

Site-selective probing of cTAR destabilization highlights the necessary plasticity of the HIV-1 nucleocapsid protein to chaperone the first strand transfer

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ABSTRACT

The HIV-1 nucleocapsid protein (NCp7) is a nucleic acid chaperone required during reverse transcription. During the first strand transfer, NCp7 is thought to destabilize cTAR, the (–)DNA copy of the TAR RNA hairpin, and subsequently direct the TAR/cTAR annealing through the zipping of their destabilized stem ends. To further characterize the destabilizing activity of NCp7, we locally probe the structure and dynamics of cTAR by steady-state and time resolved fluorescence spectroscopy. NC(11–55), a truncated NCp7 version corresponding to its zinc-finger domain, was found to bind all over the sequence and to preferentially destabilize the penultimate double-stranded segment in the lower part of the cTAR stem. This destabilization is achieved through zinc-finger-dependent binding of NC to the G₁₀ and G₅₀ residues. Sequence comparison further revealed that C•A mismatches close to the two G residues were critical for fine tuning the stability of the lower part of the cTAR stem and conferring to G₁₀ and G₅₀ the appropriate mobility and accessibility for specific recognition by NC. Our data also highlight the necessary plasticity of NCp7 to adapt to the sequence and structure variability of cTAR to chaperone its annealing with TAR through a specific pathway.

INTRODUCTION

The nucleocapsid protein (NCp7) of the human immunodeficiency virus type 1 (HIV-1) is a small basic protein

resulting from the protease-mediated cleavage of the Gag polyprotein precursor. NCp7 is formed by two highly conserved CCHC zinc-finger motifs connected by a short flexible linker and flanked by poorly folded N- and C-terminal basic domains (1,2) (Figure 1). NCp7 is critical during the early and late steps of the HIV-1 viral life cycle (3–11). Most NCp7 functions rely on its RNA- and DNA-chaperone properties that direct the rearrangement of nucleic acids into their most thermodynamically stable structure (12–15). These chaperone properties are thought to play a critical role in reverse transcription (5,16,17). For instance, during the first strand-transfer of reverse transcription, NCp7 favours the transfer of the newly synthesized minus strong-stop DNA [(–) ssDNA] to the 3'-end of the viral RNA genome, by promoting the base pairing of the complementary repeat (R) sequences at the 3'-ends of the (–)ssDNA and genomic RNA, respectively (18). *In vitro*, the stable stem-loop (SL) of the transactivation response element (TAR) sequence within the R domain of the genomic RNA does not readily anneal with the complementary cTAR sequence of the (–)ssDNA in the absence of NCp7 (18–21). Addition of NCp7 strongly increases the rate and extent of the cTAR/TAR annealing reaction (18–23) and switches the reaction pathway to direct the hybridization of these sequences through the end of their double-stranded (ds) stems (20,21). The main chaperone properties involved in promoting the first strand transfer have been characterized as two interconnected components: a destabilization activity and a hybridization-promoting activity. Promotion of the annealing of complementary sequences requires an efficient aggregation of nucleic acids (24). This is achieved by the numerous basic residues of NCp7 that decrease the electrostatic repulsion between complementary oligonucleotides by neutralizing the negatively

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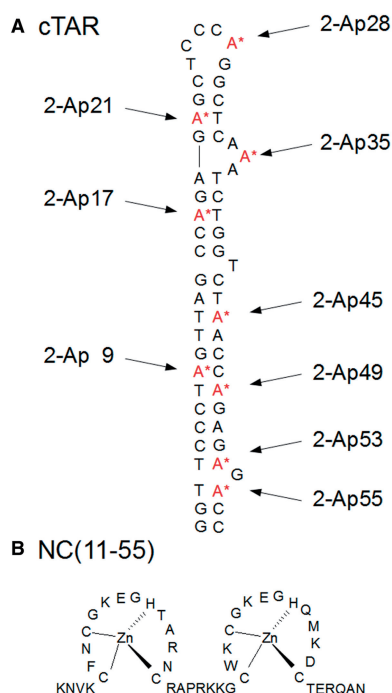


Figure 1. The 2-Ap substituted cTAR (A) and NC(11-55) (B) sequences. The cTAR sequence from the Lai strain of HIV-1 was selectively modified either at position 9, 17, 21, 28, 35, 45, 49, 53 or 55 with 2-Ap.

charged phosphate groups (5,21,25). The annealing facilitator property of NCp7 is shared with many other basic proteins that are also able to promote the cTAR/TAR DNA hybridization (26–28). On the contrary, the ability to destabilize the cTAR sequence appears to be a hallmark of NCp7. NCp7 activates the transient opening (fraying) of the lower half of the cTAR stem (29–34) constituted by a series of short ds-segments separated by a bulge and two mismatches. This activation follows a non-two-state mechanism, which results in complex spatial and temporal fluctuations of the cTAR ends that greatly facilitate cTAR/TAR hybridization. This activity has been assigned to the conserved hydrophobic platform formed by the Val₁₃, Phe₁₆, Thr₂₄, Ala₂₅, Trp₃₇, Gln₄₅ and Met₄₆ residues at the surface of the folded zinc-finger (ZF) domains (1,2,35). More specifically, the Trp₃₇ residue plays a critical role through stacking with G residues (17,36–43). This hydrophobic platform has also been found critical for binding (35,38). Nevertheless, the precise molecular mechanisms by which NCp7 destabilizes cTAR remain poorly described. Here, we selectively modified the 57-nt cTAR stem-loop sequence of the Lai strain with 2-aminopurine (2-Ap) at different positions to investigate by fluorescence spectroscopy the structural and dynamic modifications that occur within cTAR in response to NCp7 binding. In contrast to most techniques that monitor only global structural changes, the exquisite sensitivity of 2-Ap fluorescence to its local environment allows region-specific characterization of the structural modifications within the cTAR hairpin sequence. The 2-Ap has been previously used to site-specifically characterize the NCp7-induced changes in the dynamics of short

single-stranded sequences (44,45), as well as in the loop of the primer-binding site sequence of HIV-1 (46). These studies showed that binding of the intact ZF domain was responsible for a decrease of the DNA base stacking and for a strong restriction of the base mobility on a pico-second–nanosecond timescale. By selectively replacing different adenine positions of the cTAR sequence by 2-Ap (Figure 1), we aimed to picture the preferential destabilization sites and to gain insight into the molecular mechanism by which the NCp7 ZFs destabilize the cTAR stem-loop. Although NC(11–55) was found to bind all over the sequence, it preferentially restricted the dynamics and destabilized the bases of the penultimate ds-segment sandwiched between two C•A mismatches in the lower part of the cTAR stem. These NC(11–55)-induced changes are highly specific, as they were not observed when NC(11–55) was substituted by the SSHS-NC(11–55) mutant where the Cys residues in the ZFs were substituted by Ser residues to prevent the binding of zinc and the folding of the ZFs. These structural changes in this part of cTAR play likely a critical role in the ability of NCp7 to promote cTAR/TAR annealing through a specific zipping pathway.

MATERIALS AND METHODS

NC(11–55) (Figure 1) was synthesized as previously described (47) and stored lyophilized in its zinc-bound form. An extinction coefficient of $5.700 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm was used to determine its concentration. The oligonucleotides selectively labelled at different positions with 2'-deoxyribosyl-2-aminopurine (2-Ap) (Figure 1) were synthesized at a $1 \mu\text{mol}$ scale by IBA GmbH Nucleic Acids Product Supply (Göttingen, Germany) and purified by the manufacturer by double reverse-phase high-performance liquid chromatography.

