components of the anisotropic rotational diffusion tensor localized on the probe. The molecular rotational axis labels, x, y, z are the same as those of the g_x , g_y , and g_z axes (Scheme 3). $\mathbf{R}_c$ is the diffusion coefficient for global tumbling. The dimensionless parameter c_{20} refers to the axial restraining potential that couples local probe motion to overall global tumbling. S_{20} is the order parameter associated with the potential parameter c_{20} ; S_{20} varies from zero, meaning no potential, hence no local ordering, to unity, meaning perfect alignment, i.e. zero flexibility. The values of $S_{20} \approx 0.4$ in Table 1 indicates behavior intermediate between these limits. The resultant parameters are provided in Table 1, and the spectra are overlaid with the resulting simulations in Figure 9. As shown in Table 1, $\mathbf{R}_x \sim 3.3 \cdot \mathbf{R}_y \gg \mathbf{R}_z$. This means that the x-axis, and to a lesser extent the y-axis, are the fast axes for the local motion of the probe. These local fast subnanosecond motions of $\mathbf{R}_x$ and $\mathbf{R}_y$ appear to be influenced by the local nucleotide environment, where SL2, located in a region of possible lower-stem duplex fraying, has the fastest local motion and where SL3, located near the bulge but near the upper duplex region, has the slowest local motion. SL1, in a non-duplex loop region, has local motion nearly as fast as SL2. The global tumbling rate $\mathbf{R}_c$, which is more than an order of magnitude slower than $\mathbf{R}_x$ and $\mathbf{R}_y$ should be affected by the tumbling of large, multi-nucleotide segments. Approximately, the probe performs rapid, but restricted (by the axial local potential), subnanosecond rotation about its g_x axis, which is the axis pointing along the nitroxide NO bond, a direction which would also be the direction along bonds from the 2' amino group to the nitroxide. The rapid restricted motion about this direction partially averages the hyperfine and the magnetic Zeeman interactions

corresponding to the y and the z axes (Scheme 3). The slower restricted motion about the y-axis similarly partially averages x and z components, but less effectively especially given the much larger difference $(g_x \times g_z) \times 10^4 \sim 67$ than $(g_x - g_y) \sim 10^4 \sim 29$ to be averaged by the motion. This all ultimately means that the motion about the z-axis (which is unrestricted by the axial potential) is very slow. On the other hand, the g_x axis itself only slowly reorients so that there remain low field, g_x features in the 236.6 GHz spectra of SL1, SL2, and SL3.

First of all, one notes that R_c values are comparable for all three labels as they should be for the overall tumbling rate; but slow local effects could lead to small differences. Also SL3 exhibits slower R_x but faster R_z than the other two. They all have a comparable local ordering, S_{20} . In general, one finds that $W_{240} > W_{9.5}$, where W_{240} and W_9 are respectively the residual (Lorentzian) widths at 240 and 9.5 GHz, that are additional to widths due to partial averaging of the magnetic tensors by the motions (27). This could be due to local differences in solvent polarity and H-bonding (27), differences in local conformations, and/or some aggregation from undissolved labeled TAR DNA. Since the SL3 was at 250 μ M concentration, whereas the SL1 was at 100 μ M, this could be the explanation for the large W_{240} for the former.

A second SRLS approach is provided in the Supporting Information (See Table 2S and Figure 8S) in which an axial local diffusion tensor is assumed, as opposed to the rhombic tensor used above, but a rhombic restraining potential is assumed for the second SRLS approach, as opposed to the axial restraining potential above. The total number of fitting parameters was the same in these two methods of simulation, but the one in the Supporting Information was less stable requiring fixing some of the parameters. For either method, the motion of the probe was faster for SL2 than for SL1 or SL3, and the fastest local tumbling motion was about the g_x axis.

In Figure 4 a low field 236.6 GHz EPR feature emerged at 5 °C from the mini TAR RNA labeled at the 3' terminal and to a lesser extent from ψ_3 RNA labeled at the 5' terminal. The emergence of this feature implied preferential slowing of the probe motion about the g_x direction in the two RNA samples. Both the label with thio-ether attachment for the mini TAR RNA and with thio-amido attachment for ψ_3 RNA showed slower, anisotropic motion at 5 °C. The feature implying anisotropic motion was absent from the 3' probe signal from mini c TAR DNA, of which the probe tumbling motion remained more isotropic and faster. The mini c TAR DNA/TAR RNA difference would be consistent with preferential melting of the lower stem of mini c TAR DNA, which has been inferred from the absence of imino base pair proton features from the lower stem of mini c TAR DNA (6). The thermodynamic information in Table 1S of the Supporting Information shows that the spin label at position SLB of mini c TAR DNA had, in comparison with the unlabeled mini c TAR DNA, no perturbation to the melting temperature or the thermodynamic stability (ΔG) at 37 or 20 °C, so it is not the label perturbation which is causing melting. The dynamic difference shown in Figure 4 between mini c TAR DNA and mini TAR RNA suggests future comprehensive dynamic comparisons of mini c TAR DNA and mini TAR RNA label motion at numerous corresponding positions, conceivably with pure Rp and Sp diastereomers (38) of the phosphorothioate linkages.⁴

