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The Central Globular Domain of the Nucleocapsid Protein of Human Immunodeficiency Virus Type 1 Is Critical for Virion Structure and Infectivity

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The nucleocapsid protein NCp7 of human immunodeficiency virus type 1 (HIV-1) is a 72-amino-acid peptide containing two CCHC-type zinc fingers linked by a short basic sequence, ^{29}RAPRKKG^{35}, which is conserved in HIV-1 and simian immunodeficiency virus. The complete three-dimensional structure of NCp7 has been determined by ^{1}H-nuclear magnetic resonance spectroscopy (N. Morellet, H. de Rocquigny, Y. Mely, N. Jullian, H. Demene, M. Ottmann, D. Gerard, J. L. Darlix, M. C. Fournié-Zaluski, and B. P. Roques, J. Mol. Biol. 235:287–301, 1994) and revealed a central globular domain where the two zinc fingers are brought in close proximity by the RAPRKKG linker. To examine the role of this globular structure and more precisely of the RAPRKKG linker in virion structure and infectivity, we generated HIV-1 DNA mutants in the RAPRKK sequence of NCp7 and analyzed the mutant virions produced by transfected cells. Mutations that probably alter the structure of NCp7 structure led to the formation of very poorly infectious virus (A30P) or noninfectious virus (P31L and R32G). In addition, the P31L mutant did not contain detectable amounts of reverse transcriptase and had an immature core morphology, as determined by electron microscopy. On the other hand, mutations changing the basic nature of NCp7 had poor effect. R29S had a wild-type phenotype, and the replacement of ^{32}RKK^{34} by SSS (S3 mutant) resulted in a decrease by no more than 100-fold of the virus titer. These results clearly show that the RAPRKKG linker contains residues that are critical for virion structure and infectivity.

The human immunodeficiency virus type 1 (HIV-1) (6) consists of an outer envelope of cellular origin surrounding a capsid within which lies the nucleocapsid. In mature virions, the nucleocapsid contains about 2,000 nucleocapsid (NC) proteins tightly associated with the genomic 70S dimer RNA, reverse transcriptase (RT), integrase, and protease molecules (see references 14, 20, and 33 for reviews).

The NCp15 protein is derived from the C terminus of the Pr55^{gag} polyprotein precursor and is ultimately processed into NCp7 and p6 in mature HIV-1 virions (17). The highly basic NC protein is conserved among all known retroviruses and contains, except for spumaretroviruses, one or two zinc fingers of the form Cys-X2-Cys-X4-His-X4-Cys (CCHC) capable of coordinating a zinc ion (7, 9, 13, 48).

In vitro, HIV-1 NCp7, like Rous sarcoma virus NCp12 and Moloney murine leukemia virus NCp10, has nucleic acid binding and annealing activities which promote the dimerization of viral RNA and the annealing of the replication primer tRNA onto the primer-binding site (PBS) (5, 8, 40). Recent studies have also shown that NCp7 is able to promote DNA strand transfer during reverse transcription (15, 32). In addition, NCp7 can form high-molecular-weight complexes with viral RNA and DNA and is able to protect DNA and RNA from nuclease digestion, suggesting a putative role for the NC protein during integration (32).

Numerous genetic studies with NC mutants of Rous sarcoma virus (19), Moloney murine leukemia virus (25, 34), and HIV (2, 18, 24, 26) have shown that retroviruses require intact NC zinc finger proteins for the specific packaging of the unspliced dimeric viral RNA and possibly for the stability of the virus (4). In addition, substitution of neutral residues for basic amino acids flanking the zinc fingers of Rous sarcoma virus NCp12 (21) or the unique zinc finger of Moloney murine leukemia virus NCp10 (28, 39) resulted in a drastic decrease in NC protein activities in vitro and in vivo.

The ^{29}RAPRKKG^{35} region located between the two zinc fingers is well conserved in all HIV-1, HIV-2, and simian immunodeficiency virus strains. Moreover, the three-dimensional structure of HIV-1 NCp7 has revealed that the kink induced by Pro-31 of the ^{29}RAPRKKG^{35} sequence appears to be of importance in bringing the two zinc fingers into close proximity (35, 36). Substitution of DPro-31 for LPro-31 in HIV-1 NCp7 resulted in a loss of the interaction between the two zinc fingers (35). In addition, structural studies indicate that Lys-33 and Lys-34 are exposed to solvent and might be involved in binding to viral RNA (35). These observations prompted us to investigate the importance of the NCp7 ^{29}RAPRKKG^{35} linker in vivo. The results show that mutations expected to strongly modify the structure of the ^{29}RAPRKKG^{35} linker (i.e., P31L and R32G) strongly impaired virus infectivity. Moreover, the P31L mutation was found to inhibit virion maturation. In contrast, replacement of ^{32}RKK^{34} by SSS reduced the virus titer by 100-fold, while the R29S mutation had no effect on viral infectivity.

