Single Point Mutations in the Zinc Finger Motifs of the Human Immunodeficiency Virus Type 1 Nucleocapsid Alter RNA Binding Specificities of the Gag Protein and Enhance Packaging and Infectivity

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A specific interaction between the nucleocapsid (NC) domain of the Gag polypeptide and the RNA encapsidation signal (Ψ) is required for preferential incorporation of the retroviral genomic RNA into the assembled virion. Using the yeast three-hybrid system, we developed a genetic screen to detect human immunodeficiency virus type 1 (HIV-1) Gag mutants with altered RNA binding specificities. Specifically, we randomly mutated full-length HIV-1 Gag or its NC portion and screened the mutants for an increase in affinity for the Harvey murine sarcoma virus encapsidation signal. These screens identified several NC zinc finger mutants with altered RNA binding specificities. Furthermore, additional zinc finger mutants that also demonstrated this phenotype were made by site-directed mutagenesis. The majority of these mutants were able to produce normal virion-like particles; however, when tested in a single-cycle infection assay, some of the mutants demonstrated higher transduction efficiencies than that of wild-type Gag. In particular, the N17K mutant showed a seven- to ninefold increase in transduction, which correlated with enhanced vector RNA packaging. This mutant also packaged larger amounts of foreign RNA. Our results emphasize the importance of the NC zinc fingers, and not other Gag sequences, in achieving specificity in the genome encapsidation process. In addition, the described mutations may contribute to our understanding of HIV diversity resulting from recombination events between copackaged viral genomes and foreign RNA.

The assembly process of retroviruses includes the encapsidation of the genomic RNA inside the nascent virion by Gag proteins in the cytoplasm. Although this RNA accounts for <1% of the total cytoplasmic RNA, it reaches >50% of the virion nucleic acids by weight (reviewed in reference 7). This selective enrichment is the result of specific interactions between *cis*-acting packaging elements in the genomic RNA and *trans*-acting RNA binding sites in the Gag precursor.

For human immunodeficiency virus type 1 (HIV-1), many studies have demonstrated that the major encapsidation signal (Ψ) is a structured RNA sequence that lies downstream of the primer binding site and extends into the 5' portion of the *gag* gene (29, 30, 34, 44, 45). Within this region, four adjacent stem-loop structures contribute to the encapsidation of the viral RNA, with each stem-loop binding the nucleocapsid (NC) domain in the HIV-1 Gag precursor with a different affinity (2, 3, 8, 16, 17, 21, 46, 47). Two copies of a Cys-His box, containing

the consensus sequence Cys-X2-Cys-X4-His-X4-Cys, in the HIV-1 NC are crucial for an interaction with RNA. These form two “zinc finger” or “zinc knuckle” motifs (also designated F1 for the N-terminal motif and F2 for the C-terminal motif), since the conserved cysteine and histidine residues bind a zinc ion, which leads to a rigid conformation. The compact structure of each of the zinc fingers forms a direct contact with the RNA (21, 61). This structure is important for the encapsidation of the viral RNA, as point mutations in the conserved Cys and His residues cause a significant reduction in RNA packaging (1, 22, 26). Other conserved residues in the zinc fingers also appear to play a role in RNA binding and packaging. These include an aromatic and a hydrophobic residue immediately C-terminal of the first cysteine and of the histidine, respectively (21, 22). In addition, the basic nature of arginine or lysine residues that flank the zinc fingers also contributes to RNA binding and packaging by NC (14, 19, 21, 33, 53, 56). Similar observations regarding the importance of the zinc finger motifs and the basic residues in NC for RNA binding and encapsidation have been reported for other retroviruses (7), with the exception of the foamy virus subfamily (41).

Although NC mediates the specific interaction with the Ψ signal, it is not clear if other sequences within the Gag precursor contribute or assist in this interaction. In particular, evidence demonstrating a nonspecific binding of free NC to the RNA has been presented. After cleavage from the Gag