9.5 GHz study of the NCp7-related change in probe mobility

The spectroscopic differences between labels SL1, SL2, and SL3 (Figure 5A), with SL2 showing the greater mobility and SL3 showing less mobility, imply less dynamic motion in the 1 nsec time-scale near the loop (SL1) and the bulge (SL3) than in the stem (SL2).^{3, 4} Although the time scale of motion was different, the high frequency 236.6 GHz EPR findings were similar in that there was less rapid dynamic motion in the bulge (SL3). We

recognize that the ureido-2'-amino attachment of label decreased the enthalpy of melting for SL2 and SL3, where the labeling sites are in regions of base pairing. An explanation for this enthalpy change is that the ureido-2'-amino linkage, although not in the immediate vicinity of DNA bases, could disrupt sugar puckers and rotations so as to interfere with base pairing and stacking. The correlation times τ_{iso} (Figure 5B) are less than expected for the overall global tumbling time of mini c TAR DNA (taken as an approximate cylinder with 20 Å diameter and 40 Å length, leading to $\tau_{\perp} = 5.8$ ns and $\tau_{\parallel} = 2.6$ ns (53)), but still considerably slower than subnanosecond fast local probe motion observed through 236.6 GHz EPR. The nanosecond time scale of the motion would be the time for dynamic motion of a several-base segment of the mini c TAR DNA, but not of the entire molecule. The faster tumbling motion is in the lower stem (Figure 5B), suggested to be partially and dynamically melted even in the absence of NCp7 (6).

The labels in loop and bulge show more sensitivity than the ones in the lower stem to the binding of NCp7, at 1:1 and 2:1 ratios of NCp7 to mini c TAR DNA. A previous NMR study of mini c TAR DNA complexed to 11-55 NCp7, lacking the basic 1-10 tail (11) and carried out at lower ionic strength than our study, indicated a specific interaction of 11-55 NCp7 with unpaired bases in the guanidine region at the bottom of the mini c TAR DNA stem. The NCp7 which we use is 1-55 NCp7, containing both the zinc fingers which specifically recognize unpaired bases and a positively charged, basic 1-10 tail which is thought to non-specifically bind oligonucleotides. It seems less likely that 1-55 NCp7 binding to the lower stem would perturb labels above the bulge (SL1 and SL3) than below (SL2). However, binding of NCp7 to the lower stem could in principle still diminish the conformational flexibility centered at the bulge and thereby indirectly diminish motion sensed by labels at the loop and bulge in the upper part of mini c TAR DNA. The implication of the slower NCp7-induced tumbling of SL1 and SL3 compared to SL2 is either that the position of binding for the 1-55 NCp7 complex is on the loop, bulge, and upper stem, or if there is NCp7 binding to the lower stem, then immobilization and loss of conformational flexibility extend to the upper stem and loop.

The slower tumbling at a ratio of 2:1 NCp7 to mini c TAR DNA suggests that a second binding site for NCp7 impeded the motion, especially of SL1 and SL3. Two binding sites have been implied by isothermal titration and gel binding studies (11). It is possible that one or both of the NCp7 molecules that do bind are exchanging rapidly on the NMR time scale (11) but not the EPR time scale. Such exchange would impede motion on the EPR time scale but would not contribute well-defined structural features needed for NMR structural studies of TAR-NCp7 complexes.

Properties of NCp7: mini c TAR DNA complexes made at a 4:1 ratio of NCp7 to mini c TAR DNA

Significant line shape broadening shown in Figure 6 occurred at a 4:1 ratio of NCp7 c to mini DNA, which is about 1 NCp7 per 7 bases. This is the coverage where annealing also occurs (3, 4, 31) and where destabilization of the stem-loop structure has been proposed from FRET study (32, 33, 54). The 9.5 GHz EPR spectra indicated considerable immobilization of the spin label for all the locations studied. Similar behavior with a coverage 1 NCp7 to 7 oligonucleotide bases was noted in the previously reported study of ψ_3 RNA with NCp7.