Steady-state fluorescence

Experiments were performed in 25 mM Tris–HCl, pH 7.5, 30 mM NaCl and 0.2 mM MgCl₂ at 20°C. Excitation and emission spectra were recorded with a Fluorolog or FluoroMax spectrofluorometer (Jobin Yvon Instruments, S.A. Inc.). All fluorescence intensities were corrected for buffer emission, lamp fluctuations and instrumental wavelength-dependent bias. Kinetics of cTAR/TAR annealing were monitored in real-time using fluorescent doubly labelled cTAR sequences and non-labelled TAR (20). The cTAR sequences were doubly labelled with 5/6 fluorescein (Fl) and 5/6 tetramethylrhodamine (TMR), at their 3'- and 5'-ends, respectively. Excitation and emission wavelengths were at 480 and 520 nm, respectively, to monitor the Fl fluorescence restoration resulting from the formation of the cTAR/TAR duplex. Concentrations of 5'TMR-cTAR-3'Fl and TAR were 10 and 100–600 nM, respectively, to ensure pseudo-first order conditions. Both reactants in identical volumes coated by NC peptides were mixed together to trigger the reaction. The apparent rate constants k_{obs} were determined from kinetic traces, as previously described (20). All fitting procedures were carried out with the Microcal Origin 6.1

software based on non-linear least-squares methods, applying the Levenberg–Marquardt algorithm.

Time-resolved fluorescence spectroscopy

Time-resolved fluorescence measurements were performed using the time-correlated single-photon counting technique described elsewhere (46). Briefly, excitation pulses at 315 nm were generated by a pulse-picked frequency-tripled titanium-sapphire laser (Tsunami, Spectra Physics) pumped by a diode-pumped solid-state Nd:YVO₄ doubled laser (Millennia X, Spectra Physics). The emission was collected at 370 nm through a polarizer set at the magic angle and a 16-nm band-pass monochromator (H10, Jobin-Yvon). The single-photon events were detected with a micro-channel plate (R3809U, Hamamatsu) photomultiplier coupled to a pulse pre-amplifier (HFAC, Becker and Hickl GmbH) and recorded on a time correlated single photon counting board (SPC-130, Becker and Hickl GmbH). The full-width at half maximum of the instrumental response function was ~50 ps. For time-resolved anisotropy measurements, the fluorescence decay curves were recorded at vertical and horizontal positions as detailed in the Supplementary Materials. Experimentally measured fluorescence decays were de-convoluted with the instrumental response function and fitted to retrieve the most probable lifetime distribution using the maximum entropy method (48,49). As the resulting lifetime distribution evidenced four well-individualized lifetime clusters, see Supplementary Figure S3 the decays were reported as the sum of discrete exponentials according to $I(t) = \sum \alpha_i \times \exp(-t/\tau_i)$ where α_i is the relative amplitude associated to the fluorescence lifetime τ_i representing a distribution of conformations in which 2-Ap experiences similar quenching rates. The mean lifetime $\langle \tau \rangle$ was calculated according to $\langle \tau \rangle = \sum \alpha_i \tau_i$. The population α_0 of dark species was calculated by $\alpha_0 = 1 - \{ \tau_{free} / (\tau_{sample} \times R_m) \}$ where τ_{free} is the lifetime of the free 2-Ap riboside, τ_{sample} is the measured lifetime of 2-Ap within the oligonucleotide (either free or bound to NCp7), and R_m is the ratio of their corresponding quantum yields. The remaining amplitudes of each fluorescent population were recalculated according to $\alpha_{ic} = \alpha_i \times (1 - \alpha_0)$. All fitting procedures were carried out both with the maximum entropy method Pulse5 software and a software based on an iterative application of the Marquardt's method in non-linear least-squares analysis (kindly provided by G. Krishnamoorthy).

Alignment of TAR sequences

TAR sequence alignments were performed using the HIV sequence database from the Los Alamos National Lab HIV (<http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html>). A position-weighted matrix was then computed using the open-source statistical software R from the Comprehensive R Archive Network (<http://www.R-project.org/>) (50). The sequence logo (51) was plotted using the seqLogo package (<http://www.bioconductor.org/packages/devel/bioc/html/seqLogo.html>).

RESULTS

Site-specific characterization of the 2Ap-labelled cTAR sequences in the absence of NCp7

cTAR is an imperfect stem-loop characterized by a 3-nt internal loop, as well as by several bulged nucleotides and mismatches (Figure 1A). We selected labelling positions all along the cTAR Lai sequence. The 2-Ap in position 9 forms a mismatch with C within the lower part of the stem. Positions 28 and 35 are within the central and the internal loop, respectively, whereas positions 53 and 55 are flanking the G₅₄ bulge, and positions 17, 21, 45 and 49 are distributed all over the stem within the different ds-segments. The fluorescence spectra of the different 2-Ap-labelled cTAR sequences exhibited the same maximum emission wavelength (at ~370 nm) as the free 2-Ap nucleotide. In contrast, their quantum yields (QYs) were strongly reduced in comparison with the free probe (Figure 2), in line with static and dynamic quenching of 2-Ap by its close neighbours in the NAs (52–55). Although QYs were low in all labelled cTAR sequences, clear differences appeared as a function of the labelling positions. QYs were low when 2-Ap was inserted in ds-regions (positions 9, 17, 21, 45, 49, 53 and 55) that are known to favour base stacking, and thus the quenching of its fluorescence (53). The presence of guanine residues neighbouring 2-Ap was associated with a further decrease of the 2-Ap quantum yield, in full line with the most efficient quenching capabilities of Gs among bases (56). On the contrary, the presence of bulges or mismatches in the close vicinity of 2-Ap (positions 9, 53 and 55) was found to lead to somewhat higher QY values (57). By far, the highest QY value was observed when 2-Ap was inserted in the internal loop, suggesting a limited interaction of 2-Ap35 with its neighbours in this single-stranded segment of cTAR. In contrast to 2-Ap35, a significantly lower QY was observed for 2-Ap28, suggesting an efficient quenching of the probe by the G₂₉ residue in the loop. In line with nuclear magnetic resonance (NMR) data showing that the loop of cTAR is highly flexible (58), fast conformational fluctuations may drive 2-Ap28 from unstacked to stacked conformations, resulting in a low QY value. Taken together, we observed a strong dependence of 2-Ap fluorescence signals on the sequence and/or the structure of the DNA segments in which the 2-Ap is inserted. The 2-Ap signals easily discriminated stable regular ds-segments from positions destabilized by bulges or mismatches and clearly showed heterogeneous 2-Ap environments along the cTAR sequence.

To further characterize the local 2-Ap environment in the various labelled cTAR sequences, thermal melting curves were recorded using the 2-Ap fluorescence signal. These curves allow evidencing local conformations and dynamics of 2-Ap and its close neighbours (59). On melting, two types of transitions were observed. When the 2-Ap residue was located in ds-segments (positions 17, 21, 45 and 49), its fluorescence emission strongly increased during duplex melting (Figure 3A). This was ascribed to the progressive loss of base stacking as the temperature increases. The 2-Ap9 presented a similar transition pattern, suggesting that 2-Ap9 is likely base paired

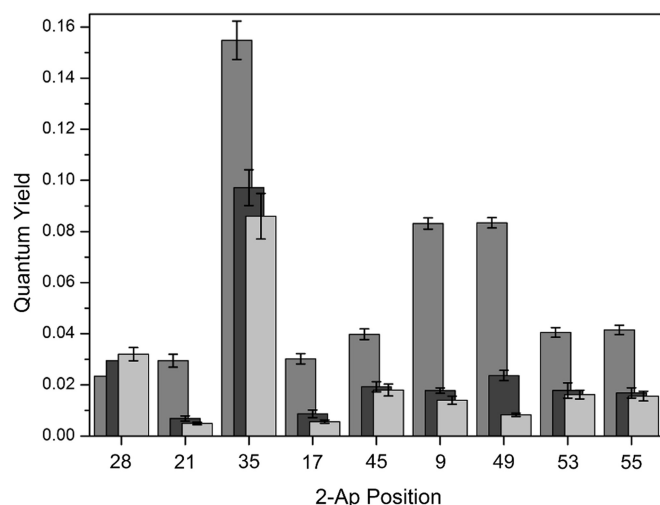


Figure 2. Quantum yields of the different 2Ap-modified cTAR sequences in the absence and in the presence of NC(11–55). The QY values of the 2-Ap-labelled cTAR sequences were determined at 20°C in the absence (light grey bars) or in the presence of 1 (black bars) or 10 (dark grey bars) equivalents of NC(11–55). In the absence of NC, QY values revealed different base stacking extent within the different cTAR segments. Addition of NC(11–55) differently increased QYs. The strongest effects were observed for positions 9 and 49, suggesting a preferential destabilization in the proximity of these positions.