MATERIALS AND METHODS

HIV-1 DNA mutagenesis and construction. The *Sph*I-*Eco*RI fragment of the pNL4.3 HIV-1 molecular clone (1) was introduced into M13mp18 (R32G and P31L mutations) or M13mp19 (R29S, A30P, and S3 mutations). Site-directed mutagenesis was carried out by Kunkel's procedure (31) with the following oligonucleotides: R29S, 5′ CCT AGG GGC CGA GCA ATT TTT GGC 3′; A30P, 5′ CCC CTT TTT CCT AGG GGG CCT GCA ATT TTT GGC 3′; P31L, 5′ C AGG GCC CTT AGG AAA A 3′; R32G, 5′ GG GCC CCT GGG AAA

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AAG 3′; and S3, 5′ CCA ACA GCC CGA TGA CGA AGG GGC CCT GC 3′. The underlined nucleotides represent the changes responsible for the amino acid substitutions. All mutations were verified by sequencing (43), and the fragments were reinserted into the pNL4.3 DNA vector.

Cell culture and HIV-1 DNA transfections. Cos7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum; 100 U of penicillin, 100 μg of streptomycin, and 100 μg of kanamycin per ml; and 2 mM glutamine. SupT1 cells (47) were grown in RPMI 1640 supplemented with 10% fetal calf serum; 100 U of penicillin, 100 μg of streptomycin, and 100 μg of kanamycin per ml, and 2 mM glutamine.

DNA transfections were performed by the modified calcium phosphate precipitation technique using 1× BBS buffer [25 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 140 mM NaCl, 0.75 mM Na_2HPO_4] and 12.5 mM $CaCl_2$ (11) mixed with 10 μg of proviral DNA added to Cos7 cells. Three days after transfection, supernatants were harvested and clarified by low-speed centrifugation (15,000 × *g* for 15 min).

Infectivity and viral titers were assessed by infecting 10^6 SupT1 human T cells with 1 ml of clarified supernatant and with 10-fold serially diluted supernatants in RPMI 1640, which was replaced 24 h later by fresh medium. Every 2 to 3 days, the cultures were checked for the appearance of cytopathogenic effects (CPE) and were diluted to maintain the cells in exponential growth. The viral titer was defined as the last infecting dilution determined by the presence of CPE and by RT activity in the cell culture supernatant (23). In order to confirm the phenotype of the mutant viruses, infectivity was also assessed by infecting HeLa cells expressing the CD4 antigen and containing the *lacZ* gene under the control of the HIV-1 long terminal repeat (named P4 cells, which were kindly provided by P. Charneau and F. Clavel) (12).

ELISA and RT assays. The amount of viral CAp24 core antigen in the culture supernatants was determined by enzyme-linked immunosorbent assays (ELISA) (kindly provided by V. Cheney and B. Mandrand, Biomérieux). Samples were diluted 10-fold serially in phosphate-buffered saline before the analysis. The assay for determination of RT activity was performed by using a modification of the protocol initially described by Goff et al. (23). Briefly, 30 μl of clarified supernatant was added to a mix containing, in a final volume of 50 μl, 50 mM Tris-HCl (pH 8.3), 0.1 mM dTTP, 60 mM NaCl, 5 mM $MgCl_2$, 20 mM dithiothreitol, 0.05% Nonidet P-40, 10 U of poly(A)-oligo(dT) per ml, and 0.5 μCi of [^{32}P]dTTP. After a 2-h incubation at 37°C, samples were filtered through a 0.45-μm-pore-size NA45 membrane (Schleicher & Schuell), extensively washed with 2× SSC (0.3 M NaCl plus 30 mM sodium citrate), and subjected to autoradiography.

Analysis of viral proteins. Clarified supernatants from transfections were ultracentrifuged through a 20% sucrose cushion at 30,000 rpm for 1 h at 4°C and then dissolved in TNE (25 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM EDTA) with 1% sodium dodecyl sulfate (SDS). For viral cytoplasmic proteins, the cells were washed twice in cold phosphate-buffered saline and scraped, and the pellet was suspended in 80 μl of 20 mM Tris-HCl (pH 8.8)–2 mM $CaCl_2$. The cells were lysed by adding 9 μl of a 10% β-mercaptoethanol–3% SDS solution and were then heated for 2 min at 95°C and immediately cooled on ice. Samples were treated with 5 μl of DNase buffer (0.5 M Tris-HCl [pH 7], 50 mM $MgCl_2$, 0.67 mg of DNase per ml) and stored at −20°C before use.

Extracellular particles and cell extracts were fractionated through a 5 to 15% gradient tricine–SDS-polyacrylamide gel (44) or an SDS–12% polyacrylamide gel and electroblotted onto nitrocellulose in Tris-glycine buffer with 40% MeOH. Proteins were analyzed by immunoblotting with a chemiluminescence system (ECL; Amersham) according to the manufacturer's instructions with anti-NCp7 (49) and anti-CAp24 monoclonal mouse antibodies and RTp66/p51 polyclonal rabbit antiserum.