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polyprotein, NC binds and covers the packaged RNA at a density of one NC molecule per six or seven nucleotides (7, 32, 63). Therefore, if NC is capable of binding the whole RNA in a nonspecific manner, then the question arises regarding what induces specific binding at the genome encapsidation stage. Experiments with chimeric or mutated Gag proteins have provided contradictory results. Some experiments have suggested that the specific interaction of Gag with the encapsidation signal is achieved solely through the NC domain. These include the following observations: a Rous sarcoma virus Gag protein with a murine leukemia virus (MLV)-derived NC domain preferentially packages MLV RNA (24), spleen necrosis virus (SNV) Gag with MLV NC can package only MLV RNA (13), an HIV-2 Gag protein with an HIV-1 NC domain can package HIV-1 vector RNA (31), an HIV-1 Gag mutant containing the Moloney MLV (MoMLV) NC domain packages RNA containing the MoMLV Ψ signal, and an MoMLV Gag mutant containing the HIV-1 NC domain preferentially packages the unspliced HIV-1 RNA over spliced HIV-1 RNAs (10). However, other experiments have suggested that additional domains contribute to the specific RNA binding activity of Gag. These include the following findings: HIV-1 Gag with an NC domain from mouse mammary tumor virus (MMTV) still packages a large amount of the HIV-1 genome, and MMTV Gag with the HIV-1 NC preferentially incorporates MMTV genomes (52). In addition, although an HIV-2 Gag protein with an HIV-1 NC domain packaged HIV-1 vector RNA, the addition of the HIV-1 SP1 (also termed p2) domain to this chimera significantly enhanced packaging (31). Moreover, mutations in the SP1 domain in HIV-1 Gag reduced the selective packaging of the genomic RNA over viral spliced forms (55). Furthermore, mutations in the basic residues in the bovine leukemia virus MA domain of Gag reduce viral RNA packaging (62). Whether these studies indicate a direct role of Gag sequences other than the NC in selective RNA packaging or were monitoring indirect effects on NC still needs to be evaluated.

From the experiments described above, it is clear that major alterations such as NC exchanges between distant retroviruses may result in changes in the RNA binding activity of the Gag precursor. However, such modifications are unlikely to take place during the course of natural infection, and it is not clear what kinds of changes in the Gag protein may occur in order to alter its RNA binding specificity. The yeast three-hybrid assay has been an effective tool for detecting mutations that influence Gag-RNA binding interactions (5, 25, 35–38). For the present study, we used a random mutagenesis approach combined with yeast three-hybrid screens to find mutations in HIV-1 Gag that alter its RNA binding specificity by increasing its affinity for the Harvey murine sarcoma virus (HaMSV) Ψ RNA. This approach should detect residues that are involved in specific RNA binding throughout the Gag domains. The mutants obtained from this screen all had mutations confined to the NC zinc finger sequences, highlighting the importance of these motifs for the specificity of RNA binding by the HIV-1 Gag precursor. In addition, the nature of these mutations (single substitutions) and their effects on RNA encapsidation emphasize the relative ease with which the HIV-1 Gag RNA binding activity can be modified. This may have a wider implication on HIV diversity due to the generation of recombinant

HIV genomes, which will be made possible by enhanced co-packaging of foreign and HIV RNAs.

MATERIALS AND METHODS

Yeast three-hybrid system. The different components of the yeast three-hybrid system were previously described in detail (5, 57). These include the *Saccharomyces cerevisiae* L40-coat strain as well as yeast RNA expression plasmids that encode bridging RNA molecules with their MS2 RNA binding sites fused to the RNA encapsidation signal of either HIV-1 Ψ , HaMSV Ψ , MoMLV Ψ , or the iron-responsive element (IRE). An additional component is the yeast expression vector pGADZX2, which carries the *LEU2* marker and encodes a fusion protein with an N-terminal Gal4 activation domain (GAL4AD) and a C-terminal HIV-1 Gag polyprotein (Gal4AD-HIV Gag). The Gag open reading frame (ORF) was derived from the infectious molecular clone HXB2 and was flanked by BamHI and SalI sites (43). pGADZX2 was modified and a linker was introduced to create pGADZX2-NotI-linker (detailed in reference 48). This plasmid was used to regenerate the pGADZX2 expression vector with a panel of Gag mutants as described below. Yeast transformations and measurements of reporter gene activation were done by a filter lift assay, and some were also done by a quantitative β -galactosidase (β -Gal) liquid assay as described below.

