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Human Immunodeficiency Virus Nucleocapsid Protein Polymorphisms Modulate the Infectivity of RNA Packaging Mutants

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The nucleocapsid protein (NC) of retroviruses is involved in viral RNA packaging and initiation of reverse transcription. NC also mediates interactions between Gag and actin filaments. We found that residues at the amino terminus of NC are involved in efficient actin binding. When alanine residues were substituted for the arginine and lysine at positions 10 and 11 of NC in HIV_{NL4-3}, these mutations decreased actin binding but had only a modest effect on virus infectivity. A similarly mutated virus based on the HXB2 clone of HIV was not infectious. Mutational analysis of NL4-3 NC residues demonstrated that NC polymorphisms modulated the phenotype of NC mutations. Conservative amino acid differences between HXB2 and NL4-3 NCs were sufficient to explain the difference in infectivity of viruses carrying the R10A and K11A mutations.

Key Words: nucleocapsid; actin; infectivity; packaging; human immunodeficiency virus.

INTRODUCTION

All of the structural proteins of the core of the human immunodeficiency virus (HIV) and other lentiviruses are derived from a single polyprotein, Gag. Assembly of HIV particles begins with aggregation of Gag polyprotein molecules at the plasma membrane. Activation of the viral protease occurs in concert with release of immature viral particles, resulting in the cleavage of Gag molecules into the matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins, as well as two small spacer peptides (p2 and p1) initially interposed between CA and NC, and NC and p6, respectively. Cleavage of Gag into these subunits leads to the formation of infectious, mature virions with a cylindrical core (Wills and Craven, 1991).

The subunits of Gag are themselves multifunctional. The NC proteins of all lentiviruses include two zinc-binding Cys–His boxes of the form $CysX_2CysX_4HisX_4Cys$, flanked by a series of basic residues (Darlix *et al.,* 1995). Alteration of these zinc-binding "knuckles" can cause loss of packaging of genomic RNA and increased packaging of spliced viral RNA and cellular mRNAs (Aldovini and Young, 1990; Gorelick *et al.,* 1999), while alterations of the basic residues seem to affect the general ability to bind RNA (Poon *et al.,* 1996; Schmalzbauer *et al.,* 1996). Among other properties, the NC protein also promotes the proper positioning of the tRNA primer for minus-strand DNA synthesis and template switching during reverse transcription (Guo *et al.,* 2000). It may also contribute to events in the integration of viral DNA (Carteau *et al.,* 1997, 1999). In addition, we and others have recently determined that residues in NC allow Gag to bind to filamentous actin (Rey *et al.,* 1996; Liu *et al.,* 1999; Wilk *et al.,* 1999). Although the biological significance of interactions between Gag (or NC) and cytoskeletal filaments remains unclear, it is conceivable that these interactions play a role in virus assembly.

During studies of the role of the cytoskeleton in HIV replication, we determined that interactions between NC and F-actin involve the basic residues that precede the amino-terminal Cys–His box of NC. We were surprised to find that changing two basic residues in this domain to alanines had little effect on the infectious titer of the NL4-3 strain of HIV, as this mutation had been previously described to interfere with the packaging of genomic viral RNA and to abrogate the infectivity of HIV reporter viruses based on the HXB2 strain (Poon *et al.,* 1996; Cimarelli *et al.,* 2000). In this report, we demonstrate that polymorphisms seen in the NC domain can have a profound impact on the infectivity of HIV-1 packaging mutants.

RESULTS

In vitro actin-binding properties of Gag proteins with mutations in NC

Previous studies demonstrated that HIV-1 Gag protein binds to actin filaments *in vitro* and *in vivo* (Ibarrondo *et*

