# Nucleocapsid Protein Chaperoning of Nucleic Acids at the Heart of HIV Structure, Assembly and cDNA Synthesis

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## The dawn of Nucleocapsid protein

Throughout the twentieth century, retroviruses led the way to remarkable discoveries fundamental to our understanding of life. The study of retroviruses has triggered exciting findings such as that of oncogenes and their implication in cancer (30,35,104,116) and the role of reverse transcribed elements in the plasticity of eukaryotic genomes (11,92,105), to name just two. The knowledge generated from the study of retroviruses has been paramount in bio-medicine and pivotal in the development of now commonplace techniques and applications such as molecular cloning, gene therapy, and novel diagnostics for cancer and viral infections. Bio-technology has come a long way since the first descriptions of oncoretroviruses (77,189), their visualization by electron microscopy (16) and the identification of the viral DNA polymerase, the celebrated reverse transcriptase (RT) (11, 204), and a small oncoviral nucleoprotein named the nucleocapsid protein (NC).

The NC story began with the isolation of ribonucleoprotein structures from oncoviruses (63,64,192) that supported cDNA synthesis (43). NC was found to be a nucleic acid binding protein with preferential binding to the dimeric 70S genomic RNA (63,64,83,159,176,193,198). In those early days not only did NC receive little attention, but results on NC-RNA interactions and NC specific binding sites on the genomic RNA of oncoretroviruses were obscured by the misidentification of the matrix protein as the specific partner of the 70S RNA (57,194; corrected in 147). In summary, data indicate that NC has a strong preference for single stranded regions flanking or within structured RNA domains and tightly binds to a small number of genomic RNA sequences rich in U and Gs, mostly located in the 5′ leader domain (147,194). Later, two series of observations dramatically enhanced our understanding of NC properties and functions, effectively kick-starting the NC story. Firstly, NC was found to be the driving force in genomic RNA dimerization and packaging into assembling RSV and MuLV virions (97,148,149). Secondly, NC was shown to chaperone viral RNA dimerization *in vitro* and primer tRNA annealing to the primer binding site (PBS) in MuLV and RSV (171,172) (for HIV-1 see refs. 13,40,41,69,82,106) . Data also suggested that NC was involved in all steps of proviral DNA synthesis. These observations have since been extensively borne out (3,9,13,37,60,61,91,101-103,180,214,215).

In the following sections we will briefly review the seminal role of NC in the fate of the HIV-1 full length RNA from mRNA encoding Gag and Gag-Pol to the genomic RNA in a dimeric form coated with hundreds of NC molecules present in the interior of the viral particle. Emphasis will be given to the implications of NC in the balance between genomic RNA translation and packaging, and on the mutual recognition between NC and the genomic RNA during virus assembly.

## Understanding the fate of the genomic RNA, from translation to packaging.

In retroviruses and HIV-1 in particular, the full-length viral RNA acts both as a messenger and genomic RNA (29,38,61,73,127). The balance between these two key roles must be maintained to allow efficient virus replication. Since a genomic RNA molecule cannot ensure both functions concomitantly, this raised the question of how genomic RNA translation and packaging are coordinated (29,39,61,135). For the sake of simplicity, the genomic RNA, either messenger or pregenomic, has been named gRNA.

How can gRNA translation and packaging be linked? As for the host mRNAs, the viral gRNA is capped and polyadenylated (176) and its translation is dependent upon the host translational machinery and is regulated by its 5′ untranslated region, the leader domain (34,42,202). The 5′ leader of HIV-1 gRNA is composed of several genetic elements that largely orchestrate virus replication (15,17,107). The processes of gRNA translation and packaging are determined by the viral 5′ Internal Ribosome Entry Segment (IRES) and its packaging signal (ψ), elements that share their primary sequences, but most probably differ in their secondary and tertiary structures (Figs. 1 and 2). Translation of the gRNA of some retrotransposons and retroviruses can proceed through a cap-independent mechanism, via an IRES (8,25,26,65,138,139161,210). The IRES directly recruits 40S ribosomal subunits in a 5′ end independent fashion. The molecular mechanism by which the host translation apparatus recognizes the retroviral IRES is unknown, but for other viral IRESes canonical initiation factors as well as specific cellular proteins, IRES transacting factors (ITAFs), participate in the recognition process (4,15,118,124,168,169186). HIV-1 harbors two IRESes, one within the Gag open reading frame (ORF) (40K-IRES) (36), responsible for the synthesis of a 40-kDa Gag isoform and the other within the 5′leader,