Mutagenesis. Mutations in pGADZX2 were introduced by two different methods, either using a mutator strain of *Escherichia coli* or by random PCR mutagenesis. Random mutations were introduced by growing the plasmid in an *E. coli* mutator strain (XL-1 Red; Stratagene), followed by amplification of the library of mutated DNAs in the *E. coli* ElectroMAX DH10B strain (GIBCO-BRL) as previously described (6). Alternatively, random mutations confined to the CA end, SP1, NC, and SP2 sequences in the gag ORF were introduced by mutagenic PCRs. Briefly, the oligonucleotides 5' HIVupPpuMI and 3' HIVdown BglII (Table 1) were used to amplify a 486-bp fragment, using the wild-type gag ORF in pGADZX2 as a template, with *Taq* polymerase in the presence of dimethyl sulfoxide and MnCl_2 (39). Four separate reactions were performed, in which a reduced concentration (2 mM) of either adenine, thymidine, cytosine, or guanine was used together with a 10 mM concentration of each of the other three nucleotides. A fifth PCR was done with UITma DNA polymerase according to the manufacturer's protocol (Perkin-Elmer), since a high error rate for this enzyme has been observed (G. Gao, personal communication). The PCR fragments were then inserted into the pGADZX2 plasmid by taking advantage of the homologous recombination capability of yeast. Specifically, each of the PCR products was independently transformed into the *S. cerevisiae* L40-coat strain expressing the HaMSV Ψ RNA (L40-coat/HaMSV Ψ), together with NotI-digested pGADZX2-NotI-linker DNA. The mutated PCR products and the linearized plasmid shared homologous ends which allowed for efficient recombination (51). This procedure regenerated circularized pGADZX2 plasmids with the complete gag ORF harboring the individual mutations confined to the NC portion, which could be selected on Leu^- medium. Overall, one library of pGADZX2 DNAs was derived following mutagenesis in the XL-1 Red mutator strain, and five independent libraries were derived from PCR-generated mutagenesis of pGADZX2. These plasmid libraries were introduced into the yeast three-hybrid screen (see above).

All other mutations in the zinc fingers were generated by site-directed mutagenesis using a two-step overlapping PCR with appropriate oligonucleotides harboring the indicated mutations, with the 5' HIVupPpuMI and 3' HIVdown BglII primers (Table 1) as the external oligonucleotides. The mutant PCR products were introduced into the pGADZX2-NotI-linker plasmid by homologous recombination as described above. Similarly, a pGADZX2 plasmid was also generated to express a fusion protein consisting of the residues from the matrix to the capsid and excluding SP1, NC, SP2, and p6 of Gag (pGADZX2-MA-CA); this plasmid was derived by introducing a premature stop codon 10 amino acids upstream of the sequence encoding the C terminus of the capsid, as described previously (48).

Isolation of Gag protein mutants with altered RNA binding specificities. Separate pools of mutated pGADZX2 DNA were transformed into the yeast strain L40-coat/HaMSV Ψ . Transformants were selected for uracil and leucine prototrophy for 3 days. Colonies from this selection were replica plated onto nitrocellulose filters, frozen at -80°C , thawed, soaked in buffer containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), incubated at 30°C , and assayed for the appearance of a dark blue color in approximately 45 min (a time in which negative colonies did not show staining or stained with only a faint light blue color [5]). Dark blue transformants were restreaked and retested, and DNAs were recovered from these positive colonies. The DNAs were used to transform *E. coli* strain KC8 bacteria (Leu^- ; Clontech), and Leu^+ transformants were selected to allow for recovery of the plasmid pGADZX2. These plasmids

nanant were used for transduction assays as described above. For each transfection, 3 ml of the remaining supernatant was treated with DNase I (Sigma D-4263; 75 Kunitz units/ml [final concentration]) for 1 h at 37°C. VLPs were purified from DNase I-treated supernatants through 25% sucrose cushions, and pellets were resuspended in 50 μ l of reverse transcriptase (RT) buffer (60 mM Tris, 180 mM KCl, 6 mM MgCl₂, 0.6 mM EGTA, 0.12% Triton X-100; pH 8.0). A portion of the samples was used in an exogenous RT assay (60) to determine the VLP content. Two volumes of RNA buffer (64.5% formamide, 22.5% formaldehyde, and 13% morpholinepropanesulfonic acid [MOPS]) was added to the remaining samples (40 μ l), which were then incubated at 60°C for 15 min followed by incubation on ice for 5 min and the addition of 1 μ l of 10 $\times$ RNA loading buffer (0.4% bromophenol blue, 0.4% xylene cyanol FF, 25% Ficoll type 400). The resulting RNA solutions, standardized to equal amounts of VLPs, were then transferred to Hybond-N⁺ membranes (Amersham Pharmacia Biotech) by use of a slot blot apparatus. The RNAs were cross-linked to the membranes by UV irradiation, and the membranes were then processed by standard Southern hybridization methods according to the membrane manufacturer's protocol, using a [³²P]dCTP-labeled GFP or GPT probe which was generated with a DNA labeling mix (Biological Industries). The GFP sequence used as a probe was digested from the pHR⁺-CMV-GFP DNA, whereas the GPT sequence was amplified by a PCR using the GPT primers listed in Table 1. These probes allowed us to distinguish between the HIVgptSVPA genome and the HIV-based vector RNA. The radioactive signals were quantified with a phosphorimager.

