Stem-loop SL4 of the HIV-1 \( \Psi \) RNA Packaging Signal Exhibits Weak Affinity for the Nucleocapsid Protein. Structural Studies and Implications for Genome Recognition

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Encapsulation of the genome of the human immunodeficiency virus type-1 (HIV-1) during retrovirus assembly is mediated by interactions between the nucleocapsid (NC) domains of assembling Gag polyproteins and a \( \sim 110 \) nucleotide segment of the genome known as the \( \Psi \)-site. The HIV-1 \( \Psi \)-site contains four stem-loops (SL1 through SL4), all of which are important for genome packaging. Recent isothermal titration calorimetry (ITC) studies have demonstrated that SL2 and SL3 are capable of binding NC with high affinity (\( K_d \sim 140 \text{ nM} \)), consistent with proposals for protein-interactive functions during packaging. To determine if SL4 may have a similar function, NC-interactive studies were conducted by NMR and gel-shift methods. In contrast to previous reports, we find that SL4 binds weakly to NC (\( K_d = (\pm 14 \text{ } \mu \text{M}) \)), suggesting an alternative function. NMR studies indicate that the GAGA tetraloop of SL4 adopts a classical GNRA-type fold (\( R = \text{purine}, N = G, C, A \text{ or } U \)), a motif that stabilizes RNA tertiary structures in other systems. In combination with previously reported gel mobility studies of \( \Psi \)-site deletion mutants, these findings suggest that SL4 functions in genome recognition not by binding to Gag, but by stabilizing the structure of the \( \Psi \)-site. Differences in the affinities of NC for SL2, SL3 and SL4 stem-loops can now be rationalized in terms of the different structural properties of stem loops that contain GGNG (SL2 and SL3) and GNRA (SL4) sequences.

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The genomes of all retroviruses encode a polyprotein called Gag that is responsible for the selection and packaging of the viral genome during virus assembly. Genome packaging is mediated primarily by interactions between the nucleocapsid (NC) domain of Gag and a \( \sim 110 \) nucleotide segment of the viral RNA known as the \( \Psi \)-site.\(^1\) Approximately 1500 copies of Gag assemble in the cytosol or at the outer cell membrane, and bud to form the immature virus particle.\(^5\) Gag specifically incorporates two copies of the full length genomic RNA into budding virions, which are non-covalently linked near their 5' ends in mature particles (see Coffin et al.\(^6\) and references there-in).

Site-directed mutagenesis experiments, chemical and enzymatic accessibility assays, and free energy minimization calculations indicate that the \( \Psi \)-site of the human immunodeficiency virus type-1 (HIV-1) contains four stem-loops (SL1-SL4) con-
nected by relatively short linkers (Figure 1). These stem loops have independent and, in some cases, redundant functions in genome selection. Stem loop SL1 serves as the primary dimer initiation site (DIS), and this overlap of dimerization and packaging functions appears to provide a mechanism for the preferential packaging of two copies of the genome during virus assembly. SL2 contains the major splice donor (SD) site, and the overlap of this signal with the gag gene initiation sequence. Both SL1 and SL2 are capable of binding to NC, and both may be recognized by the NC domain of Gag during packaging. SL3, which also binds tightly to NC, has been shown to be capable of independently directing the packaging of heterologous RNAs into virus-like particles. Note, however, that deletion of SL3 from the HIV-1 genome does not completely eliminate genome packaging, although packaging levels are significantly reduced. In addition, mutations designed to disrupt the stems of SL3 and the other PSI-site stem loops also lead to reductions in (but not complete loss of) genome packaging. Based on these and other studies, it has been suggested that SL2 and SL3 may function in genome recognition by interacting with the NC domain of Gag.

Filter binding assays indicated that NC also binds to SL4 with high affinity ($K_d = 200$ nM), suggesting that this stem-loop may also interact with the NC domain of Gag during packaging. We therefore initiated studies of NC-SL4 interactions, in an effort to refine our model of the protein-RNA recognition complex responsible for genome selection. Surprisingly, and in contrast to results obtained for NC complexes with SL2 and SL3, NMR spectra obtained for SL4 in the presence of NC exhibited broad lines and other features typical of a rapid equilibrium involving one or more weakly bound species. Subsequent native PAGE and NMR studies revealed that the affinity of NC for SL4 is more than two orders of magnitude weaker than observed for NC binding to SL2 and SL3. NMR data obtained for SL4 indicate that the tetrarope adopts a classical GNRA-type fold, and that the stem, which contains two G-U wobble base-pairs, is conformationally labile. These findings allow rationalization of the different NC binding affinities of SL2, SL3 and SL4 in terms of differences in their tetraroplo structures, and suggest that SL4 may not function during packaging as an NC binding site.