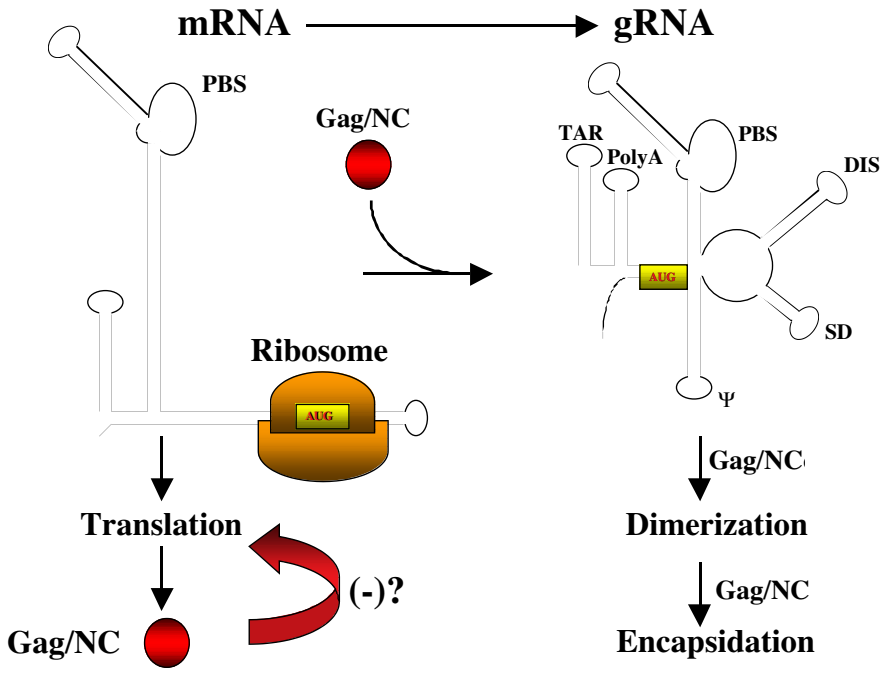


*Figure 1. Gag-NC is a major determinant in the switch from messenger to genomic RNA. Upon synthesis, HIV-1 genomic RNA is initially recognized as the messenger RNA for Gag production. Newly made Gag molecules bind the gRNA via the NC domain causing conformational rearrangements of the 5′ leader (1, 108,110,190). The structural modifications of the gRNA promoted by Gag-NC expose the cis-acting elements required for gRNA dimerization and packaging (DIS/ψ), occluding the RNA structures required for protein synthesis (right) (34). The overall effect of the RNA structural rearrangements in the 5′ leader most probably results in the inhibition of translation (AUG in the small box), promoting gRNA dimerization and packaging. In this model the gRNA structures are adapted from (1).*

the HIV-1 IRES (34). This is not without precedent as multiple IRESes have been described for c-myc (157), vascular endothelial growth factor (117) and cricket paralysis virus (212) mRNAs.

Little is known on the molecular mechanism driving the HIV-1 IRES. However, the dual mechanism of translational initiation (5′cap and IRES-dependent) added to IRES redundancy suggests that control of translation of the HIV-1 gRNA plays an essential role in the regulation of virus replication. Indeed, the HIV-1 IRES overlaps with important functional elements (34) (Fig. 1, 2): the PBS, the dimer initiation site (DIS), the 5′ SD and the major determinants of ψ (2,14, 17, 45,46,108,134,143). Similar to the IRESes of HCV (102), some members of the *picornaviridae* (33), ornithine decarboxylase (ODC) (173), c-Myc, and p58$^{PITSLRE}$ (50), the HIV-1 IRES is active during the G2/M phase of the cell cycle (34). Considering that 40S ribosome recruitment by IRESes appears to be dependent upon RNA secondary and tertiary structures, it is very likely that structural changes of the IRES are decisive in determining its activity. These structural modifications would act as a molecular switch that decides the fate of the HIV-l gRNA from translating ribosomes to Gag-NC directed packaging (19,120,121). Similar molecular models are proposed for poliovirus (90) and RSV (29,65,199). In agreement with this possibility, the 5′leader of HIV-1 gRNA can adopt two mutually exclusive secondary structures, defined as the branched multiple hairpin (BMH) and the long-distance interactions (LDI) structures (20,119,120,121). BMH promotes formation of the PBS, DIS, SD, and ψ stem-loops, while the LDI engenders alternative base pairings that disrupt the DIS hairpin loop (120,121). The region of the HIV-1 leader, that includes the Gag start codon at positions 336-338, also folds differently depending on the LDI and BMH structures. Nucleotides 334-344 form part of an extended hairpin structure in the LDI conformation (108-110), whereas in the BMH structure they are engaged in novel base pairings with the upstream U5 region (nt 105 to 115). This U5-AUG duplex (1) in the BMH structure occludes the Gag start codon, most probably preventing Gag translation and favoring gRNA packaging (1). Consequently, the BMH and LDI conformations are expected to differ in their ability to confer gRNA dimer formation and to drive Gag synthesis (Fig. 1).