Sucrose density gradient analysis of HIV-1 virions. Twelve milliliters of supernatant was harvested from transfected cell cultures, clarified by centrifugation at 3,000 rpm for 15 min, and concentrated by ultracentrifugation through a 25% sucrose cushion in TNE. The pellet was resuspended in 100 μl of TNE buffer, and 50 μl was loaded onto a premade 4-ml 25 to 60% linear sucrose gradient. After ultracentrifugation at 32,000 rpm for 2 h and 15 min at 4°C in an SW60 Ti rotor, 20 fractions of 0.2 ml were collected, starting at the top of the tube. The density of each fraction was determined with a refractometer on a tube without viruses. Each fraction was analyzed for the presence of CAp24 by ELISA and, when possible, for RT activity. The sucrose density was considered linear in the range of sucrose concentrations used for these experiments.

Genomic RNA analysis. RNAs of mutant and wild-type virus particles were isolated from concentrated transfected cell supernatants after ultracentrifugation as described above. Viral pellets were dissolved in 200 μl of 1× TMD buffer (40 mM Tris-HCl [pH 7.5], 6 mM $MgCl_2$, 10 mM dithiothreitol) containing 100 U of RQ1 DNase (Promega), 200 U of RNasin (Promega), and 50 μg of carrier RNA (tRNA *Escherichia coli*) per ml, and the buffer was incubated for 30 min at 37°C and then for 30 min at 37°C in the presence of 1% SDS and 100 μg of proteinase K per ml. RNAs were then purified by phenol-chloroform and chloroform extractions and were precipitated in 0.3 M Na acetate–ethanol. Samples were adjusted for equal amounts of CAp24 before electrophoresis of viral RNA. Viral RNAs were separated using a denaturing 0.7% agarose gel containing formaldehyde (37% [vol/vol]) and 1× MOPS buffer (20 mM 3-*N*-morpholinopropanesulfonic acid, 5 mM Na acetate [pH 7], 1 mM EDTA). RNAs were electroblotted onto a nylon membrane (Hybond N; Amersham) for 2 h at 0.8 A in a 25 mM sodium phosphate buffer at pH 6.5. HIV-1 genomic RNA was probed with a nick-translated, ^{32}P-labeled 5.3-kb *Sac*I-*Sal*I fragment of the pNL4.3 plasmid corresponding to *gag* and *pol* sequences (42).

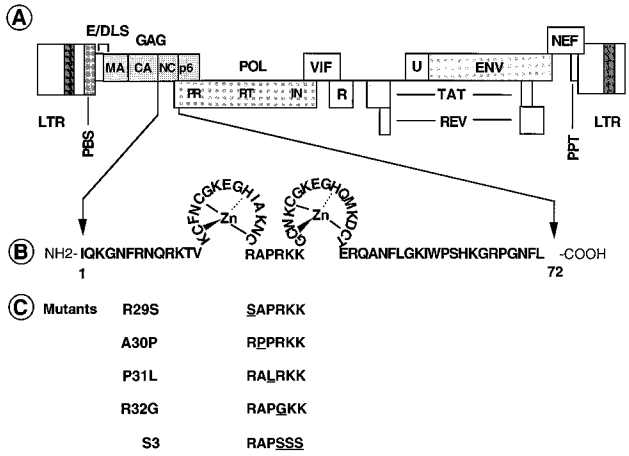

FIG. 1. Mutations of the basic region located between the two zinc fingers of NCp7 of HIV-1. (A) Schematic representation of the genetic structure of the HIV-1 provirus. LTR, long terminal repeat; E/DLS, encapsidation-dimerization sequence of the genomic RNA; PPT, polypurine track. Maturation of Pr55^{gag} yields matrix protein MAp17, capsid protein CAp24, NC protein NCp7, and p6. (B) Amino acid sequence of the HIV-1 NCp7 (pNL4.3) with the two zinc finger structures. (C) The amino acid sequences between the two zinc fingers of the five mutants are indicated, with the residues of interest underlined.

Electron microscopy. Three days after transfection, Cos7 cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.5) and treated as described previously (10).

RESULTS

Construction of HIV-1 NC mutants. To examine the role of the basic region ^{29}RAPRKK^{34} linking the two zinc fingers of HIV-1 NCp7 in virion morphogenesis and infectivity, we generated five mutants in the NCp7 coding sequence using the pNL4-3 molecular clone (1) (Fig. 1C). The importance of the proline residue in the globular structure was examined by adding a second proline (A30P) and by replacing the original proline with a leucine (P31L). To investigate the role of the basic amino acids in vivo, neutral residues were substituted in either single (R29S and R32G) or multiple (S3) mutations.