Results and Discussion

Sample preparation and purification

Recombinant NC protein samples were prepared and purified as described. Two oligoribonucleotide sequences were employed here: SL4, a 14 nucleotide construct with sequence that corresponds to residues 793-806 of the HIV-1 genome, and SL4-4, which includes four additional non-native base-pairs appended to the stem to improve transcription yields and stabilize the stem. SL4 was obtained commercially from Dharmacon Research Inc. (Boulder, CO, USA), while SL4-4 was prepared and purified by standard in vitro transcription methods with yields typically in the range of 180 optical density units (ODUs) per 30 ml of reaction solution. The identity of SL4-4 was confirmed by electrospray mass spectrometry ($MW_{\text{calc}} = 7352.2$).

The nucleocapsid protein binds weakly to SL4

Interactions between SL4 and NC were initially monitored by 1D $^1$H NMR spectroscopy. Titration of SL4 with NC resulted in a broadening and shifting of several RNA $^1$H NMR signals, but did not give rise to new sets of signals that would be indicative of tight binding. The data indicate that NC forms one or more complexes with SL4 that are in rapid exchange with the free RNA on the NMR chemical shift time scale (milliseconds). To monitor binding in a more quantitative manner, native PAGE data were obtained for SL4 as a
function of added NC. Under the conditions employed (25 mM NaCl, 25 mM sodium acetate (pH 6.5), 0.1 mM ZnCl2, 80-160 μM RNA), SL4 migrates at a rate expected for the monomeric (stem-loop) species in the absence of NC (Figure 2). Addition of increasing amounts of NC resulted in the appearance of a new band that migrates at a rate expected for an NC-SL4 complex. Bands attributed to the free SL4 RNA were clearly visible at NC:SL4 ratios greater than 1:1, and no additional bands that could be attributable to a ternary (NC2:SL4) or higher order species were observed. In contrast, in similar experiments carried out with SL2 and SL3, the band due to free RNA disappeared at NC:RNA ratios greater than 1:1, as expected for high-affinity binding ($K_d \approx 110$-170 nM, as measured by isothermal titration calorimetry (ITC)). Representative data obtained for NC binding to SL3 are shown in Figure 2. In addition, at NC:SL3 ratios in excess of 1:1, an additional band was observed for a NC2:SL3 species. Similar results have been observed by others. The fact that the second shifted band does not appear at NC:SL3 ratios below 1:1 indicates that the binding of the second NC protein occurs with affinity that is at least 100-fold less than that of the first NC protein (i.e. $K_d \approx 17$ μM).

Analysis of the gel shift data based on decreases in the band intensity for the free SL4 RNA, and using a two-state binding model, afforded an apparent dissociation constant of 47(±14) μM (reported as the mean ± standard deviation of five independent experiments). A representative non-linear least squares fit of one of the titration experiments is shown in Figure 2. The observed NC-SL4 dissociation constant differs by more than two orders of magnitude from the values determined by ITC methods for SL2 ($K_d = 110(±50)$ nM) and SL3 ($K_d = 170(±65)$ nM), and is also considerably higher than the value determined for SL4 by a filter binding assay ($K_d = 200$ μM$^2$).

ITC measurements of NC binding to SL4 were attempted in order to allow a quantitative comparison of the relative affinities for NC for SL2, SL3 and SL4. Previous ITC studies demonstrated that NC binds to SL2 and SL3 RNA with negative binding enthalpies that can be fit to 1:1 binding isotherms. However, titration of SL4 with NC did not lead to detectable binding enthalpies, even for experiments conducted at relatively high sample concentrations (50 μM RNA titrated with 10 to 250 μM aliquots of NC; data not shown).

