**Role of the N-Terminal Zinc Finger of Human Immunodeficiency Virus Type 1 Nucleocapsid Protein in Virus Structure and Replication**

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Nucleocapsid protein (NCp7) of human immunodeficiency virus type 1 is found covering the genomic RNA in the interior of the viral particle. It is a highly basic protein with two zinc fingers of the form CX2CX4HX4C which exhibit strong affinity for a zinc cation. To study the structure-function relationship of the N-terminal zinc finger of NCp7, this domain was either deleted or changed to CX2CX4H4C4. We examined virus formation and structure as well as proviral DNA synthesis. Our data show that these two NC mutations result in the formation of particles with an abnormal core morphology and impair the end of proviral DNA synthesis, leading to noninfectious viruses.

The nucleocapsid protein (NCp7) of human immunodeficiency virus type 1 (HIV-1) is a highly basic protein which is tightly associated with the genomic RNA dimer in mature viral particles to form the ribonucleoprotein complex (14). NCp7 is derived from the C terminus of the Pr55<sup>pgp</sup> precursor following proteolytic cleavage (18, 19, 29). NCp7 contains two zinc fingers of the form CX2CX4HX4C4 (49) with high affinity for a zinc cation (23, 45), and which are close to each other as shown by 1H nuclear magnetic resonance spectroscopy analyses and molecular modeling (36–38). The NCp7 protein is involved in essential steps of genome replication since it promotes annealing of the tRNA<sup>1</sup> primer to the genomic primer binding site (3, 4, 16) and minus-strand DNA transfer during proviral DNA synthesis (2, 13, 17, 28, 42). In addition, NCp7 appears to abolish nonspecific reverse transcription to self-priming that can take place either at the 3′ end or at nicks in the genomic RNA (28, 32, 34) and to enhance efficiency and processivity of the reverse transcriptase (RT) enzyme (30, 40, 42, 46, 51). These functional properties of NCp7 seem to be related to the nucleic acid annealing activity of the protein in vitro (31). In fact, NCp7 promotes rapid and extensive hybridization of two complementary nucleic acid sequences by destabilizing intramolecular duplexes and by favoring formation of the most stable intermolecular duplex (33, 47). During virion formation, NC protein, as part of Pr55<sup>pgp</sup> and/or as mature NCp7, is thought to bind to the viral RNA (11, 12), resulting in genomic RNA dimerization and packaging (14, 16) and in the formation of the virion nucleocapsid structure (8, 39, 41). Moreover, NC protein is able to stabilize dimeric RNA, converting it from the immature to the mature, stable form (22, 24).

Extensive mutational analyses of HIV-1 NCp7 have shown that substitutions of highly conserved residues thought to modify the overall conformation of the protein result in the production of viral particles defective in replication (12, 39, 41, 43). Analysis of the NC zinc finger mutant virus shows a strong defect in genomic RNA packaging (1, 20, 27, 35). Although both fingers are required for encapsidation of viral RNA and for infectivity, they are not functionally equivalent and their respective positions cannot be exchanged (26). On the other hand, substituting basic residues for neutral amino acids reduces genomic RNA packaging and results in the attenuation of NC mutant viruses (5).

In an attempt to study the structure-function relationships of the N-terminal zinc finger of HIV-1 NC protein during different steps of the viral replication cycle, the first zinc finger was either deleted (to create mutant ΔD1) or changed to a CCC motif (to create mutant H23C). Substituting H<sup>23</sup> for Cys causes structural modifications in the N-terminal zinc finger which disrupt the proximity of the two zinc fingers and result in a misfolded protein (15). The H23C substitution does not, however, interfere with the strong affinity of the mutated zinc finger for the zinc cation. This is in contrast to other mutations, such as the substitution of Cys for Ser or His for Ala, which prevent zinc coordination (1, 20, 27, 35).