Complexes with the coverage of 1 NCp7 per 7 bases have been suggested to be “fuzzy” or molten globule-like complexes (55), not amenable to NMR structural methods. A method to obtain underlying structure for such disordered systems is pulse-dipolar EPR spectroscopy (PDS) of bi-labels. The bi-labels themselves are considerably smaller than FRET probes, they do not require potentially perturbing long tethers that remove them from the site of

interest or separate donor and acceptor forms. In Figure 8 the evidence for NCp7-induced melting of the ends of c mini DNA is shown where both the 3' and 5' end have a spin label. The ~ 25 Å interprobe distance in the absence of NCp7 is due to the diameter of the duplex structure plus the length of each tether. That distance is consistent with previously determined interprobe distances between two spin labels attached to complementary, diametrically opposed phosphorothioates in the middle of a non-frayed duplex DNA (37, 56, 57). The implication is that in the absence of NCp7, the duplex structure in mini c TAR DNA below the bulge is not grossly frayed, even though its bases may undergo dynamic exchange of imino protons (6). The presence of NCp7 at a 4:1 ratio of NCp7 to mini c TAR DNA ratio clearly causes a sizable fraction of the mini c TAR DNA to come apart; the interprobe distance nearly doubles for the that substantial fraction. Quantitative details of the interprobe distribution, not simply semiquantitative evidence for destabilization and fraying, are provided by the Tikhonov reconstruction of the interprobe distance distribution (35, 36). In the ambient temperature 9.5 GHz EPR data of Figure 7 from a 4:1 ratio of NCp7 to mini c DNA there was evidence for a fraction of species having slow > 5 ns tumbling and another fraction having a tumbling time ~ 2 ns. The inter-label distance distribution of Figure 8 shows a fraction of more thoroughly frayed species having interprobe distances > 40 Å, and another less frayed fraction having an interprobe distances closer to the unperturbed 25 Å distance. It is unlikely that the fraction of bi-labeled mini c TAR DNA in Figure 8, having an interprobe distance of about 25 Å, is from mini c TAR DNA not at all bound to NCp7 because the typical dissociation constant for NCp7 from oligonucleotides is less than micromolar (11, 58), because the ambient temperature EPR of spin labeled mini c TAR DNA in the presence of a 4-fold excess of NCp7 showed no evidence for rapidly moving, unbound mini c TAR DNA (Figure 7), and because the DEER feature with peak at 25 Å in the presence of NCp7 is broadened, from destabilization by NCp7. These findings provide explicit physical evidence for the simultaneous existence of both the closed conformation and the partially open 'Y' conformation of mini c TAR DNA. It is tempting to suggest that within the condensate having a 4:1 ratio of NCp7 to mini c TAR DNA the fraction of species with the slower spin dynamics ($\tau_{\text{iso}} > 5$ ns) in Figure 7 is the thoroughly frayed fraction of mini c TAR DNA, while the species with more rapid spin dynamics ($\tau_{\text{iso}} \sim 2$ ns) is the less frayed.

Summary

In summary, ambient temperature spin label studies with 240 GHz EPR provided information on rapid subnanosecond, hindered, local anisotropic motions within the mini c TAR DNA molecule and showed differences in these motions, where the most rapid motion was in the lower stem. High frequency EPR showed a dynamic difference between the end-labeled mini c TAR DNA and the mini TAR RNA and the ψ_3 RNA, implying a more ordering environment of the label at 5 °C in the two RNAs. These dynamic differences between stem, loop, and bulge, and between mini c TAR DNA and mini TAR RNA provide insight into oligonucleotide dynamics, and they point to future comparative RNA-DNA studies.⁴ The slowing of nanosecond tumbling motions of large segments of the oligonucleotide, which are best observed by 9.5 GHz EPR, was enhanced by increasing the ratio of the nucleocapsid protein NCp7 to mini c TAR DNA from zero to one to two. A greater slowing was observed from the labels in the loop and near the bulge of mini c TAR DNA. This differential dynamic sensitivity to the binding of the NCp7, as probed by EPR, is thus a functional aspect of mini c TAR. At a 4:1 ratio of NCp7 to mini c TAR DNA there was significantly slowed tumbling of all labels, indicating, as previously with ψ_3 RNA (5), the condensation of NCp7 with mini c TAR DNA. At a 4:1 ratio of NCp7 to mini c TAR DNA, concomitant structural evidence of partial melting and a broadened 3' - 5' end-to-end distance distribution of the mini c TAR DNA was obtained by pulse dipolar EPR