with C₄₈. On the contrary, downward transitions were observed for 2-Ap53 and 2-Ap55 (Figure 3B), evidencing an extra-helical conformation of G₅₄ when the last two ds-segments are formed (60). Indeed, such an extra-helical conformation is thought to limit the dynamical quenching of G₅₄ with both 2-Ap53 and 2-Ap55, explaining a higher QY for the folded state than for the melted form. Moreover, the low melting temperature associated with the 2-Ap55 residue indicated that the last ds-segment is the least stable one. In addition, the broad melting transition observed with this residue further suggested a high propensity of the last ds-segment to transiently melt at room temperature, in line with previous reports (29,31). More surprisingly, 2-Ap28 also underwent a downward melting transition (Figure 3B), probably as the consequence of the disappearance of the structural constraints within the apical loop when the upper ds-segment melted. Taken together, the range of melting temperatures of the different ds-segments of cTAR clearly supported a non-two-state melting transition (61,62) where ds-segments melted independently and where the upper part of the stem-loop seemed more stable than the lower part (Figure 3C).

Heterogeneity of the probe environment was further investigated using time-resolved experiments. In contrast to the mono-exponential fluorescence decay of free 2-Ap, the fluorescence decays of the 2-Ap-labelled cTAR sequences were multi-exponential. A satisfactory fit was obtained with 4 lifetime components, ranging from ~100 ps to nearly 10 ns, demonstrating the large conformational heterogeneity explored by the 2-Ap residues at each position (Table 1). The longest-lived component τ_4 that likely corresponded to conformations where 2-Ap was either unstacked or extrahelical (63,64) varied from 6.1

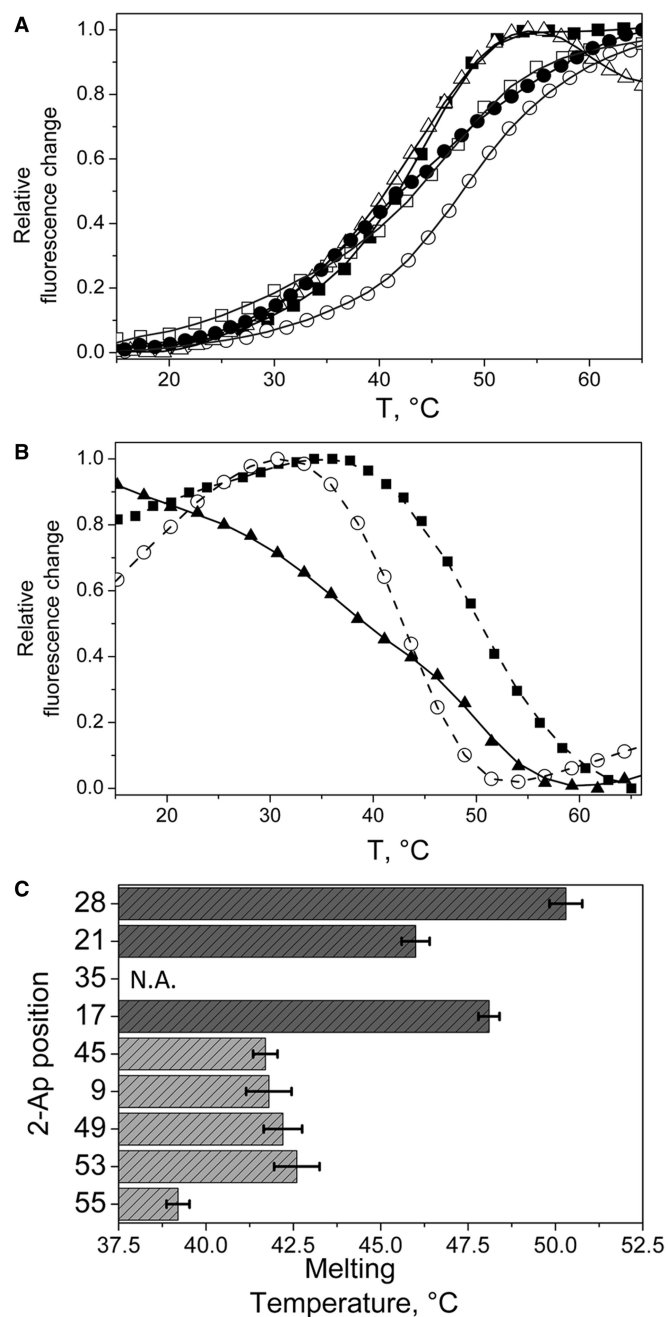


Figure 3. Site-selective monitoring of the thermal melting of the 2-Ap-substituted cTAR sequences. (A and B) Melting curves are monitored through the 2-Ap fluorescence emission. Two types of transitions were observed. (A) Upward melting curves for 2-Ap9 (closed squares), 2-Ap17 (open circles), 2-Ap21 (open squares), 2-Ap45 (open triangles) and 2-Ap49 (closed circles). (B) Downward melting curves for 2-Ap28 (black squares), 2-Ap53 (open circles) and 2-Ap55 (closed triangles). (C) Local melting temperatures recorded for the various 2-Ap positions. The melting temperatures clearly show a lower stability of the lower part of cTAR stem (light grey bars) as compared with the upper cTAR part (dark grey bars). The last ds-segment probed by the 2-Ap55 residue seemed poorly stable. No melting transition was observed for 2-Ap35, (see Supplementary Figure S1) indicating that 2-Ap in the internal loop experienced interactions with its neighbours which are similar to those in single-stranded DNA.

Table 1. Steady-state and time-resolved fluorescence parameters of 2-Ap-substituted cTAR^a