**SL4 contains a stable GNRA-type tetraloop and an unstable stem**

In an effort to understand why NC binds to SL4 with substantially lower affinity compared with SL2 and SL3, structural features of SL4 were studied using 2D 1H-1H and 1H-13C correlated NMR spectroscopy. We prepared a construct with a longer stem, designated SL4-4, which was designed to improve efficiency of in vitro transcription and stabilize the stem (see below). It should be pointed out that a high resolution NMR structure has been reported for an oligoribonucleotide hairpin that contains the same tetraloop and the two adjacent base-pairs that are present in SL4.$^{30}$ However, the stem of SL4 contains two G-U wobble pairs, and it seemed possible to us that these

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**Figure 2.** Native PAGE NC titration results obtained for (a) SL3 (160 μM), (b) SL4-4 (111 μM) and (c) SL4 (160 μM). (d) Representative non-linear least squares fit of the NC-titration data obtained for SL4 ([SL4] = 111 μM), NC binds SL3 with a dissociation constant of 170 nM,$^{23}$ and exhibits stoichiometric binding at NC:RNA ratios below 1:1. At higher NC:RNA ratios, an additional band is observed, which is attributed to an NC2:SL3 complex. These data indicate that the binding of the second NC molecule occurs with substantially lower affinity than observed for the first NC molecule. The affinity of NC for SL3 ($K_d$ of 47(±14) μM) is more than two orders of magnitude weaker than for SL3 (see the text for details).
might alter, or completely disrupt, the structure of the tetraloop. Therefore, the objective of these NMR studies was to (1) determine if the structure of the tetraloop in SL4 matches that of the determined oligonucleotide, and (2) evaluate the structural properties of the SL4 stem.

SL4 contains a tetraloop sequence of the GNRA type (denoted GN_{i+1}R_{i+2}A_{i+3}, in which R = G or A and N = A, C, G or U). A number of structures have been determined recently for RNAs that contain GNRA tetraloops, and in all cases, the guanosine residue at position i and adenosine residue at position i+3 are coplanar and poised to form a G-A base-pair with several inter-nucleotide hydrogens. The two remaining bases of the tetraloop (N_{i+1} and R_{i+2}) stack on top of A_{i+3} residue and the two G_i and N_{i+1} residues form a “U-turn” that stacks the residues at positions i+1, i+2, and i+3 on the minor groove side of the stem. Several structures have been solved for RNAs that specifically contain a GAGA tetraloop, which is identical to that of SL4. These include X-ray and/or NMR structures of the sarcin/ricin loop of the 28 S ribosomal RNA,61–65 the domain 3 of Xenopus laevis oocyte 5 S ribosomal RNA,36 and a 15-mer oligonucleotide by Pardi and co-workers (denoted 1ZIG using the PDB accession code).30

As reported by Jucker et al.,30 the folding in GNRA-type tetraloops positions the H1’ protons of the i+2 and i+4 residues in close proximity to the nucleobases of i+1 and i+3. This results in large upfield ring current-induced shifts for the 1H NMR signals of the H1’ protons of tetraloop residues at positions i+2, and i+4.30 As shown in Figure 3(a), SL4-4 also exhibits upfield-shifted signals for the H1’ protons of the G_{i+2} and G_{i+4} nucleotides. In fact, the chemical shifts observed for these H1’ protons are very similar to the shifts reported for the corresponding residues in two other oligonucleotide hairpins that contain GAGA tetraloops.30,33,34 In addition, nuclear Overhauser effect (NOE) cross-peak patterns and intensities observed for residues G6-A7-G8-A9 of SL4 and SL4-4 are very similar to those reported for both 1ZIG 30 and a dodecamer RNA from the sarcin/ricin loop,33,34 (Figure 3(b)). These results provide very strong evidence that the tetraloop of SL4 adopts the same three-dimensional structure as those reported for 1ZIG in sarcin/ricin tetraloop structures. The upper portion of the 1ZIG structure, which is shown in Figure 3(c), is entirely compatible with the NMR data obtained for SL4 and SL4-4.

Two-dimensional NOESY data obtained in 90% 1H2O/10% 2H2O and at 5°C were used to probe the structural features of the stem. Based on the proposed secondary structure, a total of seven imino proton signals are expected for the SL4 stem, including two G-H1 and two U-H3 signals for protons in the predicted GU wobble pairs (Figure 1(c)). However, only three imino proton signals were observed in spectra, which correspond to the imino protons of G1 (very weak, due to exchange-broadening), G4 and G10 (Supplementary Figure S1(a)). No imino proton signals were observed for residues G2, U3, G12 and U13 of the predicted GU wobble pairs. The absence of these signals is consistent with previous secondary structure predictions and accessibility mapping experiments, which demonstrated that G1, G2, and G10 are exposed.7,10,11 Thus, the NMR data indicate that the stem of SL4 is conformationally labile, and this is consistent with the previous findings.