To monitor the encapsidation of the MoMLV-based vector by HIV VLPs, we used an RT-PCR procedure. 293T cells in 60-mm plates were transfected with clones carrying wild-type or N17K pHIVgptSVPA (10 μ g) together with pQCXIP-gfp-C1 (7.5 μ g), and at 2 days posttransfection, the cells and culture supernatants were collected. An exogenous RT assay was used to standardize the VLP contents in the media. RNAs from 140 μ l of VLP-containing supernatant was extracted by use of a QIAamp viral RNA mini kit (QIAGEN) and resuspended in 60 μ l of diethyl pyrocarbonate-treated water. The samples were then treated with RNase-free DNase I according to the manufacturer's instructions (DNase treatment and removal kit; Ambion), and this treatment was repeated twice to ensure the removal of all contaminating DNA. First-strand cDNA synthesis was performed in a total volume of 25 μ l by using MLV RT (15 U) and random hexamers (0.5 μ g) in the presence of MLV RT buffer, RNasin (25 U RNase inhibitor), and deoxynucleoside triphosphates (1 mM). All of the above reagents were purchased from Promega (Madison, Wis.). The RT reaction was carried out at 42°C for 1 h, and the reaction mixtures were then diluted to normalize for equal virion contents. Five microliters of the RT reaction mixture or serial dilutions of the RT reaction mixture were amplified by PCR with the ExTaq enzyme (Takara) and 0.3 μ M (each) of GFP-derived primers (GFP-415F and GFP-515R; Table 1). PCR samples (up to 20%) were electrophoresed in a 2% agarose gel. This RT-PCR procedure was also utilized for RNA samples extracted from VLP-producer cells. The total cellular RNAs were extracted from cells harvested 48 h after transfection by use of an SV total RNA kit (Promega) according to the manufacturer's instructions.

RESULTS

Genetic screen for Gag mutants with altered RNA binding activities. A specific interaction between the nucleocapsid (NC) domain of the Gag polypeptide and the RNA encapsidation signal (Ψ) is required for the preferential incorporation of the retroviral genomic RNA into the assembled virion. In the past, the yeast three-hybrid system has been utilized to detect the binding of the HIV-1 Gag protein to HIV-1 Ψ (5). In this system, a fusion protein with an N-terminal GAL4-AD and a C-terminal HIV-1 Gag polypeptide (Gal4AD-HIVGag) shows specific binding to HIV-1 Ψ and only poor interactions with other RNAs, including HaMSV Ψ . For the present study, this genetic system was used to screen for random point mutations in the HIV-1 Gag protein that modulate the specificity of its RNA recognition by increasing its affinity for HaMSV Ψ (Fig. 1). Whereas the coexpression of Gal4AD-HIVGag together with HaMSV Ψ results in only a weak activation of the *lacZ* reporter gene (light blue colonies) (5), Gag mutants with higher affinities for HaMSV Ψ RNA should activate the re-

FIG. 1. Schematic representation of the yeast three-hybrid screen utilized to select for HIV-1 Gag mutants with enhanced binding to HaMSV Ψ RNA. Binding of the RNA hybrid (consisting of the MS2 coat binding sites and the viral Ψ sequence [5]) to the LexA-MS2 coat protein and to the Gal4AD-HIV Gag protein leads to the formation of a complex which activates the transcription of the *lacZ* gene. In this system, wild-type HIV-1 Gag interacts strongly with HIV-1 Ψ but not with HaMSV Ψ , resulting in minimal activation of the *lacZ* gene in the latter case (top panel). Plasmid libraries expressing random Gag mutants were screened with this system to identify mutants with enhanced binding activities for HaMSV Ψ resulting in increased β -galactosidase expression (curved arrow, bottom panel).

porter gene more strongly (dark blue colonies). Hence, such mutants can be differentiated and isolated.

Isolation of Gag mutants with altered RNA binding activities. To generate plasmid libraries of *gag* sequences with random mutations, we used the yeast plasmid pGZX2, which encodes the Gal4AD-HIVGag fusion protein, utilizing two approaches (detailed in Materials and Methods). The first approach was based on mutations of the complete plasmid, including the full-length *gag* ORF, using an *E. coli* mutator strain (XL1-Red). The second approach, PCR-based mutagenesis, specifically targeted the NC portion of the *gag* sequence. The resulting plasmid libraries of Gag mutants were expressed in an L40-coat yeast three-hybrid strain expressing HaMSV Ψ (L40-coat/HaMSV Ψ) (5).