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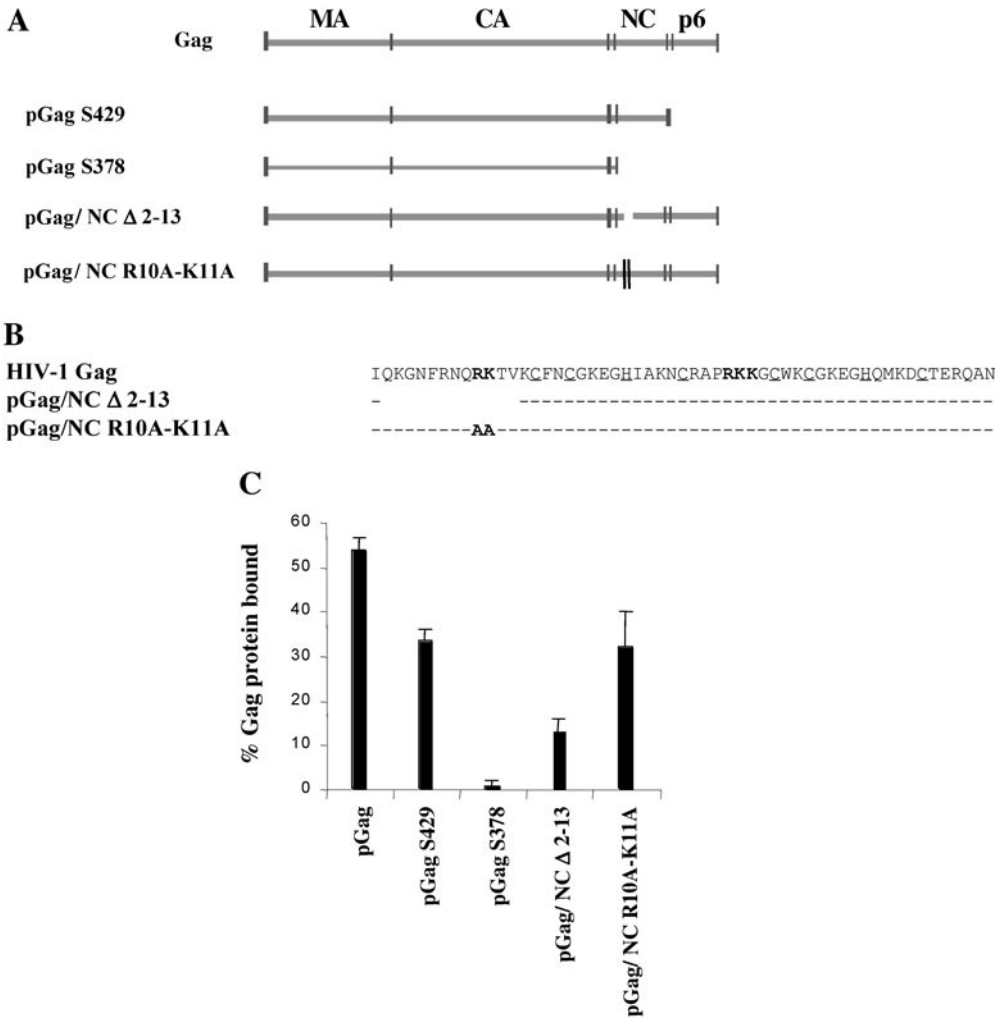

FIG. 1. F-actin binding of wild-type and mutant Gag proteins. (A) pGag S378 and pGag429 have stop codons placed after the last codon of the p2 and NC codons, respectively, to encode truncated Gag proteins. The first 12 codons of NC that precede the first zinc-binding Cys–His motif were deleted in pGag/NC Δ2-13. pGag/NC R10A-K11A encodes Gag with alanines substituted for arginine–lysine residues. (B) NC point mutations and deletions. The positions of the alanine substitution encoded in pGag/NC R10A-K11A and of the deleted amino acids in pGag/NC Δ2-13 are shown. (C) *In vitro* translated wild-type and mutant Gag proteins were assayed for binding to F-actin. Values represent the fraction of total Gag (pellet/supernatant + pellet) that icosedimented with F-actin during ultracentrifugation of binding mixtures. Gag proteins were produced in reticulocyte lysates programmed with the plasmid indicated. Background (fraction of Gag that pelleted in binding mixtures to which F-actin was not added) is subtracted. Results are the average of a minimum of three experiments.