spectroscopy (DEER) of bi-labels attached near the 3' and 5' terminals of the mini c TAR DNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- (1). Johnson PE, Turner RB, Wu ZR, Hairston L, Guo J, Levin JG, Summers MF. A mechanism for plus-strand transfer enhancement by the HIV-1 nucleocapsid protein during reverse transcription. *Biochemistry*. 2000; 39:9084–9091. [PubMed: 10924101]
- (2). Guo J, Henderson LE, Bess J, Kane B, Levin JG. Human immunodeficiency virus type 1 nucleocapsid protein promotes efficient strand transfer and specific viral DNA synthesis by inhibiting TAR-dependent self-priming from minus-strand strong-stop DNA. *J Virol*. 1997; 71:5178–5188. [PubMed: 9188585]
- (3). Vo MN, Barany G, Rouzina I, Musier-Forsyth K. HIV-1 nucleocapsid protein switches the pathway of transactivation response element RNA/DNA annealing from loop-loop “kissing” to “zipper”. *J Mol Biol*. 2009; 386:789–801. [PubMed: 19154737]
- (4). Vo MN, Barany G, Rouzina I, Musier-Forsyth K. Mechanistic studies of mini-TAR RNA/DNA annealing in the absence and presence of HIV-1 nucleocapsid protein. *J Mol Biol*. 2006; 363:244–261. [PubMed: 16962137]
- (5). Xi X, Sun Y, Karim CB, Grigoryants VM, Scholes CP. HIV-1 nucleocapsid protein NCp7 and its RNA stem loop 3 partner: rotational dynamics of spin-labeled RNA stem loop 3. *Biochemistry*. 2008; 47:10099–10110. [PubMed: 18729386]
- (6). Zargarian L, Kanevsky I, Bazzi A, Boynard J, Chaminade F, Fosse P, Mauffret O. Structural and dynamic characterization of the upper part of the HIV-1 cTAR DNA hairpin. *Nucleic Acids Res*. 2009; 37:4043–4054. [PubMed: 19417069]
- (7). Zhang Q, Sun X, Watt ED, Al-Hashimi HM. Resolving the motional modes that code for RNA adaptation. *Science*. 2006; 311:653–656. [PubMed: 16456078]
- (8). Zhang Q, Stelzer AC, Fisher CK, Al-Hashimi HM. Visualizing spatially correlated dynamics that directs RNA conformational transitions. *Nature*. 2007; 450:1263–1267. [PubMed: 18097416]
- (9). Getz M, Sun X, Casiano-Negroni A, Zhang Q, Al-Hashimi HM. NMR studies of RNA dynamics and structural plasticity using NMR residual dipolar couplings. *Biopolymers*. 2007; 86:384–402. [PubMed: 17594140]
- (10). Sun X, Zhang Q, Al-Hashimi HM. Resolving fast and slow motions in the internal loop containing stem-loop 1 of HIV-1 that are modulated by Mg²⁺ binding: role in the kissing-duplex structural transition. *Nucleic Acids Res*. 2007; 35:1698–1713. [PubMed: 17311812]
- (11). Bazzi A, Zargarian L, Chaminade F, Boudier C, De Rocquigny H, Rene B, Mely Y, Fosse P, Mauffret O. Structural insights into the cTAR DNA recognition by the HIV-1 nucleocapsid protein: role of sugar deoxyribose in the binding polarity of NC. *Nucleic Acids Res*. 2011; 39:3903–3916. [PubMed: 21227929]
- (12). Earle KA, Dzikovski B, Hofbauer W, Moscicki JK, Freed JH. High-frequency ESR at ACERT. *Magn Reson Chem*. 2005; 43(Spec no):S256–266. [PubMed: 16235203]
- (13). Qin PZ, Butcher SE, Feigon J, Hubbell WL. Quantitative analysis of the isolated GAAA tetraloop/receptor interaction in solution: a site-directed spin labeling study. *Biochemistry*. 2001; 40:6929–6936. [PubMed: 11389608]