A total of 5×10^4 and 4×10^4 yeast colonies were screened for the libraries generated by XL1-Red and PCR mutagenesis, respectively. From these screens, four (XL1-Red mutagenesis) and six (PCR mutagenesis) candidates of mutant *gag* were obtained in the context of pGZX2 plasmids. After recloning of the *gag* sequences into a fresh backbone of the pGZX2 plasmid and retransformation of fresh L40-coat yeast/HaMSV Ψ , only two of the four XL1-Red mutagenesis candidates and four of the six PCR mutagenesis candidates retained strong binding to HaMSV Ψ .

The candidate Gag mutant plasmids were examined in an X-Gal colony lift assay, and the amount of binding to several RNA molecules, including HIV Ψ , HaMSV Ψ , MoMLV Ψ , and IRE, was determined (5). Figure 2A presents the results of X-Gal colony lifts, which showed the following phenotypes: the Gag mutants showed strong interactions with the HIV Ψ RNA, as did the wild-type protein; in contrast, the Gag mutants interacted more strongly with the HaMSV Ψ RNA than did

FIG. 2. HIV-1 Gag mutants demonstrate more relaxed RNA binding specificities. (A) *S. cerevisiae* L40-coat, stably expressing the LexA-MS2 coat fusion protein, was transformed with different combinations of plasmids encoding the indicated RNA hybrids and fusion proteins. Colonies from the selected transformants were replicated on nitrocellulose filters and assayed for β -galactosidase activity (indicated by the appearance of a blue spot). To avoid color changes due to differences in density, we spotted pools of yeast transformants consisting of many colonies and then assayed the pools. The RNA binding specificities of the randomly mutated Gag proteins were compared to that of the wild-type (wt) protein. The negative control included in this assay was a fusion protein consisting of the residues from the matrix to the capsid, excluding the NC domain (MA-CA). The red color of the yeast transformed with the plasmid expressing the IRE RNA was due to the absence of the *ADE2* nutritional marker from the plasmid (57), which was irrelevant to this screen. (B) Quantitative results for β -galactosidase activities in the yeast three-hybrid system, as measured in a liquid assay. The β -galactosidase units for wild-type (wt) Gag and two of the mutants (Mut1 and -2) were calculated as described previously (5). β -Galactosidase activities following interactions with HIV-1 Ψ (white bars), HaMSV Ψ (black bars), and IRE (gray bars) are presented as means ($n = 3$). (C) RNA filter-binding assay. DIG-labeled, in vitro-transcribed HaMSV Ψ RNA was incubated with a recombinant wild-type HIV-1 Gag protein (wt), an NC deletion Gag mutant (deltaNC), or Mut1. Samples were spotted in triplicate on a nitrocellulose membrane, and bound RNAs were specifically detected with an anti-DIG antibody (α -DIG-POD; 1:1,000) by standard Western blot techniques.

wild-type Gag; finally, the Gag mutants also interacted strongly with MoMLV Ψ , in contrast with the wild type. One of the candidate mutants (Mut1) was exceptional from all the others in that it appeared to weakly bind the IRE, whereas neither the wild type nor the other mutants showed any binding. The activation of *lacZ* expression was RNA dependent, as yeast transformed with the mutated pGZX2 DNAs but without a plasmid encoding an RNA hybrid did not activate the reporter gene (data not shown), excluding the possibility of a direct protein-protein interaction between the mutants and the MS2coat-LexA fusion protein (Fig. 1).

Whereas the colony lift assay was done using pooled yeast transformants, single colonies were grown for each mutant candidate to quantify β -galactosidase activity in a liquid assay. An example of a representative assay for two of the mutants is shown in Fig. 2B. When compared to the wild-type Gag-HaMSV Ψ interaction, the results from the liquid assay demonstrated ninefold and fourfold increases in β -Gal activity for Mut1 and Mut2, respectively, confirming the results of the colony lift assay. The results also demonstrated a fourfold increase in binding of Mut1 to the IRE.