al., 2001; Rey *et al.,* 1996; Liu *et al.,* 1999; Wilk *et al.,* 1999). Truncated Gag proteins lacking p1 and p6 retained substantial actin binding (Ibarrondo *et al.,* 2001; Liu *et al.,* 1999; Wilk *et al.,* 1999). In contrast, F-actin binding was lost when Gag was further truncated to remove NC (Fig. 1). Actin-binding proteins are often rich in cationic amino acids (Vandekerckhove and Vancompernolle, 1992). In an effort to identify residues in NC that mediate Gag–actin interactions, we therefore deleted 12 amino acids of NC that precede the first Cys–His motif (NC Δ2-13); this decreased the binding of Gag to F-actin approximately twofold, from an average of 53 to 32%. Binding was decreased to a comparable extent by substituting alanine residues for the adjacent arginine and lysine residues found at NC positions 10 and 11 (R10A-K11A). Thus, residues involved in actin binding were identified in the first half of NC.

We analyzed the effect of the R10A-K11A mutations on infectivity. These mutations were introduced into the infectious molecular proviral clone pNL4-3 (Adachi *et al.,* 1986). ELISA measurements of HIV-1 capsid antigen cells revealed no significant difference in the amount of virus released from cells transfected with the pNL4-3 and pNL4-3/R10A-K11A plasmids (1120 ± 277 ng/ml versus 1222 ± 362 ng/ml; not statistically significant by paired *T* test analysis of data from 12 paired transfections).

The viral protein composition of these stocks was analyzed by pelleting virions through 15% sucrose. West-

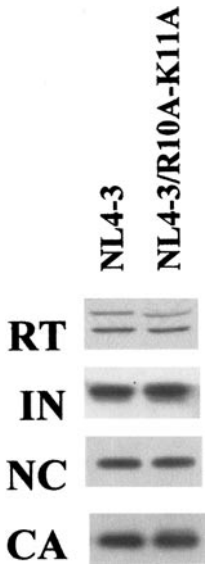

FIG. 2. Immunoblot analysis of proteins in virions. Wild-type NL4-3 and mutant (R10A-K11A) viruses released from transfected 293T cells were purified by pelleting through a cushion of 15% sucrose and disrupted in protein loading gel loading buffer. Reverse transcriptase (RT), integrase (IN), capsid (CA), and nucleocapsid (NC) proteins were detected by immunoblot. The results are representative of the analysis of virus from three separate transfections.

ern blot analysis of the pelleted virus showed that the R10A-K11A mutations did not alter the virion content of the 66- and 51-kDa reverse transcriptase proteins, the 31-kDa integrase protein, or NC relative to the HIV capsid protein (CA) (Fig. 2). The infectious titer was assessed using Magi cells (Kimpton and Emerman, 1992); the R10A-K11A mutations decreased the viral titer approximately twofold (Fig. 3).

We concluded that the reduction in actin binding due to the R10A-K11A mutations did not impair the assembly or release of virus from cells and was associated with only a small decrease in virus infectivity.

The effect of NC polymorphisms on the phenotype of the R10A-K11A packaging mutant

The infectivity results observed with pNL4-3/R10A-K11A were in striking contrast to data from earlier reports in which the R10A-K11A mutations were introduced into a reporter virus (p10-11) based on the HXB2 strain of HIV-1 (Poon *et al.,* 1996). In the background of HXB2, these mutations not only diminished the encapsidation of genomic RNA by two thirds, but also decreased virus infectivity by more than 100-fold. Moreover, transfer of a large fragment that contained the entire *gag* gene of p10-11 into pNL4-3 also abrogated virus infectivity (Cimarelli *et al.,* 2000). We hypothesized that other amino acid differences that exist between HXB2 and NL4-3 were amplifying the effects of the R10A-K11A mutations.