- (14). Edwards TE, Okonogi TM, Robinson BH, Sigurdsson ST. Site-specific incorporation of nitroxide spin-labels into internal sites of the TAR RNA; structure-dependent dynamics of RNA by EPR spectroscopy. *J Am Chem Soc.* 2001; 123:1527–1528. [PubMed: 11456739]
- (15). Edwards TE, Okonogi TM, Sigurdsson ST. Investigation of RNA-protein and RNA-metal ion interactions by electron paramagnetic resonance spectroscopy. The HIV TAR-Tat motif. *Chem Biol.* 2002; 9:699–706. [PubMed: 12079781]
- (16). Edwards TE, Sigurdsson ST. Electron paramagnetic resonance dynamic signatures of TAR RNA-small molecule complexes provide insight into RNA structure and recognition. *Biochemistry.* 2002; 41:14843–14847. [PubMed: 12475232]
- (17). Qin PZ, Hideg K, Feigon J, Hubbell WL. Monitoring RNA base structure and dynamics using site-directed spin labeling. *Biochemistry.* 2003; 42:6772–6783. [PubMed: 12779332]
- (18). Edwards TE, Robinson BH, Sigurdsson ST. Identification of amino acids that promote specific and rigid TAR RNA-tat protein complex formation. *Chem Biol.* 2005; 12:329–337. [PubMed: 15797217]
- (19). Popova AM, Kalai T, Hideg K, Qin PZ. Site-specific DNA structural and dynamic features revealed by nucleotide-independent nitroxide probes. *Biochemistry.* 2009; 48:8540–8550. [PubMed: 19650666]
- (20). Grant GP, Boyd N, Herschlag D, Qin PZ. Motions of the substrate recognition duplex in a group I intron assessed by site-directed spin labeling. *J Am Chem Soc.* 2009; 131:3136–3137. [PubMed: 19220053]
- (21). Earle KA, Freed JH. Quasioptical hardware for a flexible FIR-EPR spectrometer. *Applied Magnetic Resonance.* 1999; 16:247–272.
- (22). Freed JH. New technologies in electron spin resonance. *Annu Rev Phys Chem.* 2000; 51:655–689. [PubMed: 11031296]
- (23). Borbat PP, Costa-Filho AJ, Earle KA, Moscicki JK, Freed JH. Electron spin resonance in studies of membranes and proteins. *Science.* 2001; 291:266–269. [PubMed: 11253218]
- (24). Bennati M, Prisner TF. New Developments in High Field Electron Paramagnetic Resonance with Applications in Structural Biology. *Reports on Progress in Physics.* 2005; 68:411.
- (25). Liang Z, Freed JH. An assessment of the applicability of multifrequency ESR to study the complex dynamics of biomolecules. *J. Phys. Chem. B.* 1999; 103:6384–6396.
- (26). Polimeno A, Freed JH. Slow motional ESR in complex fluids: The slowly relaxing local structure model of solvent cage effects. *J. Phys. Chem.* 1995; 99:10995–11006.
- (27). Zhang Z, Fleissner MR, Tipikin DS, Liang Z, Moscicki JK, Earle KA, Hubbell WL, Freed JH. Multifrequency electron spin resonance study of the dynamics of spin labeled T4 lysozyme. *J Phys Chem B.* 2010; 114:5503–5521. [PubMed: 20361789]
- (28). Pitt SW, Zhang Q, Patel DJ, Al-Hashimi HM. Evidence that electrostatic interactions dictate the ligand-induced arrest of RNA global flexibility. *Angew Chem Int Ed Engl.* 2005; 44:3412–3415. [PubMed: 15861447]
- (29). Fisher CK, Zhang Q, Stelzer A, Al-Hashimi HM. Ultrahigh resolution characterization of domain motions and correlations by multialignment and multireference residual dipolar coupling NMR. *J Phys Chem B.* 2008; 112:16815–16822. [PubMed: 19367865]
- (30). Zhang Q, Al-Hashimi HM. Domain-elongation NMR spectroscopy yields new insights into RNA dynamics and adaptive recognition. *Rna.* 2009; 15:1941–1948. [PubMed: 19776156]
- (31). Vo MN, Barany G, Rouzina I, Musier-Forsyth K. Effect of Mg(2+) and Na(+) on the nucleic acid chaperone activity of HIV-1 nucleocapsid protein: implications for reverse transcription. *J Mol Biol.* 2009; 386:773–788. [PubMed: 19154740]
- (32). Hong MK, Harbron EJ, O'Connor DB, Guo J, Barbara PF, Levin JG, Musier-Forsyth K. Nucleic acid conformational changes essential for HIV-1 nucleocapsid protein-mediated inhibition of self-priming in minus-strand transfer. *J Mol Biol.* 2003; 325:1–10. [PubMed: 12473448]
- (33). Cosa G, Harbron EJ, Zeng Y, Liu HW, O'Connor DB, Eta-Hosokawa C, Musier-Forsyth K, Barbara PF. Secondary structure and secondary structure dynamics of DNA hairpins complexed with HIV-1 NC protein. *Biophys J.* 2004; 87:2759–2767. [PubMed: 15454467]
- (34). Cosa G, Zeng Y, Liu HW, Landes CF, Makarov DE, Musier-Forsyth K, Barbara PF. Evidence for non-two-state kinetics in the nucleocapsid protein chaperoned opening of DNA hairpins. *J*