**Stabilization of the stem by addition of four base-pairs does not enhance the affinity of SL4 for NC**

NMR-based structural studies have revealed that NC interacts with both stem and loop residues when bound to SL2 and SL3.20,24 In the complex with SL2, the N-terminal zinc knuckle and 3_10 helix bind to the minor groove and base triple platform of the stem,20 and in the NC-SL3 complex, the 3_10 helix binds to the major groove of the stem.24 Consistent with these findings, in vivo genome packaging studies have shown that RNA mutations designed to destabilize stem base-pairing lead to reductions in packaging, and that compensatory mutations on the opposite strand designed to restore base-pairing lead to increases in packaging to near wild-type levels.15 Collectively, these studies indicate that proper base-pairing in the stem is important for packaging. The NMR results reported here indicate that the stem of isolated SL4 is labile, and it thus seemed possible that this lability might affect the affinity of SL4 for NC. Since the stem could be constrained in the context of the intact PS-site, we attempted to stabilize the stem of SL4 by preparing a construct with four additional base-pairs (SL4-4, Figure 1(c)). In contrast to the data obtained for SL4, all of the expected imino proton signals were observed in the 1D 1H and 2D NOESY spectra of the SL4-4 stem, including the four resonances expected for the two G-U wobble pairs (supplementary Figure S1(b),(c)). The observation of NOE cross-peaks between imino proton pairs of G2-H1 (11.97 ppm)/U13-H3 (11.25 ppm) and G12-H1 (11.70 ppm)/U3-H3 (10.89 ppm), and chemical shifts characteristic of wobble base-pairs,37,38 provide evidence that the additional base-pairs do indeed stabilize the stem of SL4. In addition, essentially identical 1H NMR chemical shifts and NOE patterns were observed for the tetraloop residues of SL4 and SL4-4, indicating that the additional base-pairs do not affect the structure of the tetraloop.

NC binding studies were performed with SL4-4 to determine if the additional stem base-pairs affect the affinity of SL4 for NC. Native PAGE data obtained for SL4-4 RNA (111 μM) as a function of added NC indicate that SL4-4 also binds NC weakly, and that the oligonucleotide contains multiple weak binding sites (Figure 2). This indicates that the additional stem base-pairs, and the associ-
ated increase in the apparent stability of the stem, do not significantly affect the affinity of NC for SL4.

At this point, it is not clear why our results differ from those obtained previously using filter-binding assays. One possibility may be that the differences are due to differences in the RNA constructs employed. Parslow and co-workers studied NC binding to a 34 nucleotide construct that included residues that both precede and follow the SL4 sequence shown in Figure 1 (nucleotides 783-816). This sequence is predicted to form a bulge in the stem, and it is conceivable that this or other stem-derived structures form a high-affinity NC binding site. Alternatively, our studies indicate that RNA constructs with longer stems may contain multiple NC binding sites (e.g. SL4-4), and the filter-binding assays employed in the earlier stu-

Figure 3. (a) A portion of the 1H-13C HMQC spectrum obtained for 15N,13C-labeled SL4-4 showing ribose H1'-C1' correlation peaks (20°C, 400 μM RNA). The upfield-shifted signals observed for G8 and G10 (which are commonly observed in GNRA-type tetraloop structures) are labeled. (b) A portion of the 2D NOESY spectrum obtained for unlabeled SL4-4 (1.5 mM, 20°C) showing NOE cross-peaks associated with aromatic H5, H6, H8 and ribose H1' protons. Sequential connectivities for residues G4 through C11 of the GNRA tetraloop are indicated by continuous lines. Peaks observed for G10-H1' are not shown. This pattern of NOE cross-peaks and intensities is very similar to those reported for different RNAs containing the same GACA tetraloop.30,33,34 (c) Figure generated from PDB coordinates for 1ZIG showing residues of the tetraloop and the loop-closing base-pair, which are identical in sequence to SL4 (see the text for details). Tetraloop bases G6 (green), A7 (pink), G8 (green) and A9 (pink) are labeled. Residues A7 and A9 are shown in transparent space-filling models to indicate the ring current effects on H1' protons (white) of G8 and G10. The nucleobase of G6 is hydrogen bonded to A9, and the nucleobase of G8 is sandwiched between A7 and A9, which appears to prevent these bases from interacting with the zinc knuckles of NC. In contrast, guanosine residues in the tetraloops of SL2 and SL3 are exposed and readily available for binding to NC (see the text for details). The Figure was generated with MOLSCRIPT and Raster3D.
dies may not readily distinguish RNAs that contain a single high-affinity site from those that contain multiple lower-affinity sites. Regardless, the results presented here clearly demonstrate that nucleotides 793 to 806 form the predicted stem-loop, and that NC binds with relatively low affinity to this structure.