As an initial step, we analyzed the infectivity of the mutant pNL4-3/HX10-11. This chimeric plasmid was constructed to test the effect of the R10A-K11A mutations on infectivity of HIV when they are presented in the background of the different NC amino acid sequence encoded by the pHXB2gpt (Fisher *et al.,* 1985). Virus stocks produced with pNL4-3/HX 10-11 were 20-fold lower in titer than NL4-3 and about 8-fold lower than pNL4-3/R10A-K11A (Fig. 3B). This reduction in virus titer correlated with the relative virion content of viral RNA (Fig. 4, lanes 1–3). Inspection of the amino acid sequences of HXB2 and NL4-3 in the region encoded by the fragment transferred into pNL4-3 revealed eight amino acid differences (Fig. 3A). Two conservative changes, an asparagine to histidine change and an alanine to glycine change, exist in the capsid domain (not shown). In the p2 spacer peptide of HXB2, a serine is found in place of a proline found in NL4-3. The two strains differed at five additional amino acid positions in NC. To determine the influence of these p2 and NC polymorphisms on the phenotype of the R10A-K11A mutants, the HXB2 amino acids were introduced into the NL4-3 backbone. The serine to proline mutation in the p2 spacer region and the NC I1M and K3R mutations were introduced together (NLp2SMR) and slightly reduced virus infectivity but not genomic RNA packaging (Figs. 3 and 4). The infectivity of NL4-3/R10A-K11A was not further decreased with the addition of the SMR mutations (compare NL4-3/R10A-K11A and SMR/R10A-K11A in Fig. 3).

Three additional mutants were made in which single amino acid changes were added to the SMR/R10A-K11A background, to reflect differences between HXB2 and NL4-3 at NC positions 11, 24, and 26. Addition of the K26R mutation did not appreciably lower the infectivity of the SMR/R10A-K11A mutant of NL4-3. In contrast, adding the T12I or I24T mutations caused slight, but reproducible further reductions in the infectivity. Although it is difficult to speculate on the structural changes resulting from these mutations alone or in combination with each other, we examined the effects of adding the I24T and K26R mutations together, since these are contained in the first zinc knuckle of NC. The infectivity of the SMR/R10A-K11A-I24T-K26R mutant virus was only 5% of that of NL4-3 and was comparable to that of NL4-3/HX 10-11. There was a correlation between viral titer and RNA encapsidation, as the SMR/R10A-K11A-I24T-K26R mutant had the lowest level of RNA encapsidation of any of the NL4-3 mutants (comparable to NL4-3/HX10-11).

In summary, stepwise mutagenesis of the NC domain of NL4-3 restored the environment of HXB2 in which the R10A-K11A mutations have very deleterious effects on virus infectivity. In the context of the SMR/R10A-K11A mutations, the presence of a threonine at position 24 had the greatest impact of the single mutants studied on virus phenotype. Addition of the K26R mutation further impaired virus infectivity.