Viral proteins in HIV-1 DNA-transfected cells. Synthesis and proteolytic processing of the Pr55^{gag} polyprotein precursor from HIV-1 NC mutants in transfected Cos7 cells were analyzed by immunoblotting. The Pr55^{gag} precursor was found to be expressed in the Cos7 cells transfected with the wild-type virus as well as with the HIV-1 NC mutants (Fig. 2). Similar amounts of processed capsid protein CAp24 were found in the cells transfected with all of the HIV-1 NC mutants, except for P31L. The P31L mutant exhibited a lower level of CAp24 but a higher level of the Pr55^{gag} precursor and of Pr41^{gag} (Fig. 2, lane 3). The reduced level of CAp24 in cells transfected with the P31L mutant DNA indicates that the P31L mutation probably interfered with the processing of the Pr55^{gag} precursor.

Virion Gag proteins and RT. The ability of the wild-type and NC mutant DNAs to promote the production of viral particles upon transfection into Cos7 cells was examined by CAp24 ELISA and RT activity. The experiments were repeated at least three times, and the results indicate that the amounts of CAp24 (as the mature protein and/or as Pr55^{gag}) present in the supernatants of cells transfected with the HIV-1 NC mutants were similar to that obtained with the wild-type HIV-1 DNA and ranged between 120 and 290 ng/ml in each experiment.

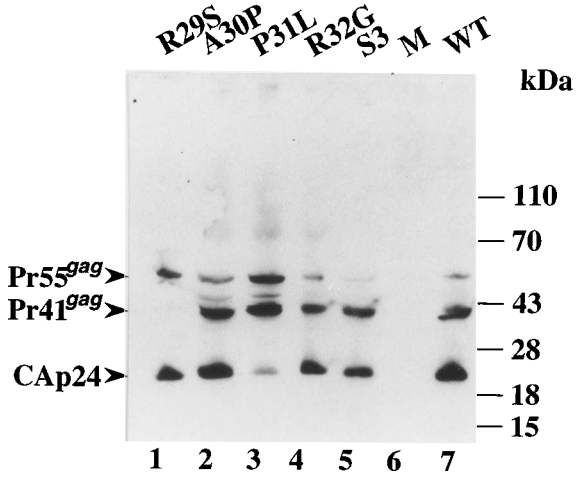

FIG. 2. Analysis of viral proteins synthesized in Cos7 cells after transfection with NCp7 mutant and wild-type proviral DNAs. Cells were lysed 72 h post-transfection, and proteins were resolved on a 5 to 15% gradient tricine-SDS-polyacrylamide gel. The cells were transfected with R29S (lane 1), A30P (lane 2), P31L (lane 3), R32G (lane 4), S3 (lane 5), or wild-type (lane 7) DNA or were mock transfected (lane 6). Viral proteins were detected by immunoblotting with antibodies against HIV-1 CAp24. The relative positions of the HIV-1 proteins are indicated on the left, and the positions of molecular mass markers (in kilodaltons) are indicated on the right.