H23C and ΔD1 were obtained by site-directed mutagenesis performed on the pNL4-3 HIV-1 molecular clone as previously described (39) with the oligonucleotides 5′-GGAAGAAA GGTTGCATAGCC-3′ (for H23C) and 5′-GAAAGACTG TTAAGGTTGGCAGGGCCC-3′ (for ΔD1). As previously reported, both mutants were completely defective in replication in SupT1 and HeLa P4 cells (15, 27).

To analyze the morphology of the NC mutant viruses, HeLa P4 cells (10) were transfected by the calcium phosphate precipitation method (44) with wild-type (wt) or mutant pNL4-3 and processed for thin-layer electron microscopy (Fig. 1). Cells transfected with the wt provirus showed numerous viral particles budding at the plasma membrane and extracellular mature virions, with morphology typical of HIV, including central electron-dense material corresponding to the core (Fig. 1, inset). However, many particles produced by cells transfected with both mutants had a mature but abnormal morphology (Fig. 1). Under higher magnification (Fig. 1, insets), a majority of mutant virions were observed to be characterized by (i) a strong...
electron density of the whole particle and (ii) an abnormal core structure which did not correspond to the typical cone-shaped core of the mature wt particles, probably due to a nucleocapsid with a modified ultrastructure. Moreover, 28 and 12% of the H23C and ΔD1 released particles, respectively, exhibited a typical immature morphology characterized by a doughnut or ring shape corresponding to a thick electron-dense outer shell and an electron-lucent center where no typical cone-shaped core was detectable. Also, NC mutant virions had a mean diameter of 134 nm, which is larger than that of wt particles (106 nm). Moreover, we observed that after ultracentrifugation, the amount of Cap24 detected by enzyme-linked immunosorbent assay was five times lower for NC mutant particles than for wt particles (data not shown), indicating that particles with an abnormal core morphology are probably unstable. These observations suggest that the first zinc finger structure of NC protein is probably critical for a stable core structure.

To determine the densities of the NC mutant virions, viral supernatants were concentrated and separated on 20 to 50% sucrose density gradients as previously described (5). The H23C and ΔD1 mutant viruses had densities of 1.179 and 1.171 g/ml, respectively, which is very close to that of the wt virus (1.18 g/ml).

Three days after transfection, virions released during a period of 24 h were pelleted through a 20% sucrose cushion and viral proteins were analyzed by immunoblotting with anti-CAp24, anti-NCp7, and anti-RTp66/p51 antibodies (Fig. 2). We observed that Pr55gag processing was affected in the H23C and ΔD1 mutants, as judged by an increase in the prominence of (i) the Pr55gag precursor itself and (ii) the p41-processed intermediate known to contain the MAp17 and CAp24/25 sequences, as well as another intermediate containing the NC region (Fig. 2A). The ratios of the precursors to mature CAp24 were approximately 10% for the wt, 50% for ΔD1, and 70% for H23C, as determined by scanning densitometry. Mature NCp7 was detected for both mutants (Fig. 2B). The use of antibodies directed against RTp66/p51 allowed us to determine no significant difference in the amount of RT protein present in NC mutant virions compared to the wt (Fig. 2C). In conclusion, the gag structural proteins and RT are present in mutant virions, although a minor defect in Pr55gag processing was observed for both mutants.

We examined the virion genomic RNA content of the NC

FIG. 1. Electron microscopy of wt and NC mutant virions. Particles derived from the wt, H23C, and ΔD1 transfected HeLa P4 cells were postfixed with 1% osmium tetroxide and embedded in epon 72 h posttransfection. Grids were counterstained with uranyl acetate and lead citrate. The arrowhead shows an immature particle with a thick electron-dense outer shell and an electron-lucent center lacking a typical cone-shaped core. The bar represents 100 nm. (Insets) higher magnification of either a normal mature particle for the wt virus (with a central electron-dense material corresponding to the cone-shaped core) or a representative example of mature particles with a typical abnormal morphology for H23C and ΔD1 virions.
mutants by slot blot hybridization, as described previously (39), by probing with a randomly 32P-labeled 5.3-kb SacI-SalI fragment of the pNL4-3 plasmid corresponding to the gag and pol sequences. Genomic RNA packaging was approximately 10% of the wt level for both mutants, which is similar to levels found in other studies involving mutations of the Zn2+-chelating residues (data not shown) (27, 35).