**Structural basis for preferential HIV-1 NC binding to GG(A/U)G tetraloops**

Structural studies of HIV-1 NC complexes with SL2 and SL3 have revealed that NC is capable of binding to different RNA targets *via* different subsets of inter- and intramolecular interactions. For example, NC binds to the minor groove of SL2 and interacts with an AUU base triple platform motif, but when bound to SL3 the protein interacts with nucleotides in the major groove. Although different studies were also observed, the NC-SL2 and NC-SL3 structures exhibited several common features. Most notable was the finding that, in both structures, the zinc knuckles of NC bind to the nucleobases of exposed guanosine residues in GNG tetraloop sequences. Since the tetraloops of SL2, SL3 and SL4 all share a GNG sequence, it seemed plausible that NC might bind tightly to SL4 in a manner similar to that observed for binding to SL2 and SL3. However, the data presented above reveal that NC binds poorly to SL4.

NMR structures reported for isolated stem loops SL2 and SL3 in the absence of NC have shown that the guanosine at position *i* in the tetraloop stacks on top of the preceding base of the stem. The first guanosine of the SL4 tetraloop stacks in a similar manner on top of the preceding cytosine of the penultimate G-C base-pair. However, the structures of the three remaining nucleotides of the SL4 tetraloop differ significantly from those observed for the NC-free and NC-bound forms of SL2 and SL3. Thus, the G*N*+1, N*i*+2 and G*i*+3 nucleotides of SL2 and SL3 exhibit conformational flexibility in the absence of NC, but become ordered upon binding to NC. In contrast, the A*N*+1,G*i*+2,A*i*+3 residues of the SL4 tetraloop exist in a single conformation, with A*i*+3 making several hydrogen bonds to G*N*. Thus, the differences in affinity for NC appear to be due, at least in part, to differences in the accessibility of the guanosine residues of the tetraloops. In SL2 and SL3, the tetraloops expose a G*N*+1,N*i*+2,G*i*+3 triple that is flexible and readily accessible to NC, whereas the tetraloop residues of SL4 are ordered and the guanosine residues at positions *i* and *i*+2 are not readily accessible for binding to NC.

**Implications regarding the role of SL4 in genome recognition**

Retroviral genome recognition occurs in the cytosol of the infected cell *via* a complex process that is not yet well understood. Deletion mutagenesis experiments have shown that stem loops SL1, SL2, SL3 and SL4 are all important for efficient genome recognition and packaging. However, the fact that individual stem-loops can be deleted or destabilized without completely eliminating packaging indicates that the stem-loops may have redundant packaging functions. Based on these and other findings, it has been suggested that genome recognition may be mediated by interactions between multiple NC domains of an assembling Gag multimer and the exposed stem-loops of a folded *Ψ*-site.

Although SL4 is clearly important for efficient genome packaging, the present findings suggest that its role does not involve direct interaction with NC. Although it is conceivable that the affinity of NC for SL4 might be greater in the context of the intact *Ψ*-site, this seems unlikely in view of the fact that the guanosine residues of the tetraloop that could potentially bind to NC are sequestered by internal hydrogen bonding and base stacking in the GNRA-type tetraloop, and are not readily accessible for binding to NC.