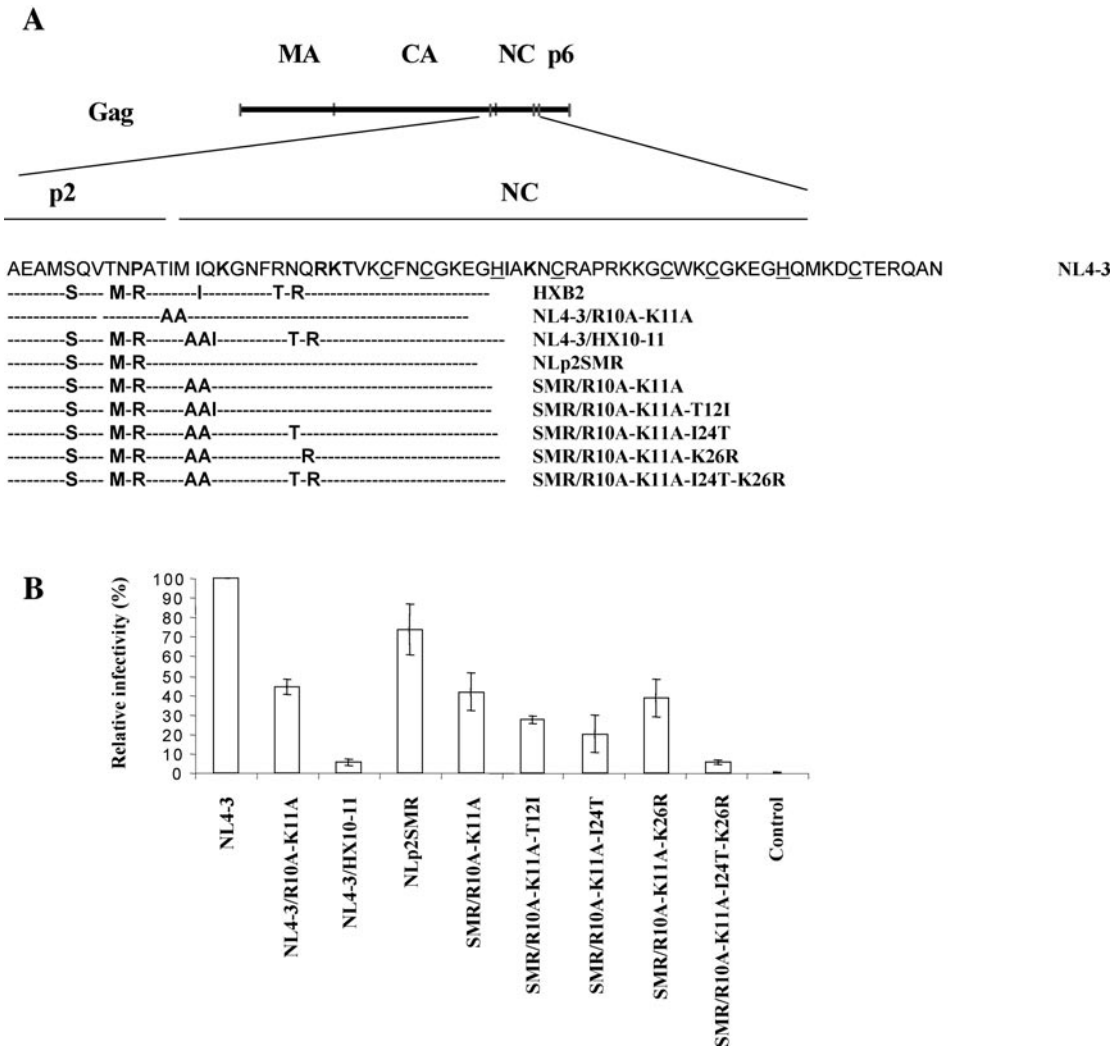

FIG. 3. Conservative changes in NC amino acid sequence affect the infectivity of NL4-3. (A) Amino acid sequence of NL4-3, HXB2, and the mutant viruses. (B) Virus titration in Magi cells. Each bar represents the average viral titer of virus stocks produced in at least three transfections. All data are normalized relative to the stock of parental HIV_{NL4-3} produced in the same batch of transfections.

DISCUSSION

In previous studies, alanines were substituted systematically for basic amino acids in the NC domain of the HXB2 clone of HIV, demonstrating that these cationic residues play an important role in the viral RNA-binding activity of NC (Poon *et al.,* 1996). Mutation of the two adjacent residues R10 and K11 of NC attenuated the infectivity of HIV to a degree comparable to the reduction of viral RNA packaging: the packaging of viral RNA was reduced by threefold but infectivity was decreased significantly more. In contemporaneous studies, these mutations were introduced in NC during studies of Gag–actin interactions. Surprisingly, the R10A-K11A mutations had much less effect on the infectivity of the NL4-3 clone of HIV, apparently due to conservative amino acid differences between it and HXB2. Simultaneously substituting serine, methionine, and arginine residues for proline, isoleucine, and lysine in p2 and the amino terminus of NC might have been expected to significantly interfere with RNA packaging and the assembly of infectious virus. Instead, these combined mutations only marginally affected infectivity. Although deletion of the p2 domain, where the proline to serine change occurred, has a profound effect on morphogenesis (Krausslich *et al.,* 1995), p2 may be unstructured and truly function as a spacer between important structural motifs found in NC and CA. While we did not specifically examine the impact of the individual I1M and K3R changes, they appeared to have little influence on the infectivity of virus containing the R10A-K11A mutations.

In contrast, the I24T mutation had a significant impact on virus infectivity, especially when combined with the K26R change. It is difficult to predict why this might be the case. Both are present in the first zinc knuckle of NC and could be involved with interactions with nucleic acids during virus assembly or reverse transcription.