	NC	QY	α_0	τ_1 (α_1)	τ_2 (α_2)	τ_3 (α_3)	τ_4 (α_4)	$\langle\tau\rangle$	Φ_1 (β_1)	Φ_2 (β_2)	Φ_3 (β_3)	θ_0
Free 2-Ap	–	0.69 ^b										
2-Ap9	–	0.014	0.71	0.1 (0.21)	0.6 (0.04)	2.6 (0.03)	10.2 (1.00)	10.2	0.08 (1.0)			90
	+	0.085	0.48	0.1 (0.16)	0.7 (0.15)	3.1 (0.12)	7.7 (0.11)	2.3	0.2 (0.20)	1.1 (0.13)	5 (0.26)	34
	M	0.043	0.56	0.2 (0.26)	0.8 (0.08)	3.0 (0.05)	7.3 (0.05)	1.5	0.2 (0.35)	1.5 (0.25)	32 (0.67)	22
2-Ap17	–	0.006	0.76	0.1 (0.18)	0.5 (0.03)	2.3 (0.01)	7.9 (0.01)	0.4	0.2 (0.22)	1.2 (0.37)	21 (0.40)	30
	+	0.031	0.67	0.1 (0.19)	0.7 (0.06)	3.1 (0.04)	8.3 (0.03)	1.4	0.2 (0.22)	2.2 (0.19)	9 (0.41)	23
2-Ap21	–	0.005	0.92	0.1 (0.06)	0.5 (0.01)	2.7 (0.01)	8.5 (0.01)	0.9	0.2 (0.33)	1.5 (0.35)	31 (0.59)	23
	+	0.031	0.68	0.1 (0.19)	0.5 (0.05)	2.9 (0.04)	8.8 (0.04)	1.5	0.1 (0.32)	2.3 (0.19)	8 (0.32)	29
2-Ap28	–	0.032	0.69	0.1 (0.21)	0.8 (0.03)	3.6 (0.02)	8.3 (0.04)	1.6	0.2 (0.48)	1.2 (0.32)	7 (0.20)	37
	+	0.022	0.79	0.1 (0.11)	0.8 (0.04)	3.8 (0.03)	8.2 (0.01)	1.6	0.2 (0.34)	1.2 (0.16)	34 (0.50)	30
2-Ap35	–	0.086	0.20	0.2 (0.34)	1.0 (0.16)	2.9 (0.26)	8.2 (0.04)	1.6	0.2 (0.20)	1.4 (0.28)	8 (0.52)	22
	+	0.156	0.14	0.2 (0.36)	1.0 (0.11)	3.5 (0.16)	7.5 (0.22)	2.8	0.2 (0.10)	1.2 (0.16)	33 (0.74)	15
2-Ap45	–	0.018	0.56	0.1 (0.34)	0.8 (0.05)	3.0 (0.03)	7.1 (0.02)	0.6	0.2 (0.36)	0.6 (0.31)	4 (0.33)	31
	+	0.039	0.71	0.1 (0.10)	0.7 (0.08)	3.1 (0.06)	7.8 (0.04)	2.0	0.2 (0.35)	2.6 (0.30)	31 (0.35)	30
	M	0.026	0.42	0.1 (0.44)	0.5 (0.06)	2.1 (0.05)	7.0 (0.03)	0.7	0.2 (0.34)	1.4 (0.32)	11 (0.34)	30
2-Ap49	–	0.008	0.85	0.1 (0.09)	0.8 (0.04)	3.0 (0.02)	6.1 (0.01)	0.8	0.1 (0.41)	1.1 (0.33)	6 (0.26)	33
	+	0.085	0.26	0.1 (0.36)	0.9 (0.18)	3.6 (0.11)	8.7 (0.08)	1.7	0.1 (0.10)	2.0 (0.33)	32 (0.57)	17
	M	0.029	0.52	0.1 (0.35)	0.9 (0.06)	2.7 (0.04)	7.0 (0.03)	0.9	0.1 (0.39)	1.7 (0.21)	15 (0.39)	32
2-Ap53	–	0.016	0.82	0.1 (0.14)	0.6 (0.01)	3.9 (0.01)	9.6 (0.02)	1.4	0.1 (0.44)	0.8 (0.30)	5 (0.26)	35
	+	0.042	0.73	0.1 (0.12)	0.7 (0.06)	3.5 (0.05)	8.8 (0.05)	2.3	0.1 (0.30)	1.9 (0.23)	24 (0.47)	28
2-Ap55	–	0.016	0.72	0.1 (0.16)	0.4 (0.09)	2.8 (0.02)	8.9 (0.02)	0.8	0.5 (0.70)		4 (0.30)	49
	+	0.043	0.62	0.1 (0.17)	0.7 (0.08)	3.0 (0.08)	7.6 (0.04)	1.7	0.2 (0.27)	1.9 (0.28)	27 (0.42)	26
	M	0.021	0.69	0.2 (0.22)	0.9 (0.05)	2.7 (0.02)	7.3 (0.02)	1.0	0.5 (0.65)		8 (0.35)	46

^aExperiments were performed with 1–5 μ M of 2-Ap-labelled cTAR in 25mM Tris, 30mM NaCl and 0.2mM MgCl₂ at pH 7.5. Fluorescence parameters were determined in the absence (–) and in the presence of 10 equivalents of NC(11–55) (+) or SSH₂NC(11–55) (M).

^bFrom Ward *et al.* (42).

τ_i (ns) are the fluorescence lifetimes and α_i their amplitudes. The amplitude α_0 of the dark species, as well as the amplitudes of the various lifetimes, was calculated as described in the ‘Materials and Methods’ section. $\langle\tau\rangle$ is the mean fluorescence lifetime. SDs for the lifetimes and amplitudes are <15%. θ_0 , the cone semi-angle describing the conformational space explored by 2-Ap, is given in degrees.

to 9.6 ns. The shortest τ_4 values likely resulted from conformational dynamics of the 2-Ap residues in their excited state, which may drive them from an unstacked to a stacked conformation. The τ_4 value is thus an indicator of the local dynamics of the labelled NA. The low- τ_4 value retrieved for positions 9 and 49 revealed highly dynamic ns-fluctuations at these positions, whereas the high τ_4 value for 2-Ap at positions 53 and 55 was fully consistent with the extra-helical G₅₄ conformation that prevented stacking with its flanking residues. The three short-lived lifetime values were ~0.1, 0.4–1 and 2.3–3.9 ns, respectively. These picoseconds–nanoseconds lifetimes could be ascribed to conformations in which collisions of the 2-Ap with its neighbours, lead to a lifetime shortening, mainly through a charge transfer mechanism (53,55). The mean lifetime of 2-Ap in cTAR (0.4–1.6 ns) was found to be 6- to 25-fold shorter than that of the free dye (Table 1). These differences in the mean lifetimes were far less than the ~10–140 ratios of their corresponding QYs, indicating a significant population of ‘dark’ species (α_0) (i.e. species with a lifetime shorter than the ~40 ps detection limit of our equipment). Dark species were previously described as stacked conformations with ultra-fast dynamic quenching (55,65). Because of the close proximity of the τ_1 value to the detection limit of our equipment, its corresponding population may be added to that of the dark species to provide the total amount of stacked conformations (Table 1). These conformations were largely dominant in all the positions, including the apical loop where they represented 90%. This confirmed that the cTAR loop exhibits high-conformational flexibility that allows

efficient quenching of 2-Ap28 by the G₂₉ residue. In sharp contrast, a much more limited population (56%) of strongly stacked conformations was observed in the internal loop, probably because of the combination of a less efficient quenching by the flanking A together with constraints in the internal loop that prevent efficient quenching by the neighbour bases.

Beside the snapshot picture of the 2-Ap conformations distribution given by the time-resolved fluorescence decays, rotational dynamics of 2-Ap monitored through time-resolved fluorescence anisotropy provide information about the local and global dynamics of the cTAR sequences. Anisotropy decays were satisfactorily fitted using a three exponential model, with the exception of 2-Ap at position 55 for which only two components were sufficient. The short correlation time (Φ_1), 0.1–0.5 ns, was assigned to the local motion of 2-Ap with respect to the strand backbone, whereas the longest rotational correlation time (Φ_3) described the whole tumbling of the molecule. The presence of an intermediate rotational correlation time Φ_2 (~1 ns) with an amplitude almost constant of about ~30% evidenced additional conformational fluctuations in cTAR. The amplitude of the short rotational correlation time reflected the conformational space explored by the 2-Ap residue during the excited time. The extent of the explored space was quantified by the cone semi-angle value θ_0 (Table 1). The β_1 and θ_0 values were low for 2-Ap residues located in ds-segments, but large for 2-Ap9 and 2-Ap49, indicating a high rotational freedom for the latter, in line with the higher propensity of mismatched bases to

