Charged Amino Acid Residues of Human Immunodeficiency Virus Type 1 Nucleocapsid p7 Protein Involved in RNA Packaging and Infectivity

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Received 23 April 1996/Accepted 19 June 1996

Interaction of the human immunodeficiency virus type 1 (HIV-1) Gag precursor polypeptide (Pr55^{Gag}) with the viral genomic RNA is required for retroviral replication. Mutations that reduce RNA packaging efficiency have been localized to the highly basic nucleocapsid (NC) p7 domain of Pr55^{Gag}, but the importance of the basic amino acid residues in specific viral RNA encapsidation and infectivity has not been thoroughly investigated in vivo. We have systematically substituted the positively charged residues of the NC domain of Pr55^{Gag} in an HIV-1 viral clone by using alanine scanning mutagenesis and have assayed the effects of these mutations on virus replication, particle formation, and RNA packaging in vivo. Analysis of viral clones with single substitutions revealed that certain charged amino acid residues are more critical for RNA packaging efficiency and infectivity than others. Analysis of viral clones with multiple substitutions indicates that the presence of positive charge in each of three independent domains—the zinc-binding domains, the basic region that links them, and the residues that flank the two zinc-binding domains—is necessary for efficient HIV-1 RNA packaging. Finally, we note that some mutations affect virus replication more drastically than RNA incorporation, providing in vivo evidence for the hypothesis that NC p7 may be involved in aspects of the HIV life cycle in addition to RNA packaging.

The retroviral RNA packaging process requires interactions between the nucleocapsid (NC) domain of the Gag precursor and the *cis*-acting RNA sequence Ψ (reference 41 and references therein). This process is highly specific, since viral RNA accounts for only 1% of the total RNA in infected cells and little host RNA is encapsidated (48). The *cis*-acting sequences required for viral RNA packaging are located at the 5' ends of the genomes of a number of retroviruses (27, 41). These sequences are necessary but not always sufficient for efficient RNA packaging. The NC domain is located at the C-terminal segment of the Gag precursor. It contains a high concentration of basic amino acid residues and, with the exception of the human spumavirus, one or two zinc-binding motifs that lie within the basic domain.

All retroviral NC proteins have a high basic amino acid content, a property shared with other RNA-binding proteins (32). The idea that the basic residues are important for retroviral RNA packaging is supported by knowledge that the NC protein of the human spumavirus lacks the zinc-binding motifs but is highly basic (35). In addition, mutations that affect the basic residues of retroviral Gag proteins affect Ψ RNA recognition and dimerization (6, 12, 13, 18, 25, 43).

A zinc-binding motif of the form CysX₂CysX₄HisX₄Cys is also a common feature of the retroviral NC protein (5). This motif occurs once in the murine retroviruses and twice in most other retroviruses studied thus far. The zinc-binding motifs have been shown to play a significant role in RNA packaging in both avian and mammalian retroviruses (8, 15, 22, 26, 39, 41, 42, 50). When two motifs are present, the first generally plays a more important role than the second (8, 22). In Rous sar-

coma virus, deletion of the first of the two zinc finger motifs abolishes RNA packaging and infectivity, whereas deletion of the second of the two motifs reduces viral infectivity about 100-fold. Both Rous sarcoma virus zinc fingers are important for 70S RNA dimer formation. Alteration of the zinc-binding motif(s) can cause loss of packaging of genomic RNA and increased packaging of spliced viral RNA and cellular mRNAs (15, 26, 39).

NC proteins have been implicated in interactions with nucleic acids other than the packaging of genomic viral RNA (13, 26, 28, 31, 39, 47). NC has a role in positioning of the tRNA primer and is a cofactor of reverse transcription. NC's DNA annealing and DNA melting activities in vitro have been proposed to play a role in facilitating strand displacement during the final stage of DNA synthesis.

The portion of the human immunodeficiency virus type 1 (HIV-1) Gag precursor involved in RNA packaging (Pr55^{Gag}) is the NC p7 domain. The role of the HIV-1 zinc-binding domains has been extensively examined in vitro and in vivo (2, 6–8, 12–14, 22, 23, 34, 37, 41). Alterations of these domains lead to loss of binding to Ψ sequences in vitro, defective genomic RNA packaging, and lack of genomic dimerization in vivo. Seventeen of the 55 amino acids that constitute HIV-1 NC p7 are basic residues. Mutational analysis of 10 of these residues has shown that 3 are more critical than others for RNA binding in vitro (43). A smaller set of the 17 basic amino acids has been studied for their potential role in RNA packaging in vivo. Simultaneous alteration of three basic residues linking the two zinc-binding motifs was found to reduce virus titer up to 100-fold (37), whereas alterations of lysine 20 (K20) and arginine 26 (R26) did not affect HIV-1 replication (14). A systematic mutational analysis of all the basic residues of the HIV-1 NC domain in vivo would provide a more thorough assessment of the importance of these residues to the viral life cycle.