Based on current understanding of Gag-NC-RNA interactions, a reasonable model that links mRNA translation and gRNA packaging is the following: (i) During the early stages of viral replication, the HIV-1 gRNA is recognized by the cellular translation apparatus as a standard 5′ capped and polyadenylated mRNA. At this stage both the 5′cap structure and the IRES element function, in an unknown fashion, to initiate synthesis of Gag and Gag-Pol polyproteins. (ii) Major changes in the cell cycle such as the viral induced G2/M growth arrest occur as viral proteins like VPR accumulate (95,123,136,144). (iii) In G2/M cap-dependent translation initiation is suppressed (174,175), but the HIV-1 IRES can ensure synthe-

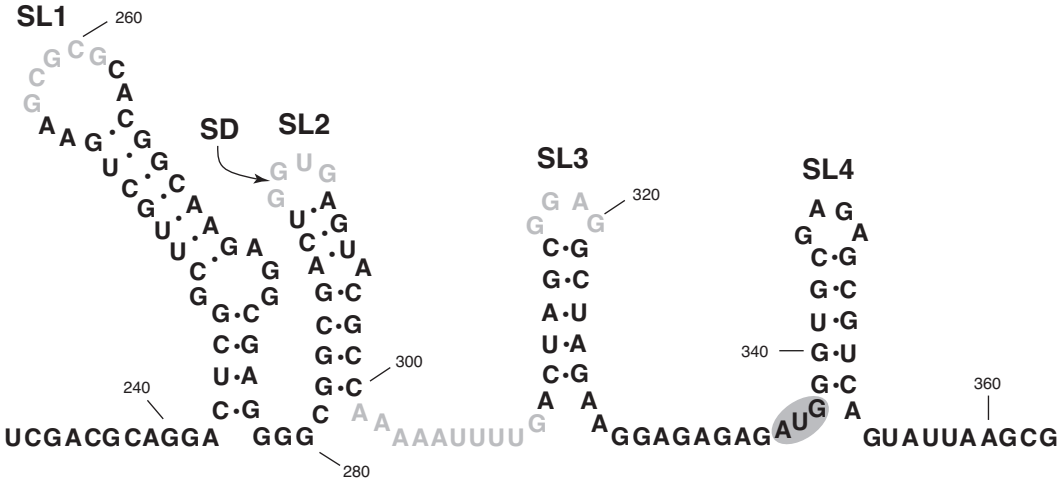


*Figure 2. The ψ signal of HIV-1 gRNA. The HIV-1 ψ is thought to be composed of four stem loops (SL). The high affinity binding sites are in gray. The Gag initiation codon is indicated. The major splice donor site (SD) is indicated by the arrow.*

sis of Gag and Gag-Pol (34,190). (iv) It is conceivable that the level of Gag will play a major role in determining the fate of the translated gRNA (124). Newly formed Gag molecules will bind the gRNA via NC, probably causing conformational rearrangements of the 5′ leader (1,120,121,126,206). Transconformation of gRNA structural elements by Gag-NC should result in the inhibition of translation as observed for RSV (29,167,199) and expose the structural elements required to chaperone gRNA dimerization and the packaging process (DIS/ψ) (1,59,120,121,184,195) (Fig. 1).