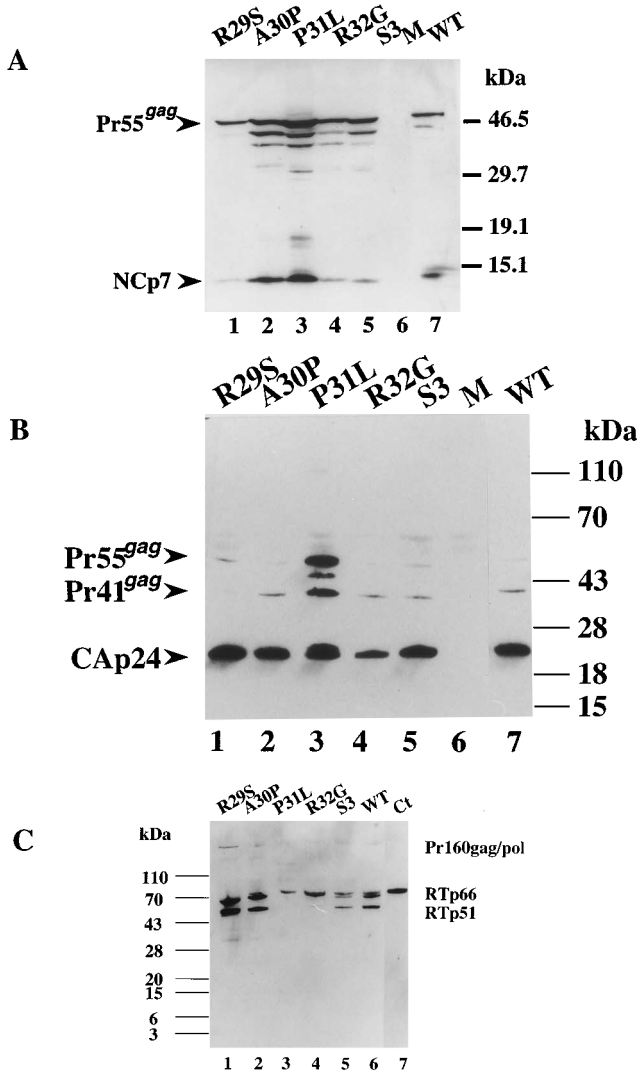

FIG. 3. Analysis of the structural Gag proteins in viral particles produced by cells transfected with the HIV-1 NCp7 mutants. Viral proteins from transfected cells were purified by ultracentrifugation through a sucrose cushion, separated on a 5 to 15% polyacrylamide-tricine-SDS gradient gel, and electrotransferred. The membrane was successively probed with anti-NCp7 monoclonal antibodies (A), anti-CAp24 monoclonal antibodies (B), and anti-RTp66/p51 antiserum (C). Lanes WT, wild type (positive control); lanes M and Ct, supernatant from mock-transfected Cos7 cells (negative control). The RT antiserum cross-reacted with a 70-kDa protein corresponding to serum albumin which can be seen in all lanes.

These results provide evidence that the mutations had no detectable effect on virus production. Furthermore, viral particles produced by Cos7 cells transfected with all NCp7 mutants except P31L had levels of RT activity similar to that of the wild type. No RT activity was found associated with the P31L mutant virions, even in virions pelleted from 1 ml of supernatant (data not shown).

To characterize the Gag proteins present in the viral particles, Western blot (immunoblot) analyses were performed using virions pelleted from the supernatant of transfected cells and antibodies directed against NCp7 (Fig. 3A) and CAp24 (Fig. 3B). A wild-type pattern of viral Gag proteins was found with all of the HIV-1 NCp7 mutant viruses except for the P31L mutant. Large amounts of $Pr55^{gag}$ and partially processed $Pr41^{gag}$ precursors were detected in the P31L mutant viral particles (Fig. 3A and B, lane 3). CAp24 protein and also pp24 (corresponding to p24 plus p2 proteins) were detected.

Immunoblot analysis using antibodies directed against RTp66/p51 indicated that all mutant viruses except P31L contained RT protein (Fig. 3C, compare lanes 3 and 6). The absence of RT protein in P31L virions (lane 3) confirms our inability to detect RT activity associated with the P31L mutant virions. In addition, a low level of RT protein was detected in the R32G mutant virions (compare lanes 4 and 6; note that the upper band seen in the negative control and all other lanes corresponds to serum albumin).

Genomic RNA content of the HIV-1 NC mutant virions. It has been shown by several groups that the NC protein promotes the specific packaging of the viral RNA genome (2, 24). More precisely, the NC zinc fingers and the aromatic amino acids present in the zinc fingers appear to be of importance for the packaging process (18). To examine the influence of the basic sequence linking the two zinc fingers of the HIV-1 NC protein on genomic RNA packaging, virion RNAs from wild-type and mutant viruses were extracted and analyzed by a Northern (RNA) blot procedure. The RNAs were analyzed under denaturing conditions and probed with a ^{32}P-labeled DNA fragment corresponding to the *gag* and *pol* sequences in order to detect the presence of unspliced genomic RNA (see Materials and Methods) (Fig. 4).

As shown in Fig. 4, all mutant virions contained genomic RNA. However, A30P, R32G, and S3 mutant viruses contained about 5 to 10 times less genomic RNA than the wild-type virus. In addition, the genomic RNA present in the R32G and A30P mutant virions was found to be more degraded than that of the wild-type virus (compare lanes 2, 3, and 6). Quantitative dot blot analysis revealed no significant difference in RNA contents between the P31L mutant virus and the wild-type virus (data not shown).

Infectivity of the HIV-1 NC mutants. To measure the viral infectivity of the NC mutants, human SupT1 cells were infected with serial dilutions of cell-free medium harvested from cell culture following DNA transfection (see Materials and

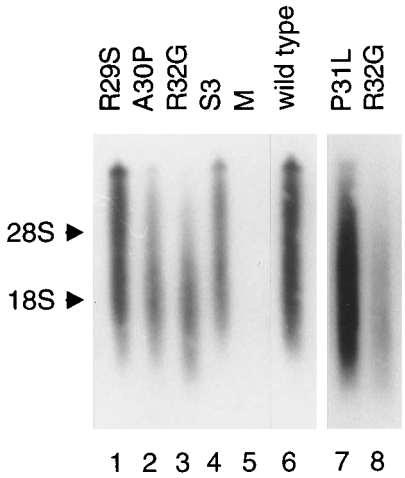

FIG. 4. RNA blot analysis of NCp7 mutant and wild-type virions. Viruses from transfected cells were pelleted by ultracentrifugation, and the RNAs were purified. Lanes: 1, R29S; 2, A30P; 3 and 8, R32G; 4, S3; 5, pellet from mock-transfected Cos7 cells (negative control); 6, wild type (positive control); 7, P31L. 28S and 18S rRNA markers are indicated.