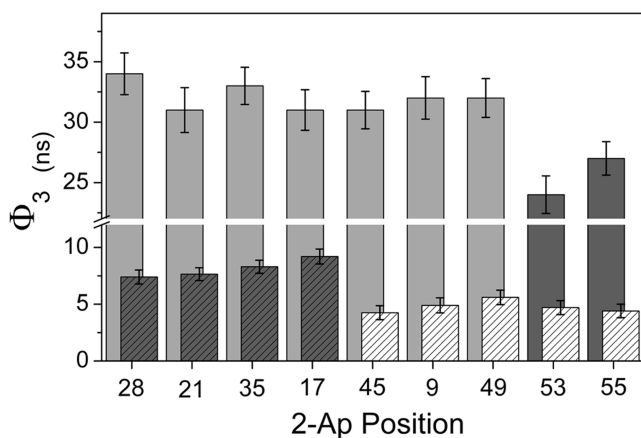


Figure 4. Representation of the longest rotational correlation time Φ_3 in the absence and in the presence of NC(11–55). Φ_3 values for the 2-Ap residues inserted in the lower half of cTAR stem (hatched white bars) are significantly shorter than the values observed in the upper part of the sequence (hatched grey bars), indicating significant segmental motions in the lower part of the cTAR stem. In the presence of saturating concentrations of NC(11–55) (non hatched bars), NC(11–55) largely decreases the segmental mobility of the DNA segments containing 2-Ap residues at position 9, 45 and 49, whereas segmental motions persist in the last ds-segments of the cTAR stem (dark grey).

explore geometries outside from the helix. The values of the long rotational correlation time (Φ_3) ranged from 4 to 9 ns, depending on the labelled position. For 2-Ap residues in the upper half-part of the hairpin loop (at positions 17, 21, 28 or 35) (Figure 4), the Φ_3 value was \sim 8–9 ns, in full line with the theoretical correlation time calculated for the tumbling motion of a cylinder rod of length 95 Å and diameter 20 Å (66). In contrast, the Φ_3 values were substantially lower (\sim 4–5 ns) for 2-Ap residues in the bottom of the cTAR sequence (positions 45, 49, 53 or 55) and were thus likely a combination of the overall tumbling of the cTAR sequence and of the segmental ‘fraying’ motion of the cTAR ends (30,34). Similarly to the Φ_3 value, the Φ_2 value was probably shortened in 2-Ap55 because of the larger segmental motions in the cTAR terminal ds-segment, making it impossible to resolve from Φ_1 . This hypothesis was further supported by the unexpectedly high β_1 value observed for 2-Ap at position 55.

Effect of NC(11–55) on cTAR

Because of their low quantum yield, high concentrations of labelled NAs were required (typically 1–5 μ M) to characterize the 2-Ap fluorescence parameters. This impeded us to use the full-length NCp7, which strongly aggregated nucleic acids, when added at these concentrations. The NC-induced destabilization of cTAR was thus investigated using NC(11–55), a mutant that lacks the major determinants of aggregation of the wild-type sequence but which is sufficient for specific binding and destabilization (24,35). Substitution of 2-Ap for an adenine in the cTAR sequence induced no detectable differences in the binding curves monitored through tryptophan quenching experiments, suggesting that 2-Ap did minimally affect NC(11–55) binding (see Supplementary Material and Supplementary Figure S4).

Fluorescence parameters of 2Ap-substituted cTAR in the presence of NC(11–55).

We investigated the effect of NC(11–55) with incremental addition of 1, 4, 7, 10 and 15 equivalents of peptide to the different 2-Ap-labelled cTAR sequences. NC(11–55) binding was confirmed by the progressive increase of the longest rotational correlation time Φ_3 , as NC(11–55) concentration increased. Φ_3 reached values between 24 and 34 ns (Table 1 and Figure 4), confirming that \sim 10 NC(11–55) molecules bound to cTAR. When the 2-Ap residue was not located at the bottom of the stem, the Φ_3 values tended to be somewhat higher than the 30 ns value expected for a sphere with the same molecular mass (72 kDa) (66), suggesting that the global shape of the NC:cTAR complexes may differ from a sphere. With such a high-rotational correlation time, Φ_3 likely described the overall tumbling motion of the whole complex, with marginal contribution of segmental motions. Shorter Φ_3 values were only observed in the lower half part of the cTAR stem for the 2-Ap residues at positions 53 and 55 (Figure 4), suggesting that only the two terminal ds-segments kept a substantial mobility in the presence of NC(11–55). As a consequence, the binding of NC(11–55) to cTAR resulted in a significant decrease of the segmental mobility of the DNA segments containing 2-Ap9, 2-Ap45 and 2-Ap49, which were shown to undergo large segmental motions in the absence of NC(11–55).

Addition of NC(11–55) also increased the 2-Ap QY. In the presence of saturating concentrations of NC(11–55), the 2-Ap quantum yields varied from almost unchanged (2-Ap28) to an increase by a factor up to nearly 14 (2-Ap49) (Figure 2). The fluorescence changes were dependent on the amount of NC bound and on the position of the 2-Ap within the sequence. Time-resolved parameters further show that NC(11–55) did marginally change the lifetime values but induced a redistribution of the 2-Ap conformational states towards the longer-lived conformations (Table 1). The extent of this redistribution was dependent on the labelling position and on NC(11–55) concentration, being the most important at positions 9 and 49, showing that NC(11–55) strongly reduced base stacking at these two positions (Figure 5A). Stacked conformations were also decreased in positions 17, 21, 45, 53 and 55, albeit to a lesser extent, showing that NC(11–55) was able to bind and to decrease the base stacking all along the cTAR sequence (67). NCp7 seemed to be thus efficient to prevent the stacking of 2-Ap with its flanking bases, especially when 2-Ap is located close to a G. This likely resulted from the insertion of the neighbouring guanine into the hydrophobic platform at the top of the folded zinc-fingers of NC, which in turn prevents its collision with 2-Ap and thus, reduces its quenching efficiency (38,39,42,44). Moreover, the reduction of the amplitude associated with the shortest rotational correlation time Φ_1 , further indicates that NC(11–55) additionally restricts the base mobility on binding. Again, the Φ_1 amplitude was the most decreased for 2-Ap at position 9 and 49, suggesting stable interactions of NC(11–55) with bases in the surrounding of 2-Ap in these two positions. The fluorescence parameters

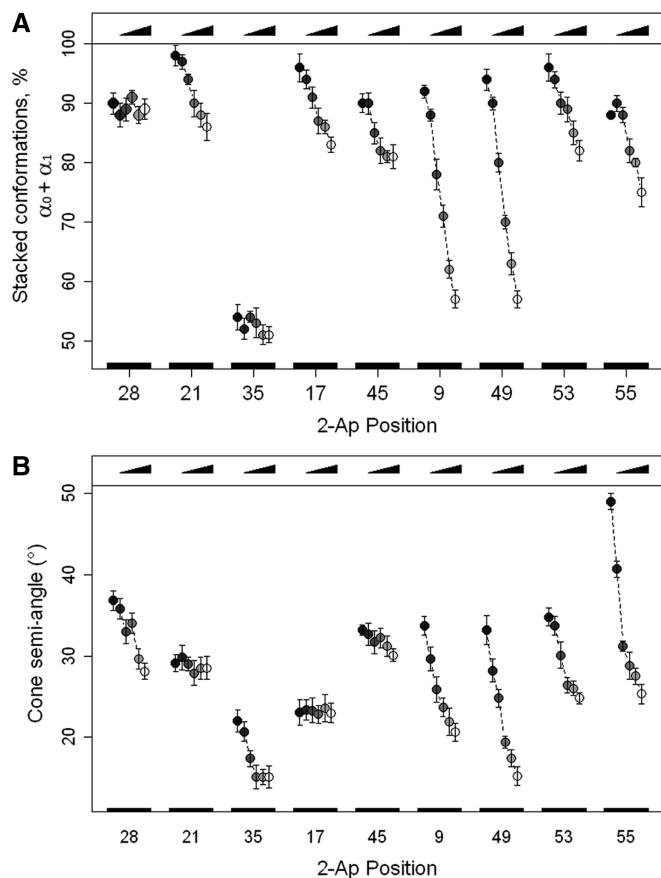


Figure 5. Dependence of the populations of the stacked-conformations (A) and the local mobility expressed as a cone semi-angle (B) of the 2-Ap residues as a function of NC(11-55) concentrations. Points from dark to light grey correspond to 0, 1, 4, 7, 10 and 15 equivalents of NC(11-55), respectively. Error bars correspond to SD of at least three independent experiments. (A) NC(11-55) generally decreased the stacked conformations, albeit to different extents, being the most efficient for residues at positions 9 and 49. (B) The cone semi-angle of the conformational space explored by 2-Ap is largely decreased by NC(11-55), when the 2-Ap residue was located in the lower part of the cTAR stem (at positions 9, 49, 53 and 55).

associated to 2-Ap9 and 2-Ap49 suggested that the collisions of the 2-Ap with their neighbouring guanines (G_{10} and G_{50} , respectively) were strongly prevented by NC(11-55).