## NC preference for single stranded domains of the ψ signal and implications for gRNA packaging.

To understand how neo-synthesized Gag-NC molecules selectively discriminate the gRNA from the large excess of cellular and spliced viral RNAs within the infected cell, the binding parameters (stoichiometry and binding constants) of HIV-1 NCp7 to the ψ signal were investigated. Since the length of ψ (about 110 nt) precludes a reliable determination of the binding stoichiometry of NC to ψ, this question has been addressed with its four individual stem-loops, SL1 to SL4. Most studies reveal a binding stoichiometry of one NC per SL (6,7,14,17,66,196,197,211), suggesting that each SL is characterized by a unique strong NC binding site. In agreement with structures revealed by NMR (6,7,66), mutational studies indicate that this NC site is located on the loop of the SL (66,142,166,196). Additional NC binding sites were observed only at high RNA concentrations (7) or in low salt conditions (142). However, the affinity of NC for these additional sites is at least 100 fold lower than that for the loop itself, confirming the preference of NC for single-stranded sequences (7,86,211). Studies with SL3 also revealed the formation of complexes composed of one NC and two SL3 RNAs, suggesting that NC may, to some extent, promote SL3 dimerization (191,196). The binding constants of NC to the different SL domains have been determined through different approaches. Originally, filter-binding assays and GST-NCp15 (46) returned binding constants of $2.5 \times 10^6$ $M^{-1}$ for SL2 and $5 \times 10^6$ $M^{-1}$ for the other SLs. Later, the affinity of mature NCp7 for the SLs was assessed by fluorescence spectroscopy (142,166,196,211), gel electrophoresis (7,66,196) and isothermal titration calorimetry (6). Even though experimental conditions somewhat varied, it can be concluded that NCp7 affinity for SL2 and SL3 is in the $10^7$ $M^{-1}$ range (6,7,142,196,211) and that for SL1 and SL4 it is approximately 3 and 10-fold less, respectively. By characterizing the binding of NC to different SL mutants, the GXG motif, which is common to the loops of all four SLs, was identified as a major determinant for NC binding (86,196,211).

## How is the strong NC-loop interaction achieved?

All NC-oligonucleotide structures so far resolved show that a major contribution is provided by NC Trp37/Guanine stacking (6,66,150). NC-oligonucleotide complexes are further stabilized by 5 to 6 ionic interactions between the basic NC amino-acids and the SL phosphate groups. Strong binding of NC to the SL also requires some conformational flexibility of the GXG motif (7). In SL4, this flexibility is prevented by the formation of additional H-bonds between G5 and A3 of the GAGA tetraloop, resulting in reduced NC affinity (see above) (7).

## NC binding sites and selection of the genomic RNA.

A consequence of the strong sequence (GXG) and structure (loop) requirements for NCp7-nucleic acid interaction is the large decrease of potential binding sites as compared with the canonical binding model where the nucleic acid is viewed as a lattice of overlapping sites with identical affinities (145). This supports the notion that no extreme binding constant is required to drive selective NC binding to a limited number of binding sites. Thus, selective recognition of the gRNA is probably achieved by Gag-NC molecules binding to SL1, SL2 and SL3 of the ψ signal (Fig. 2). As SL1 is considered to be the DIS (179), binding of Gag-NC molecules to the three loop structures is thought to chaperone gRNA dimerization. This, in turn, should increase local gRNA and Gag concentrations and change the fate of the gRNA from messenger to pregenomic.

## How do Gag-NC-SL1/DIS interactions promote gRNA dimerization?

NC-promoted chaperoning of HIV-1 gRNA dimerization was first established *in vitro* using the 5′leader RNA and NCp15 (NCp7-p6) under physiological conditions (59,69,71). Later, in HIV-1 subtype A or B, dimerization was found to rely on the inverted repeat sequence of the loop, GUGCAC or GCGCGC, respectively (154), and to proceed in a two step fashion. The first step, corresponds to an intermolecular loop-loop interaction between two SL1 motifs with six base pairs (the kissing complex, Fig. 3, left) (55,79,80). Next, the extended duplex, which involves extensive SL1/SL1 base pairing interactions, is formed between the two RNA molecules (80,94). In *in vitro* experiments carried out under physiological conditions, NC appears to promote formation of the extended stable duplex (Fig. 3 right) (82,154,185), mainly by chaperoning the most stable structure (53,110,111,209,214).