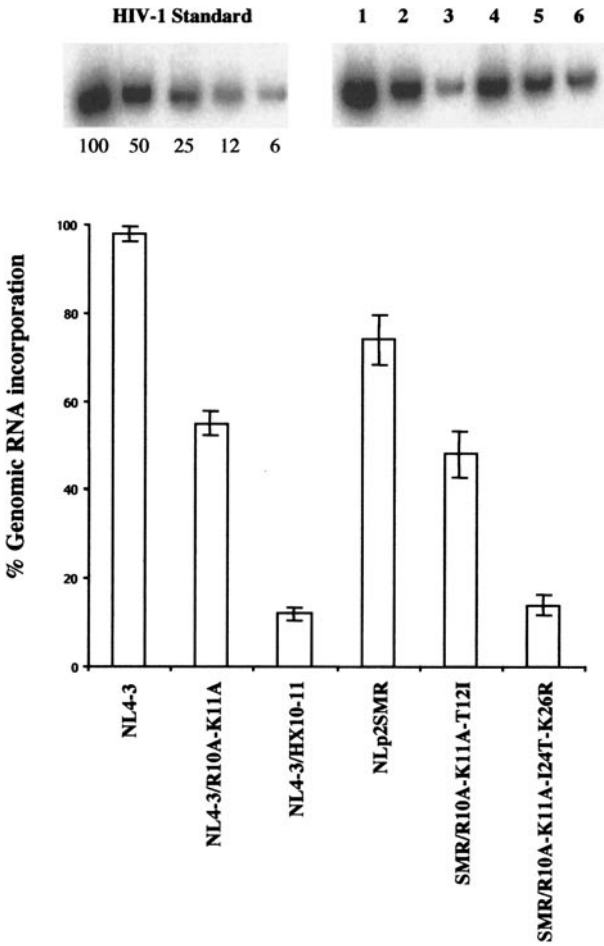

FIG. 4. Relative genomic RNA content of virions determined by RT–PCR. RNA samples from pelleted virions containing equal amounts of viral p24 antigen were reverse transcribed and subjected to PCR amplification in presence of [^{32}P]dCTP. The intensity of a band corresponding to the amplification of genomic RNA from mutated viruses was compared to the intensity of bands produced by amplifying serially diluted RNA from the wild-type parental virus NL4-3 (standards) and is reported as percentage of the wild-type virus NL4-3. The top panel shows the results of one representative experiment with a subset of the mutant viruses. In the top panel, lanes are 1, NL4-3; 2, NL4-3/R10A-K11A; 3, NL4-3/HX 10-11; 4, NLp2SMR; 5, SMR/R10A-K11A-T12I; 6, SMR/R10A-K11A-I24T-K26R. The histogram reports the mean and standard error obtained from three independent experiments, each carried out in triplicate.

However, the degree of genomic RNA packaging was not a perfect predictor of virus infectivity in studies of NC mutants (Poon *et al.,* 1996) (Gorelick *et al.,* 1999). Presumably, the loss of infectivity seen with some NC mutants that effectively package viral RNA derives from the loss of one or more other NC functions. We have not examined the actin-binding properties of Gag proteins containing the NC polymorphisms from HXB2, but it is conceivable that actin binding is an additional significant function of NC that is impaired by some of these changes.

During preparation of this paper, Cimarelli and Luban presented the results of similar studies in which they examined the effects of the R10A-K11A mutations of NC in the context of the HXB2 polymorphisms (Cimarelli and Luban, 2001). They quantified genomic RNA encapsidation by slot blot analysis and found only loose association between viral RNA content and infectivity of the R10-K11A mutant. However, they too found that the I24T mutation further diminished the infectivity of the R10A-K11A mutant of NL4-3. They also showed that this mutation diminished RNA encapsidation of NL4-3/R10A-K11A, while having little effect on encapsidation on its own. In contrast, our data reveal the additional impact of polymorphisms at other positions in NC (1, 3, 12, 26) on the single cycle infectivity of the R10-K11A mutant.