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TABLE 1. Oligonucleotide primers used in site-specific mutagenesis by overlap extension

Construct name	Oligonucleotide pair ^a
pR3.....	5'-TAATGATGCGAGGCAAGGCAATTTTA
pR7.....	5'-GAGGCAATTTTGGCAACCAAGAA
pR10.....	5'-TTAGGAACCAAGCAAAGATTGTTA
pK11.....	5'-GGAACCAAGAGCGATTGTAAAGT
pK14.....	5'-GAAAGATTGTTGCGTGTTCATT
pK20.....	5'-TCAATTGTGGCGCAGAAGGGCACA
pR26.....	5'-GGCACACAGCCGCAATTCAGGG
pR29.....	5'-CCAGAAATGCGCGGCCCTAGGA
pR32.....	5'-GCAGGGCCCTGCGAAAAAGGGCT
pK33.....	5'-GGGCCCCCTAGGGCAAAGGGCTGTT
pK34.....	5'-CCCCTAGGAAAGCGGGCTGTGGA
pK38.....	5'-AGGGCTGTTGGGCGATGTGGAAGG
pK41.....	5'-GGAAATGTGGAGCGGAAGGACACC
pK47.....	5'-GACACCAATGGCAGATTGTACTG
pR52.....	5'-ATTGTACTGAGGCACAGGCTAAT
p10-11.....	5'-AGGAACCAAGCAGCGATTGTT
pBR.....	5'-AATTGCGCGGCCCTGCGGCAGCGGGCTGT
pM1-2.....	5'-AGGGCCCCCTAGGAAAAAGGGCTGTTGGGCATGTGGAGCGGAAGGACACCAATGGCAGATTGTACTGAG
	5'GCCCTTTTCTAGGGGCCCTGCAATTTGCGGCTGTGTGCCCTTCTGCGCCACAATTGAAACACGCAACAATCTT

^a Bases that determine the change from the original amino acid to an alanine are underlined. The first listed oligonucleotide matches the virus positive-strand sequence; the second matches the negative-strand sequence. M13 forward and reverse oligonucleotides were used as outer primers in the PCR mutagenesis.

We have systematically substituted the positively charged residues of the NC domain of Pr55^{Gag} in an HIV-1 viral clone by using alanine scanning mutagenesis and have assayed the effects of these mutations on virus infectivity, particle formation, and RNA packaging. The data provide the first comprehensive in vivo analysis of the role played by each of the basic residues of the NC domain in these functions. The results of these studies also support a role for the NC domain in aspects of the HIV-1 life cycle in addition to the process of RNA packaging.

MATERIALS AND METHODS

Plasmid construction and site-directed mutagenesis. The parental viral DNA clone used in these studies is the biologically active plasmid pHXB2gpt (17). The *Pst*I-*Msc*I (bp 1419 to 2620) (40) 1.2-kb fragment was cloned into the *Pst*I-to-*Sma*I sites of plasmid vector pUC19 (plasmid pNCPM), and oligonucleotide-mediated site-specific mutagenesis by overlap extension (24) was performed on this plasmid. For each codon mutated to encode an alanine, the first two bases were changed to a G and a C while the third base was unaltered. The oligonucleotides used in the mutagenesis are listed in Table 1; standard M13 forward and reverse sequencing primers were used as outer primers. PCR products were digested with restriction endonucleases *Spe*I (bp 1506) and *Bcl*II (bp 2428) and reinserted into a similarly treated pHXB2gpt. These restriction sites are unique within pHXB2gpt and facilitated unidirectional cloning. DNA from clones pM1-2, pK20, and p10-11 was used as the template in the construction of pM1-2/BR, p20-26, and p10-11-52; mutagenesis was performed with the oligonucleotide pairs used in the construction of pBR, pR26, and pR52, respectively. To construct p14-20-26, a *Pst*I-*Apa*I (bp 1419 to 2009) 590-bp fragment from pM1-2 replaced the corresponding fragment in pHXB2gpt. To construct p10-52 and p11-52, *Pst*I-*Apa*I 590 bp fragments from clones pR10 and pK11 replaced the corresponding fragment in pR52. Construct p38-41-47 was generated by replacing the *Apa*I-*Bcl*II 420-bp fragment in HXB2gpt with the corresponding fragment from pM1-2. To construct p29-33, the *Pst*I-*Sly*I 590-bp fragment from pR29 replaced the corresponding fragment in pR33. All mutant viral constructs were confirmed by dideoxy sequencing. All DNA manipulations were performed according to standard procedures (4).

Cell lines, transfections, viral infections, and assays. The African green monkey kidney cell line cos-1 was obtained from the American Type Culture Collection and maintained in Dulbecco modified Eagle medium (GIBCO/BRL, Bethesda, Md.) supplemented with 10% fetal bovine serum (GIBCO) at 37°C under 5% CO₂. The H9 T-lymphoid cell line was maintained as previously described (38). Transfection of cos-1 cells by calcium phosphate precipitation and analyses of viral mutants were carried out as described previously (1, 3). Briefly, supernatants from transfected cos-1 cell cultures were harvested 48 h posttransfection and assayed for p24 antigen by using an enzyme-linked immunosorbent assay (ELISA) (p24 core profile kit; DuPont) and for reverse transcriptase (RT) activity (11). Viral supernatants derived from two independent transfections per construct were tested in infectivity assays. H9 cells (5×10^5)