Preferential packaging of two copies of the gRNA seems to be a general rule in the assembly of retroviruses (48,61,78,81,128,213). This phenomenon has important implications on viral variability (see last §) (48,114,115,205). It is therefore reasonable to ask if the Gag-NC directed gRNA dimerization is important in the course of HIV-1 assembly. Deleting or mutating the SL1/DIS sequence was found not only to reduce gRNA dimerization and packaging but also virus production was diminished and infectivity impaired (18,132,164,165). Interestingly, despite the reduction in gRNA dimerization, this process was far from totally abolished suggesting that Gag-NC chaperones formation of dimeric gRNA by several means. In fact, deletion of a strong NC binding site, the A-U large loop connecting SL2 to SL3 in ψ (Fig. 2), not only caused a marked inhibition of *in vitro* NC-mediated dimerization, but also impaired gRNA packaging, viral nucleoid structure and virus replication *in vivo* (45,59). These observations further support the notion that gRNA dimerization can be achieved by a number of different routes and that the dimeric genome is a major player not only in Gag-NC assembly but also in virus structure and replication (44,45,155,156).

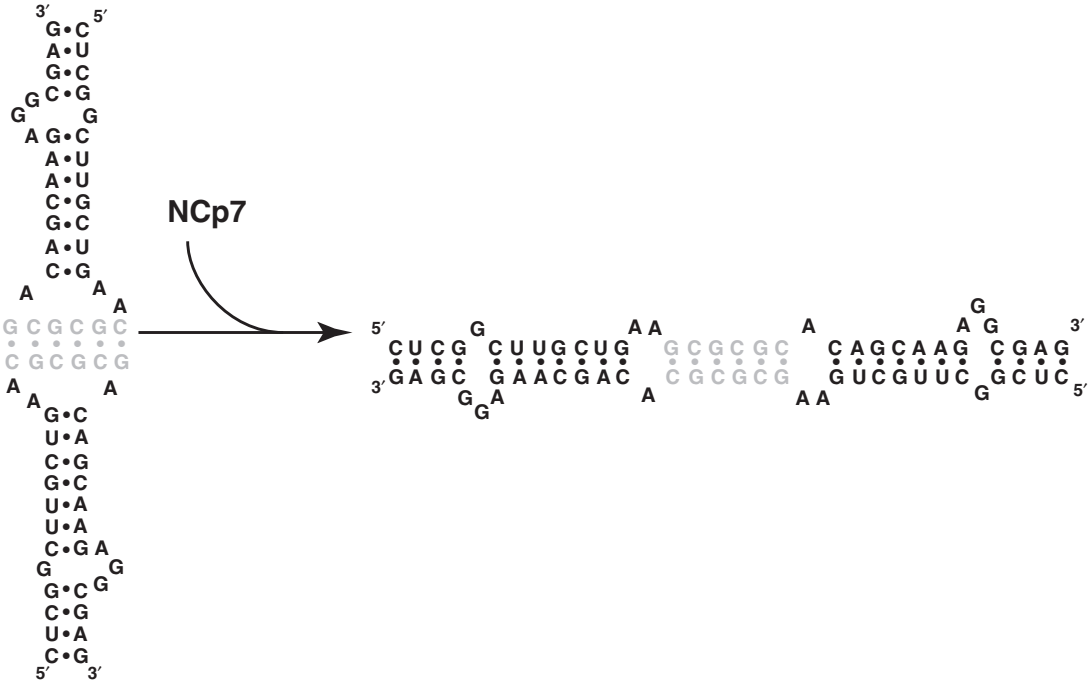


*Figure 3. Proposed scheme of DIS sequence dimerization. Schematic representation of the NC-induced structural conversion of the DIS sequence from the kissing complex to the extended duplex. The palindromic sequence involved in the formation of the kissing complex is shown in gray.*

## HIV-1 NCp7 structure and its relevance to genomic RNA recognition.

NMR-derived structures proposed for HIV-1 NCp7 and NC-RNA complexes have been instrumental in aiding our understanding at the molecular level of the process of gRNA selection by Gag-NC. All abundant retroviruses possess an NC characterized by one or two highly conserved zinc fingers (ZF) of the form CysX2CysX4HisX4Cys (51,61). In HIV-1, NCp7 has two ZFs each containing an aromatic residue, linked by the basic sequence RAPRKKG (Fig. 4). NMR and fluorescence studies of free NC in solution have shown that the affinity of Zn2+ for the fingers is very high (Ka ~$10^{14}$ $M^{-1}$) (32,146). NMR-derived structures of each ZF showed that the peptide sequence is stably folded around the ion (31,138,201) and that this structural arrangement is nearly identical for both ZFs (151,201). Similar features apply to the ZF of MoMuLV NCp10 (67). In addition, the two ZFs and the RAPRKKG linker can adopt a globular conformation leading to spatial proximity, modulated by the relative flexibility of the linker, between Phe16 and Trp37 (151). In contrast to the ZFs, the N- and C-terminal regions of NCp7 do not show any defined structure (151).