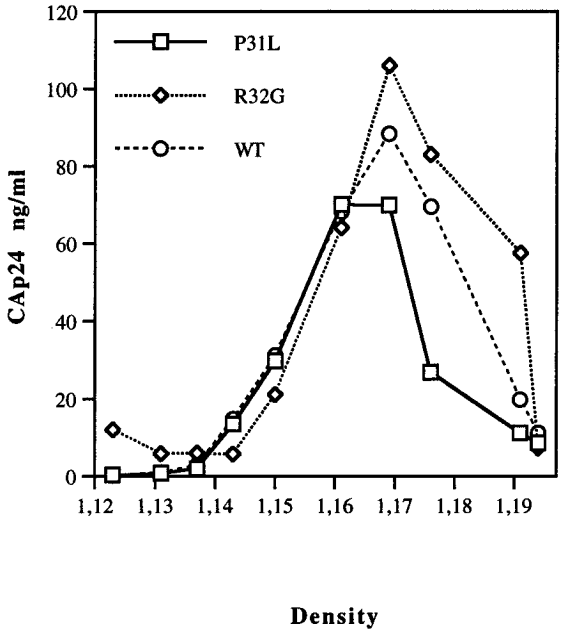

FIG. 5. Sedimentation profiles of wild-type (WT) and noninfectious NCp7 mutant viruses P31L and R32G. Sucrose gradients were made as described in Materials and Methods. Each fraction was assayed for CAp24 antigen by ELISA.

Methods). By the end point dilution methodology, the virus titer was defined as the lowest dilution of fresh harvested medium able to promote the production of virions as measured by the release of RT activity in the medium of SupT1 cells and by CPE and SupT1 syncytium formation (see Materials and Methods).

The results are presented in Table 1 and are mean values of five independent experiments, all of which gave similar results. The P31L and R32G mutant viruses were completely noninfectious, since neither CPE nor RT activity was observed 4 weeks after the addition of supernatant to SupT1 cells. Moreover, no expression of β-galactosidase was detected after the addition of supernatant to P4 cells (see Materials and Methods). Mutant A30P was very poorly infectious (approximately 1 IU/ml or 10^6 to 10^7 less infectious than the wild-type virus), since infection of SupT1 cells was observed only with the undiluted supernatant of transfected Cos7 cells. For this mutant, RT activity appeared 3 weeks after the addition of undiluted supernatant to SupT1 cells (compared with 3 to 6 days for the wild type). To investigate the stability of the phenotype of the A30P mutant, the supernatant of SupT1 cells was harvested 4 weeks after infection. This supernatant was used to infect fresh SupT1 cells under the same conditions and with the same amount of viruses (i.e., the same amount of CAp24) as those used for the first round of infection. The appearances of RT activity and CPE were still highly delayed (3 weeks), showing that the delayed infection of the first SupT1 culture was not due to a reversion.

The S3 mutant had a titer of 10^4 to 10^5 IU/ml, i.e., it was 100 times less infectious than the wild-type virus, while the R29S mutant behaved like the wild-type virus (Table 1).

TABLE 1. Virus titers of HIV-1 NCp7 mutant and wild-type viruses[a]

Virus	Titer (no. of infectious particles/ml)
Wild type	10^7
R29S	10^7
S3	10^5
A30P	1
R32G	0
P31L	0

[a] SupT1 cells were cultured with serial 1:10 dilutions of supernatant from transfected Cos7 cells. Virus titer was defined as the last infecting dilution leading to syncytium formation and culture death.

Density of the noninfectious mutant particles. A linear sucrose gradient was used to determine the densities of both P31L and R32G noninfectious mutant particles. Fractions collected were assayed for CAp24 protein by ELISA and by RT activity assaying. Fractions obtained from HIV-1 wild-type particles were assayed by both methods, and the major peak of CAp24 or RT activity was found in the fraction with a density of 1.17 mg/ml, corresponding to the normal density (38). The densities of both P31L and R32G mutant particles were found to be in the same fraction as the HIV-1 wild-type ones (1.17 mg/ml), as shown in Fig. 5. Similar results were obtained by the RT activity assay of the R32G and the wild-type fractions (data not shown).

Analysis of virus morphology. The morphologies of the wild-type and the mutant P31L NC HIV-1 particles were examined by electron microscopy. Wild-type virions presented a typical morphology, with an electron-dense core surrounded by the membrane (Fig. 6A). A section of the cells transfected with the S3 NC mutant revealed a typical budding particle (Fig. 6B). The P31L virions produced from transfected Cos7 cells always presented an immature morphology, with a broad electron-dense region underlying the viral envelope, while the central region remained electron lucent (Fig. 6C). These data are consistent with the results obtained by immunoblot analysis and support the idea that the P31L mutant has a maturation defect.