- Phys Chem B Condens Matter Mater Surf Interfaces Biophys. 2006; 110:2419–2426. [PubMed: 16471833]
- (35). Chiang YW, Borbat PP, Freed JH. Maximum entropy: a complement to Tikhonov regularization for determination of pair distance distributions by pulsed ESR. *J Magn Reson.* 2005; 177:184–196. [PubMed: 16137901]
 - (36). Chiang YW, Borbat PP, Freed JH. The determination of pair distance distributions by pulsed ESR using Tikhonov regularization. *J Magn Reson.* 2005; 172:279–295. [PubMed: 15649755]
 - (37). Cai Q, Kusnetzow AK, Hubbell WL, Haworth IS, Gacho GP, Van Eps N, Hideg K, Chambers EJ, Qin PZ. Site-directed spin labeling measurements of nanometer distances in nucleic acids using a sequence-independent nitroxide probe. *Nucleic Acids Res.* 2006; 34:4722–4730. [PubMed: 16966338]
 - (38). Grant GP, Popova A, Qin PZ. Diastereomer characterizations of nitroxide-labeled nucleic acids. *Biochem Biophys Res Commun.* 2008; 371:451–455. [PubMed: 18442474]
 - (39). Zhang Z, Xi X, Scholes CP, Karim CB. Rotational dynamics of HIV-1 nucleocapsid protein NCp7 as probed by a spin label attached by peptide synthesis. *Biopolymers.* 2008; 89:1125–1135. [PubMed: 18690667]
 - (40). Karim CB, Kirby TL, Zhang Z, Nesmelov Y, Thomas DD. Phospholamban structural dynamics in lipid bilayers probed by a spin label rigidly coupled to the peptide backbone. *Proc Natl Acad Sci U S A.* 2004; 101:14437–14442. [PubMed: 15448204]
 - (41). Karim CB, Zhang Z, Thomas DD. Synthesis of TOAC spin-labeled proteins and reconstitution in lipid membranes. *Nat Protoc.* 2007; 2:42–49. [PubMed: 17401337]
 - (42). Tummino PJ, Scholten JD, Harvey PJ, Holler TP, Maloney L, Gogliotti R, Domagala J, Hupe D. The in vitro ejection of zinc from human immunodeficiency virus (HIV) type 1 nucleocapsid protein by disulfide benzamides with cellular anti-HIV activity. *Proc Natl Acad Sci U S A.* 1996; 93:969–973. [PubMed: 8577770]
 - (43). Theimer CA, Wang Y, Hoffman DW, Krisch HM, Giedroc DP. Non-nearest neighbor effects on the thermodynamics of unfolding of a model mRNA pseudoknot. *J Mol Biol.* 1998; 279:545–564. [PubMed: 9641977]
 - (44). DeWeerd K, Grigoryants V, Sun Y, Fetrow JS, Scholes CP. EPR-detected folding kinetics of externally located cysteine-directed spin-labeled mutants of iso-1-cytochrome c. *Biochemistry.* 2001; 40:15846–15855. [PubMed: 11747462]
 - (45). Grigoryants, VM.; Scholes, CP. Variable Velocity Liquid Flow EPR and Submillisecond Protein Folding (Chapt. 5.3). In: Uversky, VN.; Permyakov, EA., editors. *Protein Structures: Methods in Protein Structure and Stability Analysis*. Nova Science Publishers, Inc.; New York: 2006. p. 47-71.
 - (46). Grigoryants VM, Veselov AV, Scholes CP. Variable velocity liquid flow EPR applied to submillisecond protein folding. *Biophys J.* 2000; 78:2702–2708. [PubMed: 10777766]
 - (47). Barnes JP, Freed JH. Aqueous SAMPLE HOLDERS for High Frequency Electron Spin Resonance. *Rev. Sci. Instrum.* 1997; 68:2838–2846.
 - (48). Budil DE, Lee S, Saxena S, Freed JH. Nonlinear-Least-Squares Analysis of Slow-Motion EPR Spectra in One and Two Dimensions Using a Modified Levenberg-Marquardt Algorithm. *J. Magn. Reson., Ser. A.* 1996; 120:155–189.
 - (49). Dzikovski B, Earle K, Pachtchenko S, Freed J. High-field ESR on aligned membranes: a simple method to record spectra from different membrane orientations in the magnetic field. *J Magn Reson.* 2006; 179:273–279. [PubMed: 16427793]
 - (50). Borbat PP, Crepeau RH, Freed JH. Multifrequency two-dimensional Fourier transform ESR: an X/Ku-band spectrometer. *J. Magn. Reson.* 1997; 127:155–167. [PubMed: 9281479]
 - (51). Borbat PP, Freed JH. Measuring distances by pulsed dipolar ESR spectroscopy: spin-labeled histidine kinases. *Methods Enzymol.* 2007; 423:52–116. [PubMed: 17609127]
 - (52). Earle, KA.; Budil, DE. Chapt. 3 Calculating Slow-motion ESR Spectra of Spin-Labeled Polymers. In: Schlick, S., editor. *Advanced ESR Methods in Polymer Research*. John Wiley and Sons; New York: 2006.
 - (53). Tirado MM, de la Torre. Rotational dynamics of rigid symmetric top macromolecules. Application to circular cylinders. *J Chem Phys.* 1980; 73:1986–1993.