were exposed to amounts of virus from transfected cells equivalent to 25 ng of p24 in 2 ml of medium. After 3 h of infection, cells were washed, resuspended in 2 ml of tissue culture medium, and maintained in 24-well plates. Cultures were fed every 4 days by removing 1.5 ml of the 2 ml of cell culture suspension and replacing it with fresh medium. Cell density increased from 5×10^5 just after feeding to 2×10^6 4 days later. At each 4-day interval, cleared supernatants were used for p24 ELISA, and harvested cells were assayed by immunofluorescence. Each time point of each culture was evaluated in duplicate in both assays. Cultures were carried for 30 days after infection. Viral supernatants collected at the end of the time course from all viruses were pelleted, RNA was extracted, and DNA encoding p7, obtained through RT-PCR, was sequenced. The sequence analysis excluded the possibility of cross-contamination among cultures. Immunofluorescence assays on H9 infected cells were carried out with a murine monoclonal antibody specific for the p24^{Gag} protein as previously described (46). Electron microscopy was carried out according to standard procedures.

Western blot (immunoblot) analysis. Cos-1 cells were transfected with each mutant, and 72 h posttransfection, the supernatants were collected, filtered through a 0.22- μ m-pore-size filter, and centrifuged through a 3-ml cushion of 15% (wt/vol) sucrose at 27,000 rpm for 3 h in an SW28 rotor (Beckman). Each pellet was resuspended in Laemmli buffer (5% glycerol, 1% sodium dodecyl sulfate [SDS], 31.875 mM Tris [pH 6.8], 0.005% bromophenol blue) (30), and the p24 content was determined by ELISA. Samples equivalent to approximately 20 ng of p24 were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and probed with HIV-1-positive human serum as described previously (45). ¹²⁵I-protein A (New England Nuclear) was used to detect HIV-1-specific proteins by autoradiography.

Particle-associated RNA analysis. After evaluation of p24 in the medium of all transfectants, supernatants containing virus particles equivalent to 15 ng of p24 were centrifuged to pellet the virions, viral RNA was extracted, and quantitative RT-PCR was performed on RNA samples as previously described (29). Briefly, the pellet was resuspended in 0.5 ml of solution D (4.2 M guanidine thiocyanate, 0.1 M sodium citrate, 0.5% SDS, 7.2% 2-mercaptoethanol) containing 120 μ g of yeast tRNA per ml as a carrier to monitor final RNA recovery. The RNA was extracted with phenol-chloroform and precipitated with ethanol. To eliminate contaminating transfection or cellular DNA, the RNA was then treated with 16 U of RQ1 DNase I (Promega) in the buffer recommended by the manufacturer (40 mM Tris [pH 8], 10 mM NaCl, 6 mM MgCl₂, 10 mM CaCl₂) in the presence of 80 U of recombinant RNasin RNase inhibitor (Promega) for 1 h at 37°C. The DNase I was denatured with solution D, 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 0.5 mg/ml. RNA samples were obtained from three independent transfections of each construct. RNA samples corresponding to 2 ng of p24 were reverse transcribed in a 30- μ l reaction with Superscript II (GIBCO/BRL), using a *gag*-specific primer (HIVc1686, 5'-ACCGGTCTACATAGTCTCTA-3'), a U3-specific primer (HIVc9134, 5'-CCACAGATCAAGGATATC-3'), and a *vpu*-specific primer (HIVc6104, 5'-GCTACTACTAAGGCTAC-3'). Three microliters of this reaction mixture was subjected to PCR with AmpliTaq DNA polymerase (Perkin Elmer) in the presence of [³²P]dCTP, employing the same primer used in the RT reaction and paired with an upstream *gag*-specific primer (HIV979, 5'-TACAA CCATCCCTTCAG-3'), a *nef*-specific primer (HIV8659, 5'-TAGCCTGCTCAA

FIG. 1. Schematic representation of alanine scanning mutations within the HIV-1 NC p7 protein. The amino acid sequence of HIV-1 NC p7 is listed above the alanine substitutions for each mutant. The specific amino acid residue(s) (aa) modified in each mutant and the name of the corresponding plasmid construct are shown on the right.

TGCCAC-3'), a *tat/rev*-specific primer (HIV6001, 5'-GCTCATCAGAACAGT CA-3'), and a 5' leader sequence-specific primer (HIV691, 5'-GGACTCGGCT TGCTGAA-3'). The negative controls included a sample from an RT-PCR performed without input RNA and an RT-PCR performed with RNA extracted from a mock-transfected supernatant. A PCR on an equivalent amount of RNA which did not undergo reverse transcription was carried out for each sample to exclude incomplete DNase I treatment. An RT-PCR using actin-related primers (ACT1 [5'-ATGGAAGAAGAGATCCGC-3'] and ACTR2 [5'-CCTCGTAGA TGGGCACCG-3']) was carried out to eliminate cellular RNA contamination. Equal volumes of RT-PCR or PCR samples were subjected to PAGE and autoradiography. The intensity of each band was quantitated with a Molecular Dynamics PhosphorImager with ImageQuant software (Molecular Dynamics).