Interestingly, in equimolar conditions where statistically one NC is bound per cTAR, a strong increase (~ 2 -fold) in quantum yield was observed for 2-Ap49, whereas only marginal changes were observed for all other positions (Figure 2). The populations of stacked conformations and the cone semi-angle θ_0 value of 2-Ap at these two positions were also significantly reduced (Figure 5A and B). These large fluorescence changes at a 1:1 stoichiometry suggest a high affinity of NC(11-55) for these positions. Therefore, it is likely that G_{10} and G_{50} constitute preferentially targeted guanines along cTAR and thus, constitute initial destabilization sites within the cTAR stem.

Effect of *SSHS*₂NC(11-55) mutant.

To confirm that the decrease in base stacking and the restriction in base and/or segmental mobility observed

on NC(11-55) binding resulted from a specific activity of NC ZFs on cTAR, we investigated the 2-Ap fluorescence parameters in the presence of (SSHS)₂NC(11-55), a NC(11-55) mutant in which all cysteine residues have been changed for serines to prevent zinc binding, and thus the folding of the ZFs (68). Addition of saturating levels of (SSHS)₂NC(11-55) affected poorly the mobility of cTAR bases, as seen from the small decrease of the θ_0 value induced by (SSHS)₂NC(11-55) at the different 2-Ap positions tested (Table 1). Moreover, the longest rotational correlation time was increased in the presence of (SSHS)₂NC(11-55) [showing that (SSHS)₂NC(11-55) was binding to cTAR] but remained significantly shorter than that observed in the presence of NC(11-55). Thus, cTAR still underwent significant segmental motions when NC(SSHS)₂ was bound. Consequently, (SSHS)₂NC(11-55) was unable to freeze cTAR dynamics on the picosecond–nanosecond timescale. Moreover, (SSHS)₂NC(11-55) was only able to moderately increase the 2-Ap QY (from 1.3- to 3.6-fold) as a result of the redistribution of the dark species (α_0 conformations) towards the τ_1 species. The most striking effect of (SSHS)₂NC(11-55) was observed on 2-Ap9 where its mean lifetime was doubled. This was mainly because of the large increase of the population of τ_4 conformations, which suggested that (SSHS)₂NC(11-55) was able to modify the conformation of the mismatched 2-Ap9 residue, in line with the ability of basic peptides to tilt non-specifically the nucleobases (69). Nevertheless, the effect of (SSHS)₂NC(11-55) on cTAR was considerably lower than that of NC(11-55), in full agreement with the severely impaired ability of (SSHS)₂NC(11-55) to destabilize nucleic acids (35). Taken together, these observations indicate that the (SSHS)₂NC(11-55) peptide marginally affects the cTAR structure and dynamics on binding, highlighting the unique role of the NC ZFs to remodel cTAR structure and dynamics.

Role of the ZF domain in the cTAR–TAR annealing reaction.

To evaluate whether the NC(11-55)-induced remodelling of cTAR could facilitate the cTAR/TAR annealing reaction, we compared the cTAR/TAR annealing kinetics in the presence of NC(11-55) and (SSHS)₂NC(11-55). We reacted 10 nM of doubly labelled 5'-TMR-cTAR-3'Fl together with TAR in pseudo-first order conditions, in the presence of either of the two peptides at a ratio of 5 nt per peptide and monitored the hybridization reaction in real-time through the Fl fluorescence restoration occurring as the ds-DNA/RNA duplex forms. As their initial part was too fast to be monitored, the kinetic curves in the presence of NC(11-55) could be fitted with a single kinetic rate constant of $2.5 \pm 0.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, close to the $1.8 \pm 0.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ value found for the NC(12-55)-promoted annealing reaction of the cTAR and TAR Mal strain sequences (20). In contrast, the kinetic curves observed in the presence of (SSHS)₂NC(11-55) were clearly bi-exponential with rate constants of $1.9 \pm 0.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $1.5 \pm 0.4 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ for the fast and slow component, respectively (Table 2). Thus, the slow component with

Table 2. Thermodynamic parameters of the cTAR/TAR annealing reaction in the presence of NC(11–55) or SSSH₂NC(11–55)

	k_{obs}^a (M ⁻¹ s ⁻¹)	ΔH^\ddagger^b (kJ/mol)	Ea^c (kJ/mol)
NC(11–55)			
Slow	$2.5 \pm 0.2 \times 10^4$	70 ± 8	72 ± 10
SSHS ₂ NC(11–55)			
Fast	$1.9 \pm 0.2 \times 10^4$	60 ± 4	62 ± 7
Slow	$1.5 \pm 0.4 \times 10^3$	108 ± 4	110 ± 6

^aDetermined at 20°C.

^bCalculated from Equation (1) and Arrhenius plot (Figure 6).

^cThe activation energy Ea is given by $Ea = \Delta H^\ddagger + RT$ for $T = 293.15$ K (70).

(SSHS)₂NC(11–55) was at least one order of magnitude slower than that observed with NC(11–55). Thermodynamic parameters of the reactions associated with the two NC mutants were determined (Table 2). The transition state enthalpy was estimated from the temperature dependence of the annealing reaction (Figure 6) according to:

$$\Delta H^\ddagger = -R \cdot d(\ln k_{obs})/d(1/T) \quad (1)$$

Compared with NC(11–55), the slow component of (SSHS)₂NC(11–55) presented a largely increased ΔH^\ddagger value, indicating that more base pairs need to be melted for the reaction to proceed (Table 2). In contrast, the thermodynamic parameters observed with NC(11–55) were close to those associated with the fast component of (SSHS)₂NC(11–55). Together with the increase of the amplitude of this fast component with temperature (Figure 6), this suggests that this component may correspond to the (SSHS)₂NC(11–55)-promoted annealing of a cTAR population, which was partly melted by temperature before it was reacted with TAR. Thus, in full line with related studies describing the effect of NC on nucleic acid thermodynamics (71,72), NC(11–55) likely decreases the transition free energy of base pair melting of cTAR.

More than destabilizing the cTAR sequence to generate the cTAR reactive species, NC(11–55) also likely increases the efficiency of the nucleation-limited reaction by promoting the competent reactive species. This could be a consequence of the ability of NC(11–55) to freeze the bottom of cTAR stem at the picosecond–nanosecond timescale into specific conformations (Figure 5B), notably through direct specific interactions with the G₁₀ and G₅₀ residues.