- (54). Liu HW, Zeng Y, Landes CF, Kim YJ, Zhu Y, Ma X, Vo MN, Musier-Forsyth K, Barbara PF. Insights on the role of nucleic acid/protein interactions in chaperoned nucleic acid rearrangements of HIV-1 reverse transcription. *Proc Natl Acad Sci U S A*. 2007; 104:5261–5267. [PubMed: 17372205]
- (55). Darlix JL, Godet J, Ivanyi-Nagy R, Fosse P, Mauffret O, Mely Y. Flexible nature and specific functions of the HIV-1 nucleocapsid protein. *J Mol Biol*. 2011; 410:565–581. [PubMed: 21762801]
- (56). Cai Q, Kusnetzow AK, Hideg K, Price E, Haworth IS, Qin PZ. Nanometer distance measurements in RNA using site-directed spin labeling. *Biophys J*. 2007; 93:2110–2117. [PubMed: 17526583]
- (57). Price EA, Sutch BT, Cai Q, Qin PZ, Haworth IS. Computation of nitroxide-nitroxide distances in spin-labeled DNA duplexes. *Biopolymers*. 2007; 87:40–50. [PubMed: 17538992]
- (58). Avilov SV, Godet J, Piemont E, Mely Y. Site-specific characterization of HIV-1 nucleocapsid protein binding to oligonucleotides with two binding sites. *Biochemistry*. 2009; 48:2422–2430. [PubMed: 19186983]

Figure 1.

A) Secondary structure of mini c TAR DNA and mini TAR-RNA and the position of spin labels used for the present study primarily of c mini DNA are shown. **B)** The primary structure of 1-55 NCp7 is shown.

Figure 2.

This figure shows the positions of SLA, SLB, SL1, SL2, and SL3 which were the labeling sites on mini c TAR DNA. SLA and SLB, which are respectively at the 5' and 3' terminals, were attached by phosphorothioate linkages using iodo precursors. SL1, SL2, and SL3, which are respectively at cytidines of the loop, lower stem, and bulge region, were attached at a 2' amino group on cytidine sugars via a ureido-2'-amino linkage.

Figure 3.

ESR spectra of mini c TAR DNA labeled at positions SL1, SL2, SL3, SLB, mini TAR RNA labeled at its 3' (SLB) terminal, and ψ_3 RNA labeled at the 5' terminal. Concentrations of labels were approximately 100 μM (except SL3 which was 250 μM) in pH 7.5 20 mM Hepes, 20 mM NaCl, 0.2 mM Mg^{++} , temperature 20 °C. The spectra are compared at EPR frequencies of 9.5 GHz and 236.6 GHz to show the resolving power of high frequency EPR for differences in motion. EPR conditions are given in the Methods Section. The features labeled Mn^{++} are due to low level Mn^{++} impurities whose line shapes are vastly sharpened at high frequency and provide effective internal field markers.

Figure 4. 236.6 GHz EPR spectra at 20 and 5°C are presented to show temperature-dependent differences in line shape that were observed for end-labeled SLB mini c TAR DNA, SLB mini TAR RNA, and ψ_3 RNA. The difference in the enclosed low field region is largest at 5 °C. Besides the temperature, conditions for obtaining these spectra are as in Figure 3.

Figure 5.

A. 9.5 GHz EPR signals from mini c TAR DNA single spin labels titrated with zero, one, and two equivalents of NCp7. Temperature 20 °C, field modulation = 1.3 G. Spectra were normalized to the same number of spins by double integration.

B. Simplified isotropic correlation times (τ_{iso}) of spin labeled c TAR DNA species derived from the above spectra in **Figure 5A** by fitting spectra by the NLSL routine (48, 52).

Figure 6.

This figure compares the absorption 9.5 GHz EPR line shapes of the spin labeled mini c TAR DNAs at low ionic strength. The NCp7 to mini c TAR DNA ratio was increased to 4, where a slowly tumbling complex forms. Spectra were normalized on the second integral. Sample conditions: 20 mM HEPES, 20 mM NaCl, 0.2 mM MgCl₂, pH 7.5. Temperature 20 °C. The mini c TAR DNA concentration was 100 μM.