RESULTS

Construction of viral mutants. The portion of HIV-1 Pr55^{Gag} which is processed to produce NC p7 in the mature virion is rich in basic amino acids. To investigate the relative contribution of each of these charged residues to RNA packaging, we have systematically substituted the NC arginine and lysine residues with alanine. Mutations were introduced into pHXB2gpt, a biologically active HIV-1 DNA clone which produces infectious virus when transfected into cos-1 cells (16). Alanine substitution was chosen because alanine has a neutral charge, it is a relatively conservative change at any residue that is not buried in the hydrophobic core of the structure, it rarely affects protein stability, and it is a proven and effective method of identifying functionally important amino acid residues (10, 19, 49).

The mutations that were generated are summarized in Fig. 1. Each lysine or arginine mutation was introduced into an individual clone (constructs pR3 through pR52). In some cases, mutations affecting multiple basic residues were introduced. Histidine substitutions were not investigated since these residues were previously shown to be critical for RNA encapsidation (14, 21).

Biological activities of viral mutants. We screened all of the mutant constructs in an infectivity assay to determine which

mutations had a substantial effect on viral replication kinetics. To assess the biological activities of the HIV-1 particles produced by transfection of cos-1 cells with the wild-type and mutant constructs depicted in Fig. 1, supernatants adjusted to contain comparable levels of p24 were used to infect H9 cells, which are susceptible to infection and permissive for HIV-1 replication (38). A time course analysis of infections by all viruses was carried out for 30 days after infection, during which the percentage of HIV-1 antigen-positive cells and the level of particle-associated p24 were measured.

The mutants could be assigned to three groups based on differences in kinetics of propagation in culture (Fig. 2). The kinetics of mutants derived from constructs pK20, pR26, pR29, pR32, pK33, pK34, pK41, pR52, p20-26, and p29-33 were similar to those of wild-type virus, and these mutant viruses were designated group A. At the other extreme, mutants derived from constructs pR3, pK14, pM1-2/BR, pM1-2, p38-41-47, p14-20-29, pBR, p10-11-52, and p10-11 were persistently negative in cellular p24 immunofluorescence or particle-associated p24 assays, and these viruses were placed in group C. Lack of infectivity of the mutants assigned to group C was confirmed by performing infections with higher amounts of p24 in the viral supernatants (data not shown). Viruses from all of the remaining mutants exhibited delayed kinetics and were placed in group B (Fig. 2). In this group, both the percentage of infected cells and p24 production were substantially reduced early in the course of infection. For example, at days 6 and 10, the values of the two assays were 10- to 100-fold lower for viruses in group B than for those in group A.

The mutant viruses which were replication competent and grew in culture for 30 days were examined for the presence of the specific mutation(s) introduced in the viral clones at the beginning of the experiment. Thirty days from the initial infection, viral RNAs were extracted from particles and subjected to RT-PCR. DNA encoding p7 was sequenced. In all

FIG. 2. Time course analysis of mutant virus infectivity in H9 cells. Beginning at day 6, and at each 4-day interval over 30 days, supernatants and cells were removed and used in an ELISA and in an immunofluorescence assay to test for virus-associated and cellular p24, respectively. Each time point of each culture was evaluated in duplicate in both assays. The mutant viruses could be placed into three groups (A, B, and C) based on their replication kinetics. Group A includes the wild-type virus HXB2gpt and mutant viruses derived from plasmids pK20, pR26, pR29, pR32, pK33, pK34, pK41, p20-26, and p29-33. Group B includes viruses derived from plasmids pR7, pR10, pK11, pK38, pK47, pK52, p10-52, and p11-52. Group C includes viruses derived from plasmids pR3, pK14, pM1-2/BR, pM1-2, p14-20-26, p38-41-47, pBR, p10-11, and p10-11-52. (A) Kinetics of viral replication for group A, B, and C viruses in H9 cells, as measured by percentage of p24 antigen-positive cells. Striped (group A) and shaded (group B) areas cover the range of values detected in the cell immunofluorescence (IF) assay for each group of viruses. Upper and lower values at each time point are represented with an open circle for viruses in group A and with a closed circle for viruses in group B. (B) Range of values of percentage of p24 antigen-positive cells obtained for viruses within each group at each point in the time course. (C) Range of values of virus-associated p24 obtained for viruses within each group at

cases, the original mutation(s) was detected, indicating that reversion of the mutation(s) had not occurred during culture.

Analysis of particle-associated viral proteins and virion morphology. Since some of the mutants were not infectious in culture, we investigated whether the cells transfected with the different constructs released particle-associated proteins at a level comparable to the wild-type level. Mutant viral constructs and the parental wild-type pHXB2gpt plasmid were independently transfected into cos-1 cells, and culture supernatants were pelleted and assayed for the presence of p24 and RT. For each of the mutants that exhibited an altered phenotype in the infectivity assay, the levels of p24 ranged between 25 and 33 ng/ml and the level of RT activity was 60 to 100% of the wild-type level (Table 2). These data suggest that the mutations did not substantially affect particle assembly.

Particle protein content was also analyzed by Western blot analysis for the mutants that were replication incompetent. Viral protein lysates obtained from sedimented supernatants of cos-1 cell transfections which contained equal amounts of p24 were analyzed by Western blot analysis using a human HIV-1-positive serum. Analysis of lysates from pHXB2gpt, pR3, pK14, p10-11, p10-11-52, p14-20-29, p38-41-47, pBR, pM1-2, and pM1-2/BR revealed that the protein content of the replication-deficient particles was not significantly different from the wild-type content (Fig. 3).