Early studies reported that substitution of a Ser or Ala for Cys in position 23 caused a loss of the high affinity for the zinc ion, resulting in ZF unfolding. Mutant viruses produced by DNA transfection of cells were replication defective (68,99,162). Mutations in which Cys23 was substituted by His did not modify the affinity for the zinc ion but affected ZF folding, abrogating the spatial proximity of the fingers (61,68,72,98,99) (Fig. 4) as well as virus infectivity (61,99). A number of other ZF mutations aimed at disrupting folding of the fingers (177,178) also lead to modifications of virion nucleoid structure with a concomitant loss of virus infectivity (2,61,72,99,162). These findings support the notion that a *bona fide* three dimensional structure of the NC central globular region plays a key role in virus replication (61,68,99,162).

As described above, NC has strong nucleic acid binding and chaperoning properties (53,70,110). Genomic RNA (gRNA) recognition is probably initiated by interactions between the ZFs of Gag-NC and the ψ determinants thought to be the unpaired sequences within the SL determinants of the ψ signal. This raises questions concerning the molecular mechanisms governing NC binding to the ψ element. As discussed, it is likely that more than one NC molecule at a time recognizes ψ, rendering the recognition process very selective. However, the structural analysis of such a high molecular mass NC-RNA complex is considered to be extremely difficult. Currently, only the 3-D structure for one molecule of NC bound to SL3 or part of SL2 in a DNA form is available (Fig. 5) (66,150). In these complexes, the backbone of each ZF is preserved and the spatial proximity between the Phe16 of the first ZF and Trp37 of the second ZF is stabilized (66,150). Interestingly, the rather large flexibility of the RAPRKKG linker appears to be the way by which NC can interact with either single or double stranded nucleic acids. In the NC(1-55)/SL3 complex, the ZF preferentially interacts with the single stranded loop as in the NC/d(ACGCC) complex. In the NC(1-55)/SL3 structure the basic N-terminal region of NC, non-structured in the free form, adopts an helical conformation fitting the double-stranded stem. Comparison of the two 3D conformations emphasizes the critical role of Trp37 in the second ZF since it was found stacked to a guanine residue in both complexes (Fig. 5) (66,150). Therefore Trp37 appears to be a key NC determinant for nucleic acid recognition in general, and of the gRNA in particular (74,181). The role of the RAPRKKG linker (150,162,177) and basic N-terminus (27) can be envisaged as reinforcing and modulating nucleic acid recognition and subsequent chaperoning (70,110,130,207). Thus Trp37 would select the theme and the basic regions would perform 'art variations'.

## Virus assembly; the current view.

Abundant published data on the fate of the genomic RNA and NC in respect of virus formation have tried to answer key questions on how and where viral assembly takes place. On this basis it is possible to postulate the following picture of gRNA and Gag-NC interactions: (i) the full length viral RNA is translated by a combination of Cap and IRES driven mechanisms to generate Gag and Gag-Pol. Accumulation of VPR arrests infected cells in G2/M (10,100,123,144,152,179) favoring gRNA synthesis and IRES mediated translation initiation. (ii) Newly made Gag molecules interact with the gRNA via NC (21-24,54,62,141), inhibiting translation and targeting the gRNA to the cell membrane (34,84,85).