The implications of NCp7 in the reverse transcription process in vitro prompted us to use a PCR-based system to analyze the major steps of proviral DNA synthesis in vivo. Infection of SupT1 cells was performed by addition of 24-h cell-free virus produced 3 days after transfection in the presence of 3 U of RQ1 DNase (Promega) per ml and concentrated 10 times in a Biomax OSI column. After extracting DNA 2, 6, and 24 h postinfection, we used PCR and a corresponding set of primers to detect R-U5 DNA (which consists mainly of strong-stop cDNA), the end of minus-strand DNA synthesis, and second-strand transfer (Fig. 3). The absence of plasmid pNL4-3 was confirmed with primers specific for the pUC vector (data not shown). Our results show that the levels of R-U5 DNA were similar for the wt and the NC mutant viruses, as were the extents of minus-strand DNA and second-strand transfer (Fig. 3). To detect possible defects at the very end of proviral DNA synthesis, we used a primer localized at the 5′ end of the long terminal repeat (LTR) U3 sequence (Fig. 3). The absence of plasmid pNL4-3 was confirmed with primers specific for the pUC vector (data not shown). Our results show that the levels of R-U5 DNA were similar for the wt and the NC mutant viruses, as were the extents of minus-strand DNA and second-strand transfer (Fig. 3). To detect possible defects at the very end of proviral DNA synthesis, we used a primer localized at the 5′ end of the long terminal repeat (LTR) U3 sequence (Fig. 4) (6). This revealed an amplified fragment of the appropriate size in cells infected with wt virus, but no product was detectable for either NC mutant, suggesting that although reverse transcription of the viral genome was complete for the mutants (as indicated by observable second-strand transfer) (Fig. 3), proviral DNA synthesis leading to the formation of the 5′ LTR was incomplete.

Previous work indicated that one- and two-LTR circle forms are generated within the nucleus, probably by host activities since incubation of deproteinized linear HIV-1 cDNA with cell extracts leads to the formation of both forms, thus excluding NC protein from this process (7, 21, 48, 49, 50). We used these findings to further examine proviral DNA synthesis. The quantity of DNA used for PCR was adjusted for equal amounts of

![FIG. 3](image-url)

FIG. 3. Analysis of early and late steps of viral DNA synthesis by PCR. Infection of HeLa P4 cells was performed with wt and mutant viruses adjusted to the same level of viral RNA. Viral DNA synthesis was analyzed by PCR with primer pairs as follows: 5′ R (5′-GGTCTCTGTTAGACCA-3′) and 3′ US (5′-CTGCTA GAGATTTCACACAC-3′) to generate R-US DNA, 3′ PBS (5′-ACTTGAAAAGGTAAGGAC-3′) and MA (5′-TGATGACACAAGGAGGAC-3′) to detect the end of synthesis of minus-strand cdNA, and 5′ R and MA to study second-strand transfer. DNA was extracted from either total cellular extract (R-US DNA and synthesis of the minus-strand DNA) or nuclear fraction (second-strand transfer) as previously described (5). The localization of each primer pair relative to the viral genome is represented on the left. The absence of plasmid pNL4-3 was confirmed by using primers localized within the flanking DNA (5′ check [5′-AGGCAG TCTAGTCCCCAG-3′]) and pUC (3′ check [5′-AGGCAG TCTAGTCCCCAG-3′]) and pUC (3′ check [5′-AGGCAG TCTAGTCCCCAG-3′]). The cycling times were 5 min at 94°C for denaturation, 30 s at 58°C for annealing, and 30 s at 72°C for extension. Thirty cycles were performed. Ct, mock-transfected cells; PBS, primer binding site.