The morphology of wild-type virus and mutant HIV-1 particles from p10-11-52 and pM1-2/BR was examined by electron microscopy (Fig. 4). Scoring of the sections indicated that the electron-dense core typical of HIV-1 was not observed in mutant particles. Analysis of mutant virus derived from transfection of pR3 indicated the presence of comparable amounts of mature and immature particles (data not shown). These analyses of particle morphology and particle-associated viral proteins indicate that the mutations studied did not affect viral particle formation.

RNA content of mutated viral particles. To determine whether the defects in viral replication exhibited by mutants can be attributed to altered RNA packaging efficiency, RT-PCR was performed on RNA samples derived from wild-type and mutant viral particles containing equivalent amounts of p24. RT-PCR was chosen because it permits detection of minute amounts of genomic viral RNA. To assess the percentage of RNA incorporation by mutant viruses relative to the parental wild type, the intensity of a band corresponding to the amplification of genomic RNA from mutated viruses was compared with the intensity of bands produced by amplifying serially diluted RNA from the wild-type parental virus HXB2gpt. The results are summarized in Table 3, and a representative set of the data is shown in Fig. 5.

When the single amino acid substitutions were analyzed, the results indicated that K14 (pK14 construct) and K11 were more critical than other basic amino acids for RNA encapsidation, as particle RNA incorporation was 22 and 25%, respectively, of that of the wild-type virus (Table 3). For the viruses assigned to infectivity group A, RNA incorporation was in the range of the wild-type levels (data not shown). All other mutant viruses containing single alanine substitutions incorporated RNA at levels between 50 and 100% of the wild-type levels (Table 3). Thus, among the basic residues subjected to

each point in the time course. The values reported in panels B and C include results obtained in two independent experiments. For viruses in group C, values were persistently negative and no variation was observed. For viruses in groups A and B, the variation of values measured in each assay for the same mutant at any given time point was at most 20%.

TABLE 2. Quantitative analysis of HIV-1 proteins in supernatants from cells transfected with NC mutants^a

Construct	p24 (ng/ml)	RT activity (% of wt)
pHXB2gpt	31.41 ± 1.76	100
pR3	28.71 ± 3.46	70.85 ± 2.14
pR7	30.05 ± 2.02	89.89 ± 1.85
pR10	30.62 ± 2.02	82.84 ± 0.94
pK11	29.02 ± 0.61	85.02 ± 2.74
pK14	26.84 ± 3.47	64.52 ± 1.67
pK38	32.28 ± 0.73	95.48 ± 2.24
pK47	33.41 ± 0.18	99.98 ± 1.47
p10-11	24.36 ± 1.12	65.56 ± 1.68
p10-52	30.37 ± 1.53	86.22 ± 1.47
p11-52	28.47 ± 1.73	80.40 ± 0.81
p10-11-52	28.47 ± 1.18	61.93 ± 1.93
p14-20-29	27.03 ± 2.42	72.37 ± 2.01
p38-41-47	27.50 ± 3.35	70.14 ± 2.62
pBR	25.40 ± 3.41	60.07 ± 1.90
pM1-2	26.00 ± 4.12	64.51 ± 2.74
pM1-2/BR	24.59 ± 2.75	63.58 ± 2.17

^a p24 and RT values are reported for mutants included in infectivity groups B and C. p24 content is given as the average of three independent experiments ± standard error. RT activity is given as percentage of wild-type (wt) activity, and the average of three experiments ± standard error is reported. All samples used for RT activity had been adjusted to contain equivalent amounts of p24. p24 values for group A viruses were similar to values for mutants assigned to groups B and C. RT values for group A viruses were 87 to 109% of the wild-type value.

mutagenesis in this study, NC K14 and K11 are the most critical for efficient RNA packaging.

We reasoned that the loss of single basic amino acid residues might not fully reveal the contributions of each residue to RNA packaging if other positive charges in the immediate vicinity can compensate for the loss of a single charged residue. For this reason, we investigated the effect of substituting alanine for all 10 arginine and lysine residues in the segment containing the zinc-binding domains and their linking peptide (mutant pM1-2/BR [Fig. 1]). These residues account for >50% of the total charged residues of NC. Analysis of M1-2/BR mutant particles revealed that they contained less than 1% of the viral RNA that was incorporated into wild-type virus (Table 3). Thus, elimination of the 10 arginine and lysine residues in this domain leads to a drastic reduction of genomic viral RNA incorporation.

To further dissect this domain and identify the contributions of sets of charged residues in RNA packaging, additional mutants were analyzed. In these mutants, alanine residues replaced all or some of the basic residues in (i) both zinc-binding motifs (pM1-2), (ii) the first zinc-binding motif only (p14-20-26 and p20-26), (iii) the second zinc-binding motif only (p38-41-47), (iv) the peptide linking the two motifs (pBR and p29-33), and (v) the basic residues flanking the two motifs (p10-11-52, p10-11, p10-52, and p11-52) (Fig. 1). The effects of these mutations on RNA packaging efficiency were evaluated as described above.