Separate structural models have been developed for NC bound to synthetic RNA molecules that correspond to two stem loop structures found in HIV-1 genomic RNA (SL2 and SL3) (De Guzman *et al.,* 1998; Amarasinghe *et al.,* 2000). In both models, NC amino acids 3 to 11 form a 3_{10} helix that packs against the N terminal zinc knuckle. However, the intraprotein interactions differ in the two models. Among these differences, in the NC-SL2 structure, the aromatic side chain of the proline at position 6 is buried between the side chains of V13 and I24, while in the NC-SL3 structure it is largely exposed and packs against I24. Moreover, the orientation of the two zinc knuckles of NC relative to one another is different in the two structures. These models demonstrate the flexibility of NC and its adaptive nature. These properties may have clinical significance if nucleocapsid targeting drugs are successfully developed for the treatment of HIV infection (De Clercq, 2000).

METHODS

Construction of plasmids

Site-directed mutagenesis was used to mutate the nucleotide sequence of pGag (previously called pGem3Z-Gag) (Rey *et al.,* 1996), which contains the complete Gag open reading frame of HIV-1_{NL4-3} (Adachi *et al.,* 1986) using previously published procedures (Krogstad and Champoux, 1990). Using the oligonucleotide 5′ GCT ACC ATA ATG ATA TGT TTC AAT TGT GGC 3′, the codons for the 12 amino acids of NC that precede the first Cys–His box were deleted to produce the vector pGag/NCΔ2-13. Similarly, missense mutations were introduced to produce vectors encoding Gag proteins in which alanine residues substitute for positively charged amino acids found preceding the first zinc finger of NC (R10 and K11) (pGag/NC R10A-K11A). Site-directed mutagenesis was also used to introduce stop codons in the NC and p1 coding domains, to produce plasmids (pGag S378 and pGag S429) that encode truncated Gag proteins (Ibarrondo *et al.,* 2001) (Fig. 1). The nucleotide sequence of the mutations was verified by dideoxynucleotide sequencing. RNA was transcribed from these vectors and

used to produce radiolabeled, truncated Gag proteins in reticulocyte lysates following the manufacturer's instructions (Promega, Madison, WI). pGag/NC R10A-K11A was digested with *Spe*I and *Apa*I restriction endonucleases to release a 503-bp fragment, which was ligated into pNL4-3 (Adachi *et al.,* 1986) to produce a proviral vector with the NC R10A-K11A mutations (pNL4-3/R10A-K11A). Other mutations were subsequently made using primer overlap PCR mutagenesis (Ausubel *et al.,* 1987). Using pNL4-3 as the template, 1396s (5′ GAG ACC ATC AAT GAG GAA G 3′) and 2081A (5′ GCC TGT CTC TCA GTA CAA TC3′) as outside primers, and overlapping, internal mutagenic primers, the proline residue in the p2 domain and the isoleucine and lysine residues at NC positions 1 and 3 were changed to serine, methionine, and arginine. The DNA product of this amplification was digested with *Spe*I and *Apa*I, and the gel-purified 503-bp fragment obtained from this digestion was ligated into pNL4-3. This construct, pNLp2SMR, was used as the template for subsequent rounds of mutagenesis in which additional missense mutations were introduced in the NC coding domain, as discussed in the text. The following mutants, whose individual mutations are described in Fig. 3A, were generated: SMR/R10A-K11A, SMR/R10A-K11A-T12I, SMR/R10A-K11A-I24T, SMR/R10A-K11A-K26R, and SMR/R10A-K11A-I24T-R26K. The sequences of the mutagenic oligonucleotides will be made available on request. Also, a *Spe*I–*Apa*I fragment from p10-11 (Poon *et al.,* 1996) was ligated into pNL4-3 to produce the plasmid pNL4/HX 10-11. The nucleotide sequences of the *Spe*I–*Apa*I fragments were confirmed after cloning them into the NL4-3 proviral DNA, prior to transfection of cells to produce virus stocks.