Conservation of the cTAR sequence and structure

If G₁₀ and G₅₀ residues are critically involved in NCp7 recognition sites needed for efficient strand transfer, it is likely that these positions must be conserved. To check whether these positions corresponded to conserved motifs within the HIV subtypes, we aligned the cTAR sequence presented in Figure 1 to the LANL HIV subtype reference database (<http://www.hiv.lanl.gov>). The 587 sequences retrieved from the QuickAlign procedure were used to calculate a position weight matrix from which a sequence logo was derived (Figure 7). This

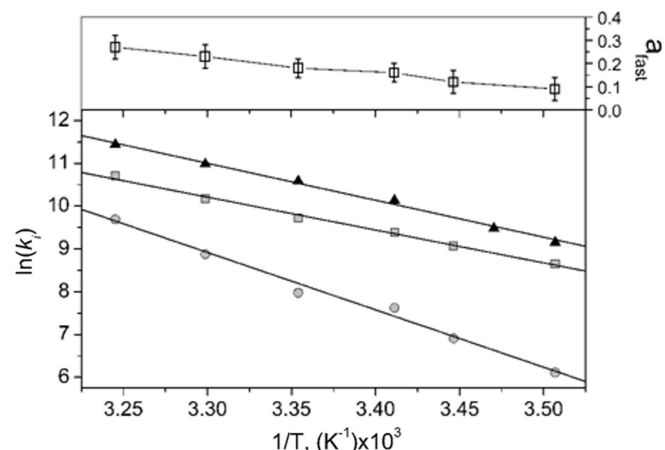


Figure 6. Arrhenius analysis of the cTAR/TAR annealing reaction in the presence of NC(11–55) or (SSHS)₂NC(11–55). The temperature dependence of the cTAR/TAR annealing reaction was determined by reacting 10 nM of the doubly labelled TMR-5'-cTAR-3'-Fl species together with 200 nM of TAR in the presence of NC(11–55) (closed triangle) or (SSHS)₂NC(11–55) (squares correspond to the fast component and circles to the slow one) added at a ratio of one peptide per 5 nt. The retrieved thermodynamic parameters are reported in Table 2. In the presence of (SSHS)₂NC(11–55), the fraction α_{fast} of the fast component (upper panel open square) increased with temperature.

sequence logo is drawn such that the overall height of the stack indicates the information content at each position (derived from the Shannon entropy), whereas the height of the base symbols within the stack indicates their relative frequency (51,73,74). The information content, which can reach a maximal theoretical value of 2 bits for nucleic acids, is usually interpreted as an indicator of evolutionary conservation. The cTAR sequence seems to be well conserved, including the G₁₀ and G₅₀ residues. In contrast, residues A₉ and T₁₁ flanking G₁₀ were associated to low information content as compared with the other residues of the cTAR ends, suggesting that the nature of the bases at these positions is of limited importance. As a result of this low base conservation, A₉ was replaced by a T in one-third of the sequences. In addition, the C₄₈ residue was frequently replaced by an A or a T. Therefore, the A₉–C₄₈ mismatch is barely as probable as the canonical A–T base pair at this position (Figure 8B). However, a C₁₁–A₄₆ mismatch was also frequent, indicating that the position of the distal CA mismatch seems to be exchangeable on both sides of the G₁₀–C₄₇ base pair (data not shown). Similarly, a C₈–A₄₉ mismatch was also frequently found when the C₆–A₅₁ mismatch was absent. It resulted that albeit in different positions, CA mismatches were largely conserved, in close proximity of the conserved C₇–G₅₀ and G₁₀–C₄₇ base pairs, highlighting that their presence is required to finely tune the thermodynamic stability of the two terminal ds-segments of cTAR (75).

DISCUSSION

In this work, we investigated the steady-state and time-resolved fluorescence properties of cTAR labelled

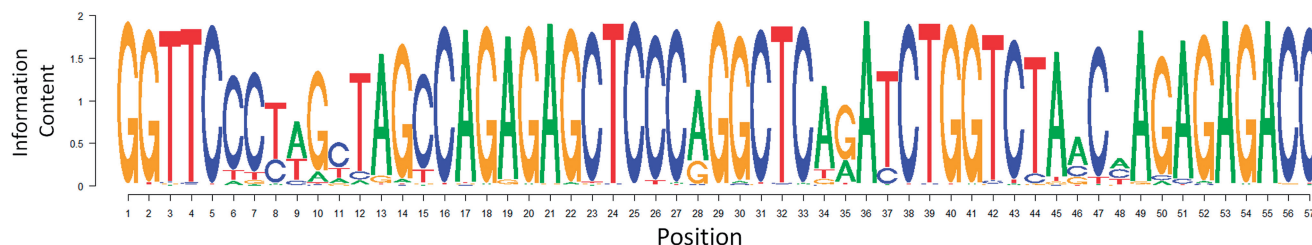


Figure 7. Sequence logo of cTAR. Sequence logo resulting from 587 aligned cTAR sequences showed the globally strong conservation of the cTAR residues. Residues flanking the G₁₀-C₄₇ base pairs seemed to be among the least conserved.

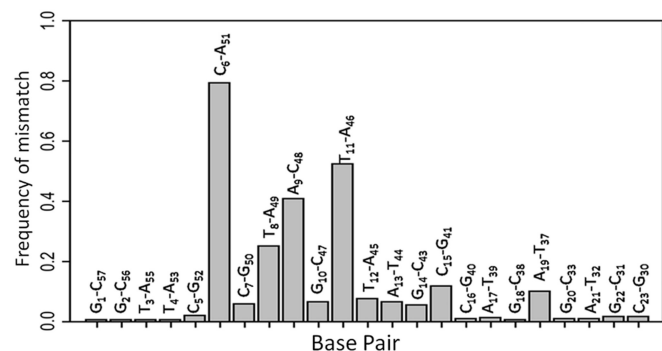


Figure 8. Histogram of the mismatch frequencies for the different base pairs within cTAR. Mismatch base pairs are preferentially flanking the two well-conserved G₁₀ and G₅₀ residues identified as directly interacting with NC(11-55). These mismatch base pairs are conserved, suggesting a critical role of the mismatches for NC recognition.

by 2-Ap residues at different positions. Fluorescence decay kinetics enabled the visualization of various 2-Ap conformers as snapshot pictures, whereas time-resolved fluorescence anisotropy provided complementary information on the associated rotational dynamics. This approach was thus well suited to monitor the structure and dynamics of the cTAR stem-loop and to probe the structural and dynamical perturbations induced on NC(11-55) binding. The base stacking patterns of 2-Ap seemed to be highly heterogeneous, adopting at least five conformations where the most stacked ones were the most represented. cTAR seemed to be highly dynamic at the picosecond-nanosecond timescale, especially in its lower half part that underwent segmental motions as the consequence of the fraying process that governs the dynamics of the cTAR stem ends (30,32,34). The terminal loop was found highly flexible, whereas the internal loop seemed to be highly constrained, in agreement with the slow micro-to-millisecond motions of the residues of the internal loop reported by NMR (58). Taken together, our data fit well with the NMR data of the top-half of cTAR (58) and the chemical probing experiments (67), as well as with the m-fold structure prediction of cTAR (76), except for the last ds-segment of cTAR that we found transiently annealed at 20°C in our conditions. Our data constitute thus a refined structural and dynamical model on the environment and the available conformational space for the base within the cTAR sequence.

Binding of NC(11-55)-induced local fluorescence changes that depended on the location of the 2-Ap

residue within the sequence and on the amount of protein molecules bound to cTAR. Although at a 1:1 NC(11-55)/cTAR ratio, the peptide shows a preferential binding to the ds-segments encompassing residues 9 and 49, NC(11-55) molecules were shown to bind all over the cTAR sequence at saturating concentrations, suggesting that they can adjust to the heterogeneous structures and dynamics present all along cTAR (Figure 9). Nevertheless, major NC(11-55)-induced changes in the cTAR structure and dynamics were observed in the lower half of the stem and notably at positions 9 and 49. The dramatic changes in the 2-Ap fluorescence signals at these two positions were similar to those attributed to the stacking interaction of the aromatic Trp37 residue of NC with a Guanine flanking a 2-Ap residue on the interaction of NC(11-55) with small 2-Ap-labelled oligonucleotides or (-)primer-binding site (44,45,77). Thus, 2-Ap9 and 2-Ap49 fluorescence parameters strongly suggest that G₁₀ and G₅₀ residues directly interact with NC(11-55), likely through a stacking interaction with the Trp37 residue of NC. The resulting 'freezing' of cTAR at these two positions is thought to constitute a prerequisite for the NC-induced destabilization of cTAR stem. Indeed, this NC-induced restriction of the local and segmental flexibility of the NA likely prevents fast transitions back to the stably stacked conformations, and thus allows the formation of locally destabilized domains with longer lifetime and probably with exposed bases that are competent for annealing with the complementary TAR sequence. The specific restriction of the local DNA mobility by NC ZFs likely constitutes a key component of the NC destabilization mechanism that is required to further facilitate the cTAR/TAR hybridization. This hypothesis is confirmed by the fact that the (SSHS)₂NC(11-55) mutant, which is unable to constrain the cTAR structure and dynamics, seems to be much less efficient than NC(11-55) to promote the cTAR/TAR annealing reaction.