The results reveal that substituting the basic residues in the second of the two zinc finger motifs has the greatest effect on RNA packaging levels (Table 3). Virus produced from the p38-41-47 construct, in which the basic residues of the second motif are substituted, incorporated RNA at less than 1% of wild-type levels. In contrast, virus produced from the p14-20-26 construct, in which the basic residues of the first motif are substituted, incorporated RNA at 12% of wild-type levels. Residue K14 appears to be responsible for much of the RNA packaging defect exhibited by virus from the p14-20-26 con-

struct because virus from construct p20-26 incorporated RNA at levels comparable to those for wild-type virus. When the six charged residues present in the two zinc-binding domains (K14, K20, R26, K38, K41, and K47) were simultaneously substituted, the virus produced by mutant pM1-2 incorporated RNA at 1% of the level of the wild type, consistent with the severity of the defect exhibited by p38-41-47 alone.

Single amino acid substitutions of the basic residues in the seven-amino-acid peptide linking the two zinc-binding domains had little effect on RNA packaging, but the loss of multiple positive charges had a substantial effect. Simultaneous loss of two charges (p29-33) allowed wild-type levels of viral genomic RNA incorporation, but the simultaneous loss of all four charged residues (pBR) reduced RNA incorporation to 14% of wild-type levels. Thus, the presence of the population of basic residues in the linker peptide appears to be important for efficient RNA packaging.

The simultaneous mutation of a charged residue on each side of the zinc-binding motifs (K11 and R52 or R10 and R52) can have a moderate effect on RNA packaging efficiency, as RNA incorporation in particles produced by construct p11-52 and p10-52 was reduced to 56 and 48% of wild-type levels. The combination of the R10 and K11 mutations (p10-11) also had an effect on RNA packaging (32% of wild-type levels). When these two mutations were combined with R52 (p10-11-52), incorporation of genomic RNA dropped to only 10% of wild-type levels. These data support a role in RNA packaging for the combination of basic residues that flank the two zinc-binding motifs.

In general, these data indicate that a reduction of RNA packaging of two- to fourfold of wild-type levels results in slower replication kinetics, while a reduction of greater than fourfold results in lack of infectivity.

Lack of nonspecific RNA incorporation by NC mutants. Previous reports indicate that mutations in NC that reduce genomic RNA incorporation lead to increased incorporation of spliced viral mRNAs (7, 15, 33). For this reason, we investigated whether the NC basic residue mutations that have the most adverse effects on genomic RNA packaging cause nonspecific incorporation of spliced viral RNAs (Fig. 6). To do this, we compared the results of RT-PCR analysis using a set of primers specific for genomic RNA (Fig. 5A) with the results of the same analysis performed with primers that would detect simultaneously genomic, singly spliced, and multiply spliced viral RNA (Fig. 6A). A diagram of the RT-PCR strategies is shown in Fig. 6D. The four different sets of primers showed comparable efficiencies of reverse transcription and amplification when evaluated on known amounts of RNA and DNA (data not shown). The ratio of spliced and unspliced mRNAs and the amounts of total viral RNA present were similar in cells transfected with wild-type and mutated proviruses (data not shown). The data indicate that the amount of RNA detected in the mutant viruses is approximately the same when the assay is carried out with primers that detect genomic RNA alone and when it is carried out with primers that detect both genomic and spliced viral RNA, indicating that the contribution of spliced RNAs to the signal in Fig. 6A is very small.

The RT-PCR experiment was repeated with the same particle and cellular RNAs, using primers that would generate DNA fragments of specific sizes if spliced mRNAs were present (Fig. 6B and C). The results show that these fragments were clearly detected in RNA from cos-1 cells transfected with pHXB2gpt but were not observed in significant levels in mutant particle RNA preparations. Insignificant amounts of the DNA fragments representative of spliced mRNAs were amplified from the wild-type particle preparation and from some of

FIG. 3. Analysis of the protein content of HIV-1 mutant particles by Western blotting. Viral protein lysates, normalized to contain comparable amounts of p24, were subjected to SDS-PAGE (10% gel), transferred to nitrocellulose, and probed with HIV-1-positive human serum. The construct used to generate each lysate is indicated above the relevant lane. (A) Viral proteins visualized after 12 h of autoradiography. (B) Envelope proteins from viral lysates. Autoradiography was for 72 h, except for lanes 2 and 3, which were exposed for 96 h. The amounts of gp120 varied somewhat in various preparations of mutated and wild-type virus. The variation in gp120 is likely due to differential shedding of this protein during preparation of the viral lysates.

the mutants (Fig. 6B and C). We infer that the alanine substitutions of basic residues in these mutants may have affected the general RNA binding properties of the NC domain.

DISCUSSION

The HIV-1 NC protein appears to have roles at multiple stages of the HIV-1 life cycle. As part of the precursor Pr55^{Gag}, it is involved in particle assembly and RNA packaging (reference 41 and references therein). The processed NC p7 may be involved in early steps of the infection process (13, 26, 28, 31, 39, 47). We reasoned that the basic nature of this protein might be critical for its functions and have systematically substituted the basic amino acid residues of the NC domain in an HIV-1 viral clone by using alanine scanning mutagenesis. With this approach, we have identified the basic residues that contribute to HIV-1 replication and RNA packaging *in vivo*.