Actin cosedimentation assays

Binding assays to examine interactions between recombinant Gag protein and radiolabeled *in vitro* translated Gag protein and F-actin were performed as previously described (Rey *et al.,* 1996) using 95 or 99% pure rabbit skeletal muscle actin purchased from Cytoskeleton Inc. (Denver, CO). Briefly, radiolabeled protein from reticulocyte lysates were incubated with *in vitro* polymerized actin in 150 μl of binding buffer at 0°C for 1 h and then subjected to ultracentifugation (50,000 *g* for 60 min in a Heraeus Biofuge (Hanau, Germany)). The supernatant was removed, and one-fifth volume of 5× SDS–PAGE sample buffer was added. The pellets were disrupted in 30 μl of 5× SDS–PAGE sample buffer, to which binding buffer (120 μl) was subsequently added. Equal volumes of these supernatant and pellet fractions were separated by SDS–PAGE and quantified using a Phosphoimager SI and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Virus preparation and infectivity assays

Virus stocks were produced by calcium phosphate transfection of 293T cells, which were maintained in Dulbecco's modified Eagle's medium. The infectious titer was determined using cells that express β-galactosidase under the control of the HIV-LTR (Kimpton and Emerman, 1992). Triplicate wells were infected with virus stock containing 10 to 100 ng of HIV p24 antigen content. The number of blue foci in three fields was counted in each of three wells.

Viral protein analysis

Filtered supernatants of transfected 293T cells were placed over 15% sucrose cushions (100 mM NaCl, 10 mM Tris, pH 8.0) and centrifuged for 2 h at 150,000 *g*. Pelleted virions were resuspended in PBS, and p24 viral capsid antigen was measured by ELISA. Lysates containing equal amounts of p24 were separated in SDS–PAGE gels and transferred to membranes. The membrane was separated into four fragments for immunoblot analysis. Pooled patient antiserum was used to detect the 24-kDa CA protein. Integrase was detected using rabbit antiserum kindly provided by Dr. Duane Grandgenett. Reverse transciptase proteins were detected using a monoclonal antibody (Catalog No. 716) obtained from the AIDS Research and Reference Reagent Program [Division of AIDS, NIAID, NIH (supplied by Dr. Paul Yoshihara)]. NC was detected using antiserum generously supplied by Dr. Stuart LeGrice.

Particle-associated RNA analysis

Supernatants from transfected cells containing 50 ng of viral p24 antigen were centrifuged to pellet the virions, pellet-associated p24 was reevaluated, and viral RNA was extracted from equal viral amounts. Quantitative RT–PCR was performed on RNA samples according to a previously described procedure (Poon *et al.,* 1996). Briefly, the viral pellet was resuspended in 0.5 ml of TRIZOL (GIBCO BRL) containing 100 μg/ml of yeast tRNA as a carrier to monitor final RNA recovery. The RNA was extracted with phenol–chloroform and precipitated with ethanol. In order to eliminate contaminating transfection or cellular DNA, the RNA was then treated with 20 units of RQ1 DNase I (Promega) in the buffer recommended by the manufacturer (40 mM Tris, pH 8, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$) in the presence of 40 units of recombinant RNasin Ribonuclease Inhibitor (Promega) for 1 h at 37°C. The DNase I was denatured with stop solution provided by the manufacturer (20 mM EGTA, pH 8.0) and the RNA was precipitated with ethanol. DNase-I-treated viral RNAs were resuspended in diethyl pyrocarbonate-treated water and the yeast tRNA concentration was adjusted to 1 mg/ml. RNA samples were obtained from three independent transfections of each

construct. RNA samples corresponding to 6 ng of p24 were reverse transcribed in a 20-μl reaction with Superscript II Reverse Transcriptase (GIBCO/BRL) using a *gag*-specific primer (HIVc1159: 5′-GTCCTGTGTCAGCTGCTGCTTG-3′). Two microliters of this reaction was subjected to PCR with AmpliTaq DNA Polymerase (Perkin–Elmer), in the presence of [^{32}P]dCTP, employing the same primer used in the RT reaction paired with an upstream *gag*-specific primer (HIV910: 5′-CTAGAACGATTCGCAGTTAATCC-3′). The negative controls included a sample from an RT–PCR reaction lacking input RNA and a RT–PCR reaction with RNA extracted from a mock transfected supernatant. A PCR reaction on an equivalent amount of RNA, which did not undergo reverse transcription, was carried out for each sample to exclude incomplete DNase I treatment. Equal volumes of RT–PCR or PCR samples were subjected to polyacrylamide gel electrophoresis and autoradiography. The intensity of each band was quantitated using a Molecular Dynamics PhosphorImager with ImageQuant software (Molecular Dynamics).

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