Moreover, our data further suggest that the cTAR destabilization process is the result of the progressive coating of cTAR by NC(11-55). In contrast to 2-Ap9 and 2-Ap49 residues, which show subsequent changes in at least one of their fluorescence parameters at already low NC(11-55)/cTAR ratios, the 2-Ap45 residue exhibited fluorescence signal changes only for higher concentrations of NC(11-55), suggesting that the upper ds-DNA segment probed by 2-Ap45 can be destabilized only when its lower part is destabilized by NC(11-55). Moreover, as position 45 is only a few nucleotides apart from positions 9 or 49, the

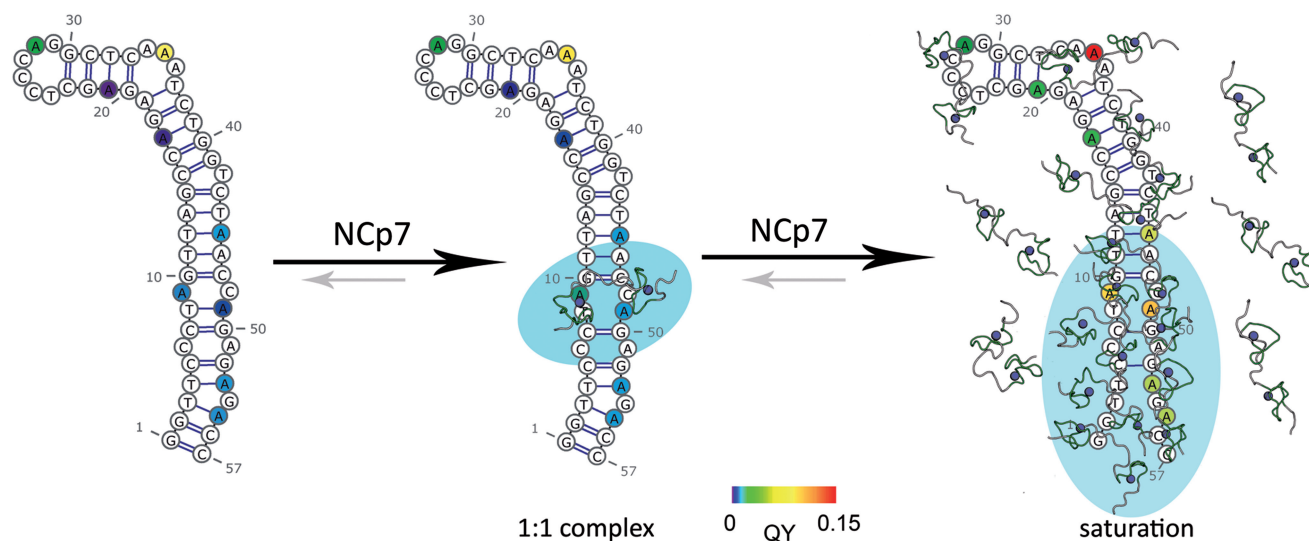


Figure 9. Proposed mechanism for NC-induced destabilization of cTAR. NC(11–55) preferentially recognizes G₁₀ and G₅₀ and initiate the destabilization of cTAR. In the presence of higher concentrations, NC(11–55) can adjust to the heterogeneous structures and dynamics to bind all along cTAR and generate the reactive species needed to anneal the complementary TAR sequence.

effects of NC on cTAR seem to be highly localized around its initial binding site. The latter is a prerequisite for transient interaction (78,79). Another consequence of this local NA remodelling is that effective chaperone activity requires a sufficient amount of bound NC, a situation which is achieved in the viral nucleoprotein complex context. The requirement for a sufficient level of coating to induce NA destabilization distant from its initiation site is in line with the fact that NC shows optimal chaperone activity when the target oligonucleotides are fully coated (21,29). This requirement also suggests that the initial and limited destabilization of cTAR can be extended by the binding of additional NC molecules, resulting in larger destabilized domains that likely persist long enough to efficiently react with the complementary TAR sequence. A progressive destabilization of cTAR stem as NC concentration increases was previously described (29) and likely explains the NC-coating dependent progressive switch in the cTAR/TAR annealing mechanism from a loop/loop mechanism to a zipper mechanism through the ends of their ds-stems (21).

The preferential binding of NC(11–55) to G₁₀ and G₅₀ residues showed that NC can discriminate and specifically target these two G residues within cTAR. Surprisingly, these guanines were located within motifs with low information content, suggesting that the nature of the bases flanking the targeted G seemed to be of limited importance for NC(11–55) recognition. However, the CA mismatches on the side of the G₁₀–C₄₇ and C₇–G₅₀ base pairs likely destabilize these base pairs and confer to the G₁₀ and G₅₀ residues the high degree of mobility (80), which was recently shown to be essential for recognition by NC (81). Thus, these intrinsic destabilizing CA motifs within the target DNA may facilitate the initial interaction of NC with the NA. The presence of mismatch base pairs seemed to play a more critical role in G recognition and discrimination than the nature of the NA sequence *per se*.

Similar conclusions have been previously made on MuLV Gag and NC showing that local context can considerably increase NC affinity for particular nucleic acid motifs (82). The conservation of the low stability of cTAR stem, probably co-evolving with NC chaperone properties, was shown to be critical for cTAR destabilization (31). This was confirmed by the early restoration of CA motifs within revertant viruses, which further highlights the importance of these destabilizing motifs in cTAR stem for the virus infectivity (83). Thus, this substantiates the hypothesis that NC destabilizes preferentially motifs with low stability, in line with the weak destabilizing activity of the NC ZFs (71,84). This finely tuned match between NC activity and the stability of the target NA sequence appears as an elegant way for NC to selectively chaperone a limited set of sequences within the HIV RNA genome and its DNA copies.

Moreover, the variability of the CA mismatch positions around G₁₀ and G₅₀ within the HIV-1 subtypes further highlights the necessary plasticity of NC to efficiently chaperone the first strand transfer. The flexible nature of NC must ensure sufficient plasticity to facilitate the correct and specific intercalation of the ZF aromatic residues to initiate the destabilization of cTAR, irrespective of the relative positions of the target G residues in respect with the CA mismatches. The poor sequence specificity and the high flexibility of NC likely ensure that NC can chaperone its various target nucleic acid sequences in the context of the high mutation rate of the virus. In addition, the high plasticity of NC and its unique ability to destabilize NA must provide NC a preferential and specific role in respect to other viral or cellular proteins (5,26,85,86) to timely and specifically enhance the cTAR/TAR annealing and to prevent self-priming (19). As HIV-1 has evolved to select such a complex interplay between NC and TAR structures and properties, the ability of NC to melt cTAR and to induce a specific

annealing pathway through the bottom of these sequences is probably of major importance for the production of stable vDNA. As a result, therapeutic agents disturbing the fine-tuned equilibrium between cTAR stability and NC activity could probably significantly alter the specificity of the annealing pathway during the first strand transfer, and thus compromise the faithful and timely production of viral DNA (87–89).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–5 and Supplementary Methods.

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