Analysis of viral clones with single substitutions of lysines and arginines revealed that when all other basic residues remain intact, lysine 14 and 11 are the most critical basic amino acid residues for RNA packaging. However, the analysis of viral clones with multiple substitutions indicates that clusters of positively charged amino acids in three different subdomains are necessary for efficient RNA packaging. The loss of multiple basic amino acids in any of three independent domains—the zinc-binding domains, the basic region that links them, and the residues that flank the two zinc-binding domains—can affect the efficiency of HIV-1 RNA packaging. The importance of

FIG. 4. Electron micrographs of transfected cos-1 cells. (A) Particles derived from construct pM1-2/BR. (B) Particles derived from construct p10-11-52. (C and D) Particles derived from construct pHXB2gpt. The fields shown in panels A to C were selected to show viral morphology, and that in panel D was selected to show the mature viral core. Pictures were taken at magnifications of $\times 40,000$ for panels A and B, $\times 17,800$ for panel C, and $\times 115,000$ for panel D.

TABLE 3. Effects of alanine substitutions in NC p7 on viral genomic RNA incorporation and infectivity^a

Construct	RNA incorporation (% of wt)	Infectivity	Group
pHXB2gpt	100	+++	A
pR3	81.63 ± 11.46	—	C
pR7	52.80 ± 5.22	+	B
pR10	47.20 ± 4.29	+	B
pK11	24.90 ± 3.13	+	B
pK14	22.30 ± 1.67	—	C
pK38	63.90 ± 2.58	+	B
pK47	59.46 ± 1.71	+	B
pM1-2/BR	0.33 ± 0.33	—	C
pM1-2	1.33 ± 0.23	—	C
p14-20-26	12.56 ± 2.51	—	C
p38-41-47	0.70 ± 0.35	—	C
pBR	14.03 ± 2.12	—	C
p10-11-52	10.30 ± 3.85	—	C
p10-11	32.83 ± 5.18	—	C
p10-52	48.03 ± 3.15	+	B
p11-52	56.76 ± 9.22	+	B

^a Relative nucleic acid content of viral particles was measured by RT-PCR. RT-PCRs using gag-specific primers were carried out to detect viral genomic RNA incorporation. The average of values ± standard error from three independent experiments is reported for each mutant. The infectivity group to which each mutant was assigned is also listed. wt, wild type.

charge in the peptide that links the HIV-1 NC zinc-binding domains has already been noted (37), but this is the first evidence for a requirement of charge within the HIV-1 Cys-His boxes and in the flanking peptide regions in vivo. The in vivo analysis reported here leads to conclusions about the importance of specific basic residues for RNA packaging in vivo somewhat different from those obtained from previous in vitro analysis (43). Our study indicates a significant role for residues K14 and K11 in genomic RNA incorporation in vivo that was not revealed by studies of residues important for RNA binding in vitro. Replacement of R7, R32, and K33 with asparagine, serine, and asparagine, respectively, had a particularly striking effect on RNA binding in vitro (43). However, we found that alanine substitution of R32 and K33 does not produce a discernible phenotype in vivo, while substitution of R7 resulted in a twofold reduction of RNA incorporation in viral particles and reduced replication kinetics.

Mutations in the basic residues of NC that significantly reduced genomic RNA incorporation did not result in increased incorporation of other viral RNAs, as has been seen with mutations affecting certain residues of the zinc fingers or with HIV-Moloney murine leukemia virus chimeric NC proteins (7, 15, 33). It is possible that the basic residues of the NC domain contribute to specific RNA binding and to nonspecific interactions with nucleic acid residues. Thus, the substantial decrease in RNA incorporation observed with some mutants may be due, in part, to loss of nonspecific RNA binding properties

FIG. 5. Relative nucleic acid content of viral particles determined by RT-PCR. RNA incorporation was measured for all mutant viruses in three independent experiments using this assay, and the average values are summarized in Table 2; the results of one representative experiment with a subset of the viruses are shown. (A) RNA samples from pelleted virions containing equal amounts of p24 were reverse transcribed by using primer HIVc1686 and subjected to PCR amplification in the presence of [³²P]dCTP by using the HIVc1686 primer paired with primer HIV979. As a positive control, a sample from an RT-PCR with RNA extracted from cos-1 cells transfected with a wild-type HIV-1 plasmid was included. The negative control included a sample from cos-1 cellular RNA. Equal volumes of RT-PCR samples were subjected to PAGE and autoradiography. The intensity of a band corresponding to the amplification of genomic RNA from mutated viruses was compared with the intensity of bands produced by amplifying serially diluted RNA from the wild-type parental virus HXB2gpt (standards). (B) An RT-PCR using actin-related primers was carried out to detect the presence of cellular RNA contamination. RT-PCRs with RNA samples from transfected or mock-transfected cos-1 cells were included as positive controls. (C) A PCR with RNA which did not undergo reverse transcription was carried out for each sample to exclude incomplete DNase I treatment. A PCR on pHXB2gpt plasmid DNA was included in the first lane as a positive control.