Virus stability. The stability of the virions was assessed by incubating the cell-free supernatant at 37°C for 24 h before infection. This approach was used to examine virus stability, i.e., the ability of the mutated NCp7 molecules to protect the RNA genome within the virions against the RNase activities contained in culture fluids. The infectivity of the wild-type virus

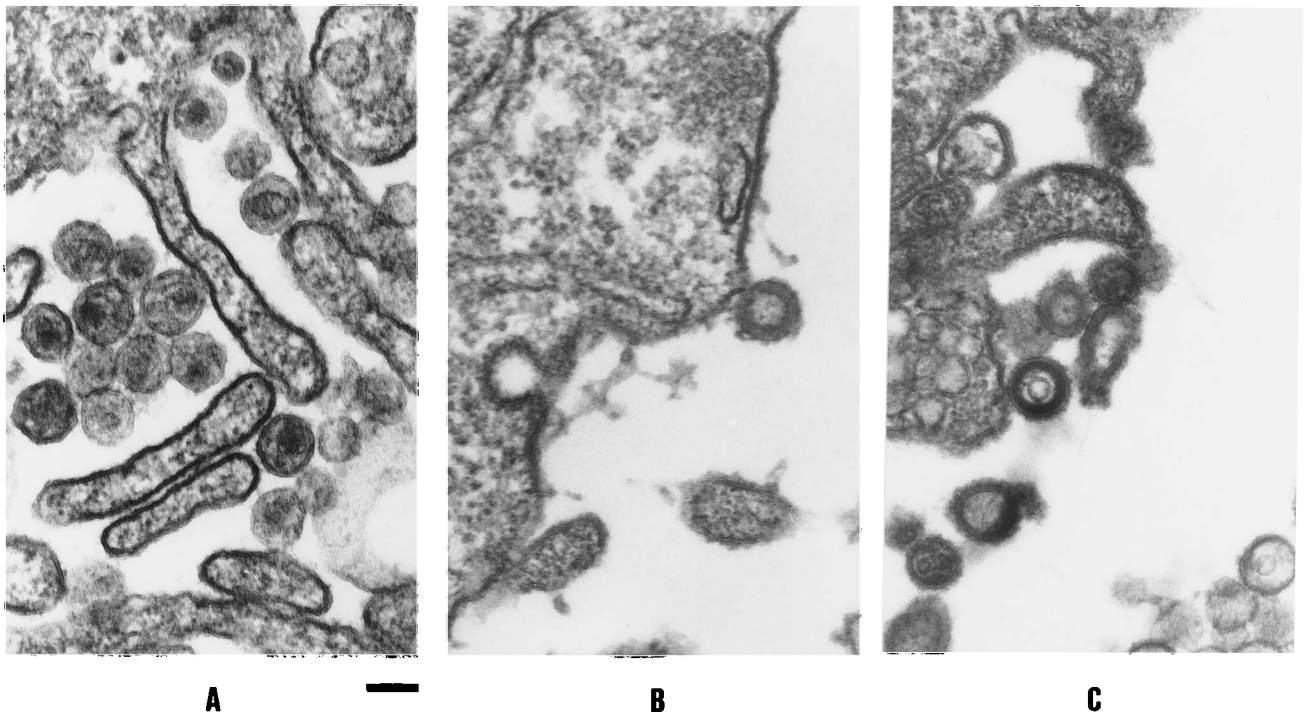

FIG. 6. Electron microscopic analysis of virions produced by wild-type and NC mutant proviruses. Representative virions produced after transfection with wild-type (A) and NCp7 P31L mutant (C) provirus are shown. A budding virion produced after transfection with NCp7 S3 mutant provirus (B) is also shown. Bar, 100 nm.

when the supernatant of transfected cells was incubated for 24 h at 37°C decreased by 100-fold compared with those in which the supernatant was not preincubated for 24 h at 37°C prior to infection. The decreases in infectivity for the R29S and S3 mutant viruses were 1,000 and 100,000, respectively, corresponding to 10^4 and 1 IU/ml. Therefore, the HIV-1 R29S and S3 mutants are less stable at 37°C than the wild-type virus.

DISCUSSION

The retroviral NC protein has both structural and functional roles, since it is an integral part of the virion NC structure and has nucleic acid binding and annealing activities in vitro that promote the annealing of the primer tRNA to the PBS and genomic RNA dimerization (14, 40). Moreover, the hybridase activity of the NC protein appears to direct DNA strand transfers that take place during reverse transcription (3, 15).

Genetic analyses of the NC protein in the viral context indicate that deletions and amino acid substitutions in the NC coding sequence result in the production of poorly infectious or noninfectious viruses (4, 21, 26–28, 34). More precisely, amino acid substitutions in the zinc fingers of NCp7 have shown that the zinc fingers are required for genomic RNA packaging and thus for the production of infectious viruses (18, 24). In addition, substitution of neutral residues for the basic amino acids flanking the zinc finger of Moloney murine leukemia virus NCp10 was shown to cause a strong reduction in virus infectivity, most probably because of a defect in RNA packaging and annealing of the primer tRNA to the PBS (28).