![FIG. 4](image-url)

FIG. 4. PCR analysis of the end of proviral DNA synthesis. (A) Schematic representation of 3′ LTR synthesis and localization of the sense primer SU3 (5′-GCACCATCAGAGGAGGTCAG-3′) and the antisense primer ASPBS (5′-CGTCTGTTAGACCAATTTTATCAG-3′). The dashed line indicates the DNA strand displacement necessary for 3′ LTR synthesis. (B) DNA extracted from infected cells (5) was analyzed by PCR as described in the legend for Fig. 3. Molecular size markers are indicated on the left.
plus-strand DNA in infected cells. The nuclear fraction was analyzed by PCR 20 h after infection, using primers which amplify the one- or two-LTR DNA circles (Fig. 5A). No circle forms were detected after amplification in cells infected with either the H23C or D1 mutant virus, while they were detected for wt HIV-1 (Fig. 5B and C). This experiment suggests that mutant proviral DNA is unable to form DNA circles because of defective ends and/or inefficient translocation in the nucleus.

In the present study, the importance of the correct spatial arrangement of the proximal CCHC zinc finger has been assessed with respect to the biological functions of HIV-1 NC protein. Electron micrographs reveal that the majority of the NC mutant particles had either an immature or an abnormal core morphology compared to that of the wt virus, although no significant variation was detected in their respective densities. This impairment of the viral core structure may be related to defects in polyprotein precursor processing observed for both NC mutants but not observed in the wt. We assume that a misfolded NC domain within Pr55\(^{gag}\) could negatively influence the conformation of the precursor and/or the stability of gag and gag-pol oligomers. This could be related to a defect in viral assembly.

Early reverse transcripts, such as R-U5 DNA, were detected by PCR for both NC mutants in infected cells, indicating that neither virus entry into cells nor the beginning of reverse transcription was affected. Moreover, viral DNA synthesis seemed to be complete as far as the second-strand transfer for H23C and D1 mutants, providing evidence for a fully functional involvement of the mutated NC proteins during reverse transcription in vivo. Interestingly, we observed that the final step of viral DNA synthesis leading to synthesis of the 5' LTR did not occur.

FIG. 5. PCR analysis of one- and two-LTR proviral DNA forms in infected HeLa P4 cells. (A) Schematic representation of the various forms of unintegrated circular HIV-1 viral DNA and the positions of the primer pairs used for PCR. Viral DNA nuclear import was analyzed by PCR with primers Nef (5'-GTTCCTCAGTCACACCTCAGG-3') and 3' ASPBS (5'-CTCCTCGCTTTACTTTTCG-3') to detect the single-LTR circular form (1-LTR) (B) and primers SU5 (5'-GACCGTTTAGTCAATGTTGG-3') and ASU5 (5'-CCAGAATCACACACACAGAG-3') to detect the double-LTR circular form (2-LTR) (C). Molecular size markers are indicated on each panel.
not proceed correctly for either the H23C or the ΔD1 variant. This is interesting in light of the observation that neither of the one- and two-LTR circle forms could be detected for these mutants; due to defective ends, these molecules were unable to generate circle forms or to integrate. We assume that the H23C and ΔD1 mutations affected the stability of the reverse transcription complex and led to a defect in DNA strand displacement necessary for 5’ LTR synthesis, which has been reported to be slow and inefficient (25), and/or the loss of protection of viral DNA against exonucleases.

Moreover, correct integration may require functional cooperation between the NC and integrase (IN) proteins, as observed in vitro (9). Similarly, putative interactions between NC protein and cellular proteins involved in this process could also be affected. Further investigations are needed to determine whether NC mutant proteins and/or viral DNA is present within the nuclei of infected cells.

Taken together, these results show that the conformation of the NC protein is critical not only for virus assembly but also for complete proviral DNA synthesis and/or integration. This suggests that the NC protein acts as a chaperone protein during the course of the viral life cycle, mediated by its multimeric organization and enabling the production of infectious particles.

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