FIG. 6. Detection of different viral RNAs in mutant viral particles by RT-PCR. RNA samples corresponding to equal amounts of p24 were reverse transcribed by using primer HIVc9134 (A and C) or primer HIVc6104 (B). Equivalent aliquots of the RT reaction were subjected to PCR amplification in the presence of [³²P]dCTP. As a positive control, a sample from an RT-PCR with RNA extracted from cos-1 cells transfected with a wild-type HIV-1 plasmid was included. The negative controls included a sample from an RT-PCR with RNA extracted from untransfected cos-1 cells (A to C) and an RT-PCR lacking input RNA (B and C). Equal volumes of RT-PCR samples were subjected to PAGE and autoradiography. Three independent experiments on RNA samples derived from three independent transfections were performed, and the results shown in the panels are from one set of RNAs. (A) Simultaneous detection of both genomic and spliced RNAs in a subset of mutant viruses. Primer HIVc9134 was paired with primer HIV8659 to amplify a fragment of 475 bp. The intensity of bands in panel A was quantitated as described for Fig. 5. (B) Preferential amplification of singly spliced viral RNAs. Primer HIVc6104 was paired with primer HIV691. Multiple bands of 1243, 766, 500, 427, 378, 323, and 317 bp are generated when singly spliced RNAs are present, depending on the splice acceptor site used (36). This set of primers could in principle also amplify the genomic RNA to generate a DNA fragment of 5,413 bp, but fragments of this length are not amplified efficiently under the conditions used in this RT-PCR. Background bands are visible in the cos-1 lane that differ in size from the major bands obtained in the HXB2-transfected cos-1 cells. (C) Preferential amplification of multiply spliced viral RNAs. Primer HIVc9134 was paired with primer HIV6001. Two bands of 914 and 799 bp are generated when multiply spliced RNAs are present. This set of primers could in principle also amplify the genomic and the singly splice viral RNAs to generate a DNA fragment of 3,133 bp, but fragments of this length are not amplified efficiently under the conditions used in this RT-PCR. A background band is visible in the cos-1 lane that does not align with the two major bands in the HXB2-transfected cos-1 cells. (D) Diagram of the RT-PCR strategies. Representation of viral RNAs is simplified, and the diagram is not to scale. Oligonucleotide primers used in the RT-PCRs are indicated with arrows, and their positions in the genome are indicated by numbers under the HIV-1 genome, each corresponding to the position of the first nucleotide according to the numbering by Ratner et al. (40). The figure in which results are shown for each pair of primers is indicated. The structure of genomic and spliced viral RNAs is adapted from reference 36. LTR, long terminal repeat.

which normally contribute to the affinity of genomic RNA binding.

None of the mutations studied here appear to affect virus assembly, as particle-associated proteins could be detected at comparable levels for all of the mutants. Electron microscopic analysis of replication-incompetent viruses revealed that with the exception of virus from pR3, for which mature morphology could be documented, the viral particles produced upon transfection were almost exclusively of immature phenotype. The immature particle morphology appears to correlate with reduced packaging efficiency of the mutant virions. Lack of an electron-dense core could be due to the absence or incorrect positioning of the RNA and/or to less efficient precursor processing. It is possible that the presence of an RNA dimer is critical for achieving the correct morphology of the particle, as the RNA might function as a scaffold in particle assembly and maturation (9). Maturation of the particle is linked to post-budding processing of the viral precursors Gag and Gag-Pol. It is possible that the rate of precursor processing is affected in the mutants with reduced RNA incorporation, as efficient *in vitro* processing of p15 has been reported to be RNA dependent (44). Western blot analysis did not show substantial differences in the processing of mutant and wild-type particles, but more sensitive experiments need to be carried out in order to document a possible processing delay in these mutants.

Previous *in vitro* experiments have indicated that mature NC protein may contribute to a variety of functions, including initiation of reverse transcription, strand displacement during the final stage of DNA synthesis, improved efficiency of integrase, and protection of viral RNA from nuclease activity (13, 26, 28, 31, 47). The results described here suggest an *in vivo* function in addition to RNA packaging for the HIV-1 NC protein. Virus replication was absent when R3 was substituted with alanine, despite the ability of this mutant to incorporate viral RNA at 81% of wild-type levels (Table 3). Virus from p10-11 incorporates RNA at levels comparable to those of virus from pK11, but unlike the latter, it is not infectious. It is possible that the NC domain is crucial for RNA packaging when present in Pr55 and for other steps of the virus life cycle as mature NC p7. Analysis of Moloney murine leukemia virus NC mutations *in vivo* has led to similar conclusions (20). Further detailed investigation will be necessary to establish whether, for instance, the HIV-1 NC mutations described here lead to impaired tRNA primer annealing and initiation of reverse transcription or impaired strand displacement during the final stage of DNA synthesis.

Systematic substitution of the positively charged residues of the NC domain of Pr55^{Gag} in an HIV-1 viral clone revealed the basic residues that have roles, either singly or in combination with other basic residues, in virus infectivity and RNA packaging *in vivo*. Further analysis of these and other NC mutations may lead to a more sophisticated understanding of the multiple functions of the NC domain in the life cycle of HIV-1.

ACKNOWLEDGMENTS

We thank Michael Eck, Robert Husson, and Richard Young for critical reading of the manuscript.

This work was supported by NIH grant AI 36060.

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