GNRA tetraloops exist widely in biology, and it is now known that their primary function is to form long-range RNA-RNA tertiary interactions. Numerous examples exist from phylogenetic, biochemical, and 3D structural studies, in which GNRA tetraloops stabilize a global RNA tertiary fold by forming hydrogen bonds with nucleotides in helical and stem-loop RNA sub-structures. In view of this fact and our finding that SL4 binds weakly to NC, it seems likely that SL4 may serve a similar structural role in genome packaging. In this regard, Parslow and co-workers examined a series of RNA constructs that contained extensions and deletions of the HIV-1 *Ψ*-site in an effort to identify determinants of dimerization. One of the constructs examined in that study contained stem loops SL1-SL3, but lacked stem-loop SL4. Interestingly, this construct migrated under native PAGE conditions as a broad, diffuse band with the mobility of a dimer, whereas a narrow band was observed for an extended *Ψ*-site construct that contained all four stem-loops (nucleotides 632-810; see Figure 7 of reference Clever et al.). We have obtained similar results with *Ψ*-site constructs that contain only stem-loops SL1-SL4 (nucleotides 694-807, which migrates as a narrow band consistent with a dimer) and SL1-SL3 (which migrates as a diffuse series of bands) (Banky and M.F.S., unpublished results). These results indicate that HIV-1 *Ψ*-site constructs that lack SL4 can form dimers, but that the structures formed are conformationally heterogeneous.

Based on the above findings, we propose that SL4 functions in genome packaging not by binding to NC, but instead by stabilizing the tertiary structure of the *Ψ*-site. This proposal is consistent with the observations that (a) SL4 interacts weakly with NC, (b) the SL4 tetraloop adopts a classical GNRA-type conformation, (c) the primary function of GNRA tetraloops is to partici-
within the 5’ region of the HIV-1 genome are underway.

Materials and Methods

Sample preparation

The recombinant 55-residue NC protein was expressed from pRD2, containing the NC coding region of NC from HIV-1NL4-3 subcloned into pET3a (Novagen, WI, USA), and purified under non-denaturing conditions as described. The 14mer SL4 RNA was obtained from Dharmacon Research (Boulder, CO, USA) with 2’-o-bis (acetoxyethoxy)-methyl (ACE) protection. The 2’ACE deprotection was performed following manufacturer recommendations and ethanol precipitated using standard protocols prior to use. The 22 nucleotide SL4-4 RNA was synthesized in vitro transcription, purified by denaturing PAGE to single nucleotide resolution as described, and ethanol precipitated prior to use. Uniformly 13C-/15N-labeled RNA was prepared as described. NMR samples were prepared either in 100% D2O or 95% H2O/5% 2H2O solutions (Cambridge Isotopes, Andover, MA, USA). Methods similar to those used for SL4-4 RNA synthesis were used to prepare SL2 and SL3 RNA samples.

NC titration experiments

Native PAGE assays to evaluate NC binding were carried out using samples with constant RNA concentrations of 80-160 μM and varying NC protein concentrations. RNA samples were heated to 95°C for three minutes and snap cooled on ice prior to use. Titration experiments were performed in NC NMR buffer (pH 6.5) and incubated for 15 minutes at ambient temperature prior to electrophoresis. An equal volume of 50% (v/v) glycerol was added to each sample prior to loading onto native 12% polyacrylamide gels, and the gels were pre-run at 100 V for ten minutes in 0.5x TB buffer (20 mM Tris-boric, pH 7.5) operating at 100 V prior to sample loading. Gels were stained for five minutes in ethidium bromide (10 μg/ml) and de-stained in H2O for ten minutes prior to imaging with a Kodak DC 820 digital camera equipped with an ethidium bromide filter (Eastman Kodak Company, New Haven, CT, USA). Analysis of the imaged data were performed using the public domain NIH Image program (developed at the US National Institutes of Health). A lower limit for the NC:SL4 dissociation constant was obtained by fitting data derived from the intensity of the free SL4 RNA as a function of added NC. The amount of bound RNA (NC:SL4) was calculated as the difference between the total RNA intensity in the absence of NC minus the total free RNA after addition of NC. With this model, 100% of the signal loss is attributed to the 1:1 NC:RNA complex, and the analysis is not complicated by differences in the abilities of SL4 and NCSL4 to bind dye. This approach was employed only for titrations with SL4, which did not exhibit evidence for the binding of additional NC molecules in the presence of excess NC. Titration curves were analyzed by non-linear least-squares fitting using the MicroCal Origin 5.0 software (MicroCal, Northampton, MA, USA). $K_d$ values were calculated from four independent experiments, and were determined from the midpoints of the fitted titration data.

NMR spectroscopy

The 2D 1H homonuclear-correlated NOESY and TOCSY NMR data, and 2D 1H-13C correlated HMQC spectra, were collected using Bruker AVANCE.
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