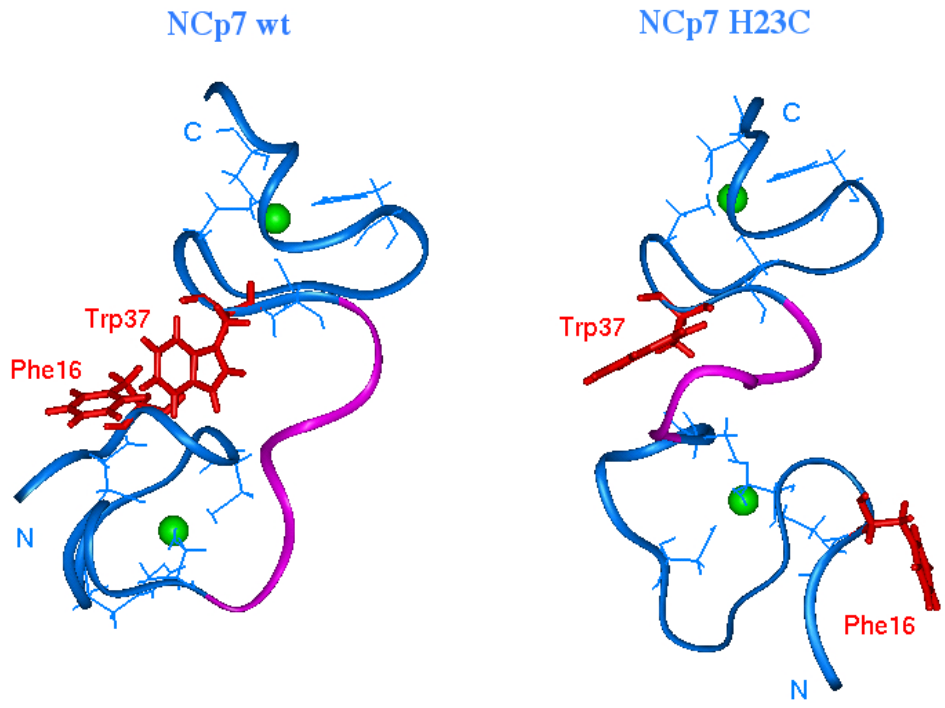


*Figure 4. 3-D structure of NCp7 and mutant H23C. The central domain of NCp7 wild type (wt) is shown on the left. The N-terminal zinc finger (ZF1) is at the bottom while the C-terminal zinc finger (ZF2) is at the top. The Zn2+ ion is represented by a small green marble. The RAPRKKG linker is in purple. Note the proximity of Phe16 (ZF1) and Trp37 (ZF2) in the structure (aromatic rings in red). Mutation H23C, shown on the right, causes a disruption of the central globular structure as evidenced by the opposing arrangement of Phe16 and Trp37. Note that this mutation results in the production of non infectious viral particles with structural abnormalities within the viral core.*

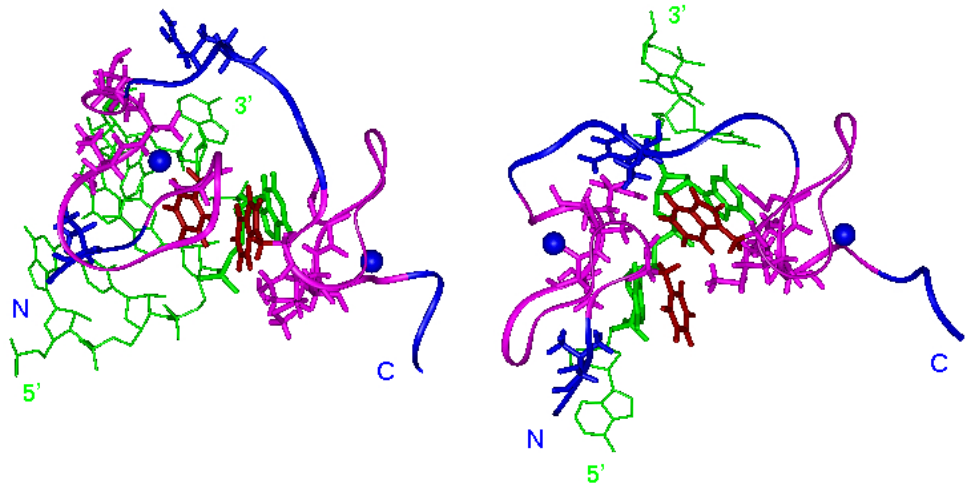


*Figure 5. 3-D structure of NCp7-SL3 RNA and NCp7-dACGCC complexes.*
*The structures of NCp7-SL3 RNA and NCp7-dACGCC complexes are shown on the left and right, respectively. The nucleic acids are shown in bright green and oriented bottom (5′) to top (3′). NCp7 is oriented from left (N) to right (C). Note the stacking of Trp37 to the guanine residue in both structures. In addition, the N-terminal domain of NCp7 and the RAPRKKG linker both interact with the nucleic acid molecule (dark blue).*

At this early stage, the selection of the gRNA is probably mediated by Gag-NC molecules through recognition of the ψ signal by the NC zinc fingers, stabilized by ionic interactions between NC basic residues and the ψ sequence. The multiple SL determinants of HIV-1 ψ (21-23,54,134,170,184) that are positioned between the 5′ SD and AUG of Gag ensure NC-mediated selection of the gRNA only (2,14,17,45,46,47,61,143,217). The mutual gRNA/Gag recognition can be viewed as a zip-like process since it probably requires one NC per SL and thus several NC per ψ signal and, in addition, several NC-RNA interactions for each ψ SL. (iii) Finally, accumulation of Gag-NC molecules on the gRNA will chaperone the conformational rearrangements of the 5′ leader, gRNA dimerization and capture into the growing viral globule (22,23,46,76,126,127,130,133,141,217).