Recently (35), we reported preliminary data on the modification of the NCp7 structure achieved by means of the replacement of L-Pro-31 by D-Pro or L-Leu. NCp7 was found to be poorly active in vitro, and virus infectivity was found to be strongly impaired. Here, we describe the extensive characterization of the P31L mutant virus as well as the effects of other mutations in the linker region that were expected to alter the three-dimensional structure of NCp7 as determined by molecular modeling (mutants A30P and R32G). In addition, the effects of mutations changing the basic nature of NCp7 (R29S and $RKK^{34}\rightarrow SSS$-S3 mutants) (Fig. 1) were also examined.

The results show that replacement of one basic residue by a neutral one, the R29S mutant, did not modify virus production or infectivity. Replacement of three basic residues by neutral ones, the S3 mutant, had no effect on virus production but resulted in a 100-fold decrease in virus infectivity (Table 1). Interestingly enough, the S3 mutant was found to be unstable, since the virus was very poorly infectious after 24 h at 37°C (1 IU/ml), in contrast to the wild-type virus (10^5 IU/ml). It is possible that the S3 mutation renders the virion NC structure unstable, probably by modifying the interactions between the genomic RNA and the NCp7 molecules. This is presently being investigated in vivo and in vitro.

The results show that mutations that are thought to alter the NCp7 structure (A30P, P31L and R32G) led to the formation of very poorly infectious viruses (A30P) or completely noninfectious viruses (P31L and R32G). It is interesting to note that the R32G virus was unable to infect SupT1 cells while the S3 mutant virus was still infectious as the $^{33}KK^{34}\rightarrow SS$ mutant (36a). These findings suggest that structural modifications impair the functions of NCp7 more drastically than replacement of one or more basic residues by neutral ones. The very low level of intact genomic RNA in R32G virions indicates that this mutation probably affects both the stability of the NC structure and the efficiency of genomic RNA packaging (4, 24, 26, 34).

Surprisingly enough, the P31L mutation appears to partially modify the assembly process, since the virions were found to be immature by electron microscopy and to lack RT (Fig. 3C). Therefore, the P31L mutation probably impairs the assembly process during which Gag–Gag-Pol interactions are essential

for the formation of infectious mature virions. As already reported, the $Pr55^{gag}$ precursor alone is able to promote the formation of retrovirus-like particles (22, 29, 41, 46) and also to direct the coassembly of the unmyristylated Gag-Pol polyprotein (46). The incomplete maturation of $Pr55^{gag}$ in the P31L virions probably results from a low level of protease activity due to the few encapsidated Gag-Pol precursor molecules (50). The absence of the RT protein in NCp7 P31L virions could be explained by the inability of the P31L mutated Gag precursor to stably interact with a Gag-Pol precursor and, therefore, to direct the coassembly of Gag and Gag-Pol precursors or, alternatively, by the instability of the Gag-Pol precursors. In addition, the R32G mutation resulted in a low level of RT that was present in the mutant virions (Fig. 3C), suggesting that this mutation can partially impair the interactions between the Gag and Gag-Pol precursors. Since most of the Gag precursors were found to be processed in the R32G viruses, this suggests that only a low level of protease is sufficient to achieve Gag processing in the course of capsid formation (Fig. 2). It is also possible that partially processed Gag precursor molecules and/or mature Gag proteins participate in the assembly process taking place under the cellular membrane (Fig. 2) (27). In agreement with this, it is interesting to note that about 50% of Gag proteins were processed in the P31L viruses (Fig. 3) that appear to be immature by electron microscopy (Fig. 6).

These results favor the idea that the NCp7 domain of the Gag precursor is probably involved in Gag and Gag-Pol precursor interactions during virion formation. Similarly, deletion of amino acids 112 to 500 of RT leads to an impairment of virus maturation (36a) that closely resembles the effect of the P31L mutation. As clearly indicated by several studies, different domains of Gag and Gag-Pol precursors must have tight contacts in order to mediate Gag and Gag-Pol precursor maturation and capsid formation. Morphological defects leading to immature viral particles resembling a protease-defective mutant virus (30, 37) were also observed with *gag* (27) and integrase mutants (45) as well as with *pol*-defective mutants (our unpublished data).

The results present here indicate that an additional region of Gag, the NC domain, is also important for the correct assembly of HIV-1 viral particles. More precisely, the data on P31L and R32G mutants favor the idea that the overall structure of NC is critical for virion structure and infectivity. In agreement with this idea, a slight modification of the zinc finger structure achieved by the His→Cys mutation at position 23 in the first zinc finger (16) and the His→Cys mutation at position 44 in the second zinc finger (36a) resulted in the production of noninfectious viral particles.

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