Figure 7.

First derivative 9.5 GHz EPR spectrum obtained at room temperature from SLB-labeled mini c TAR DNA in the presence of a 4:1 mole ratio of NCp7 to mini c TAR DNA. Spectrum showed evidence for two differently immobilized species, one with $\tau_{\text{iso}} \sim 2.3 \text{ ns}$ and the other with $\tau_{\text{iso}} \sim 6.8 \text{ ns}$.

Figure 8.

Doubly labeled mini c TAR DNA (BLUE) + 4:1 NCp7 (RED). Structural evidence for fraying of ends of stem-loop is obvious from PDS carried out at a 4:1 ratio of NCp7 to bi-end-labeled mini c TAR DNA and low ionic strength. A) shows the normalized PDS signals. B) provides the comparison of the interprobe SLA-SLB distance distribution in the absence and in the presence of a 4:1 ratio of NCp7 to bi-labeled mini c TAR DNA. The experimental spectrometer conditions are provided in the Methods Section. Sample conditions: 20 mM HEPES, 20 mM NaCl, 0.2 mM $MgCl_2$, pH 7.5. Samples were frozen in 10 % glycerol to prevent tube breakage.

Figure 9.

This figure shows the combined simulations to 9.5 GHz and 236.6 GHz spectra to provide evidence for anisotropic diffusion tensor of probe provided through the parameters for SL1, SL2, and SL3 in Table 1. $\mathbf{R}_x$, $\mathbf{R}_y$, $\mathbf{R}_z$, c_{20} , and $\mathbf{R}_c$ are the major fitting parameters.

Scheme 1.

This scheme shows the reaction **(a)** of a phosphorothioate sulfur with 3-Iodomethyl-(1-oxy-2,2,5,5-tetramethylpyrroline) in blue to form a phosphorothioate linkage. A similar reaction **(b)** was performed with 3-(2-Iodoacetamino) proxyl spin-label shown in red.

Scheme 2.

Reaction of 4 - isocyanato TEMPO spin label with 2' - amino-cytidine to form a ureido-2' - amino linkage

Scheme 3.

High frequency EPR better resolves anisotropic probe motion. It distinguishes x, y, or z rotations because of its greater sensitivity to g_x , g_y , g_z anisotropy. Specifically for SL1, SL2, SL3 which have a TEMPO nitroxide, we take $g_x = 2.00893$, $g_y = 2.00604$, $g_z = 2.00224$; $A_x = 6.9$, $A_y = 7.6$, $A_z = 36.5$ G.

Table 1

Simultaneous Fitting to the 9.5 - 236.6 GHz EPR Spectra of SL1, SL2, and SL3. $\underline{R}_x$, $\underline{R}_y$, $\underline{R}_z$, c_{20} , and $\underline{R}_c$ are major fitting parameters.^a

Spin Label	SL1	SL2	SL3
$\underline{R}_c$ (10^7 s ⁻¹)	2.6 ± 0.1	3.2 ± 0.1	3.3 ± 0.1
$\underline{R}_x$ (10^7 s ⁻¹)	72.4 ± 6.5	75.3 ± 6.6	54.7 ± 4.9
$\underline{R}_y$ (10^7 s ⁻¹)	19.2 ± 0.8	22.0 ± 0.9	19.5 ± 0.8
$[(\underline{R}_x + \underline{R}_y) / 2]$ (10^7 s ⁻¹)	45.8 ± 3.3	48.9 ± 3.3	35.0 ± 2.5
$\underline{R}_z$ (10^7 s ⁻¹)	0.6 ± 0.2	0.6 ± 0.2	1.5 ± 0.2
c_{20}	1.86 ± 0.02	1.70 ± 0.02	1.67 ± 0.02
S_{20}	0.41	0.38	0.37
$W_{9.5 \text{ GHz}}$ (G)	0.94	0.89	0.90
$W_{240 \text{ GHz}}$ (G)	1.3	4.4	7.8

^aThe parameters used are those listed in **Tables 1** here and 2S in the Supporting Information. All parameters were varied in the fitting process except for the ones labeled “fixed” in Table 2S. No additional parameters were included to obtain the numbers in **Table 1** or Table 2S. β_d (β_d), the angle between the nitroxide magnetic tensor and the fast diffusion tensor, was not included; in fact $\beta_d = 30^\circ$ was tried and the fit was poorer. Errors in parameters were estimated by randomly starting the fitting process within ± 10% of the parameter values in **Table 1** (or Table 2S) and then recording the resultant variation in the fitted parameters.