## Where?

It seems that two platforms orchestrate virus assembly: firstly, the membrane, or outer platform, in which HIV-1 Gag molecules are anchored via the N-terminal part of the matrix domain (44,84,85,219). Secondly, the gRNA or interior platform acting as a scaffold molecule, nucleating Gag oligomerization and assembly (45,76,156,203). Finally, the Gag-Pol precursor is recruited into the viral globule (93,163) during assembly resulting in protease activation most probably due to the high local concentration of Gag-Pol. The protease-directed processing of Gag results in the genome being entirely coated with mature NC molecules that will later chaperone the annealing of primer $tRNA^{Lys,3}$ to the PBS and the initiation of cDNA synthesis by reverse transcriptase (9,13,40,41,56,60,69,74,106,133,140,208,201,218). Lastly, NC will chaperone nucleoid condensation and stability (81,87,160,200). At the early stages of infection, NC will chaperone all the steps of the RT-mediated replication of the gRNA, from primer tRNA annealing to the PBS to the synthesis of the complete double stranded proviral DNA as well as viral genetic re-assortments by recombination in the course of cDNA synthesis (5,9,49,96,112,114,115,125,131,158,183,187,188). Also, a significant body of evidence favors the idea that NC can guide the integration process by preserving the LTR ir sequences and activating the integrase enzyme (37,91,129).

## Conclusion and future prospects

In conclusion, NC either as Gag-NC or as the mature protein appears to be the 'high fidelity' partner of the genomic RNA, chaperoning functional rearrangements of the RNA structure, stability and *bona fide* conversion to proviral DNA by RT (53,61,99,131,180).

Addressing the following key areas in the years ahead should significantly advance our understanding of the molecular mechanisms underlying HIV-1 assembly: (i) a comprehensive view of gRNA selection by Gag-NC will require deciphering of the 3D structure of HIV-1 ψ in the monomeric and dimeric forms, alone or associated with NC molecules. The affinity of the immature and mature forms of NC (Gag-NC, NCp15, NCp7(1-71), NCp7(1-55) (71,109) for SL, ψ and ψ mutants will need to be compared. Since SIV gRNA can participate in HIV-1 Gag assembly (although not reciprocally) it will be of interest to compare the affinity of HIV-1 Gag and SIV Gag for SL and ψ, in both homologous and heterologous combinations. (ii) Little is known on the extent of translational regulation in HIV-1 and in retroviruses generally. It would seem beneficial therefore to further investigate the binding of cellular translation initiation factors (e.g. eIF4G) to HIV-1, SIV and other retroviral IRESes and to determine in greater detail their modulatory effects on viral translation during the different phases of the cell cycle. (iii) The 5′ HIV leader contains multiple genetic determinants essential in all stages of virus replication. Secondary structures within the leader have been proposed, however the possibility of long distance interactions between the leader and the 3′ UTR requires greater attention as these are potentially of key importance in gRNA structure and replication, as suggested by evidence from RSV and retrotransposons of yeast (52,58,88). Such 5′-3′ gRNA interactions could greatly facilitate translation, assembly and reverse transcription. (iv) Little is known about the dynamics of the viral particle between completion of assembly and budding out of the cell. Evidence that reverse transcription begins in newly made particles (56,141,208,216,218) favors the notion these are fully functional as soon as their interior is formed. However, little is known about the cellular route(s) particles follow to leave the cell.

Finally, in view of the fundamental role of NC during the HIV life-cycle, a concerted effort aimed at the development of drugs to abrogate its functions would seem amply justified (28,75,182).

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Abbreviations. HIV, human immunodeficiency virus. IRES, internal ribosome entry signal. MuLV, murine leukemia virus. NC, nucleocapsid protein. RT, reverse transcriptase. ZF, zinc finger. RSV, Rous sarcoma virus. SIV, simian immunodeficiency virus.

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