To further confirm the enhanced binding to HaMSV Ψ

RNA, we examined Mut1 RNA binding in an independent filter-binding assay. We expressed in bacteria the wild-type and the Mut1 Gag protein, in which six histidine residues (His tag) replaced the p6 domain at the carboxy terminus of Gag. The elimination of the p6 domain was shown previously to stabilize the HIV-1 Gag protein in bacteria (12, 50), and the histidine stretch enabled us to purify the Gag proteins by affinity purification using nickel resin (50). An additional His-tagged Gag mutant with a deletion of the entire NC domain (Δ NC) was generated to serve as a negative control for the filter-binding experiments. Equal amounts of purified proteins were incubated with DIG-labeled HIV Ψ or HaMSV Ψ RNA in the presence of excessive amounts of carrier tRNA. The RNA-protein complexes were bound to nitrocellulose filters, and the bound RNA was detected with an anti-DIG monoclonal antibody. Whereas binding to HIV Ψ RNA was the same for the wild-type and mutant Gag proteins (data not shown), Fig. 2C shows that more HaMSV Ψ RNA was retained on the filter after incubation with the Mut1 Gag mutant than after incubation with the wild-type Gag protein. The binding results for this assay are probably only semiquantitative, as purified Gag proteins aggregate in solution (50; our unpublished observation),

FIG. 3. Schematic representation of HIV-1 Gag and zinc finger sequences demonstrating the positions of random mutations. The random mutants that were generated and selected for enhanced binding to HaMSVΨ all had mutations in the zinc finger motifs of the NC, either as single point mutations or double mutations. For two of the mutants (M46K and M46V), a zinc finger mutation was accompanied by an additional mutation in the carboxy region of the CA domain.

a feature that complicates this analysis. Taken together, these results indicate that while the different Gag mutants retained their interaction with HIVΨ, they showed increased binding to other retroviral packaging signals. Furthermore, the loss of specificity in the RNA binding experiments was more severe for the Mut1 candidate, which was also able to interact with a structured RNA derived from a nonviral origin (IRE).

Gag mutants with altered RNA binding specificities harbor point mutations in their zinc finger motifs. In order to determine the mutations present in the selected clones, we sequenced the *gag* ORF (Fig. 3). This revealed that all clones carried mutations in the NC zinc finger motifs, including the N17K mutation (numbering starts at the first amino acid residue of NC, and henceforth mutants will be referred to by their substitution mutations) in the F1 motif and the following mutations in the F2 motif: E42K, E42G/D48N, M46V, and M46K. The two mutations generated by the mutator bacterial strain both encoded the same point mutation, E42K. The M46V and M46K mutations were accompanied by an additional single point mutation in the carboxy terminus of CA (Fig. 3). To evaluate the contributions of the CA mutations to the altered RNA binding specificities, we recreated the CA and NC mutations separately in pGZX2 plasmid DNA. An analysis of these mutants revealed that the NC mutations and not the CA mutations caused enhanced RNA binding to HaMSVΨ (data

not shown). The double NC mutant E42G/D48N demonstrated weak binding to HaMSVΨ, and furthermore, the separation of these mutations to make single point mutants did not significantly alter the RNA binding capability (data not shown).

A point of interest regarding the mutants is that most of the mutations caused an overall increase in the positive charge by the introduction of a positively charged amino acid (N17K and M46K) or the replacement of negatively charged residues (E42K and E42G/D48N). The exception to this was the M46V mutant, which harbored a substitution of one neutral amino acid for another.

The introduction of single lysine residues to the zinc fingers in several positions alters the RNA binding activity of the Gag protein. Our data so far appeared to implicate a change in the charge as a modulator of RNA binding specificity. To test if an increase in the positive charge of the Cys-His boxes would affect the RNA binding specificity, we created lysine substitutions by site-directed mutagenesis. We used the existing mutations in one zinc finger as a guide for the introduction of lysine substitutions at equivalent positions in the second zinc finger (Fig. 4A). This procedure generated four new *gag* alleles with the following point mutations in the context of the pGZX2 DNA: E21K, A25K, N27K, and D48K. When tested in the yeast-three hybrid system, these mutations also interacted more strongly with HaMSVΨ RNA than the wild-type Gag protein did (Fig. 4B), confirming the importance of the positive charge addition.

The majority of Gag molecules with NC mutations showed normal particle assembly. The NC domain of Gag is important for virion assembly, as many mutations in this domain reduce particle production (14, 20, 22, 24, 64). Importantly, part of the assembly determinants is a small sequence called the interaction (I) domain that overlaps the zinc finger motifs (11). To examine the effect of the NC mutations on particle assembly, we introduced each of the mutations into the *gag* ORF of the pHIVgptSVPA plasmid and named the resulting clones after the mutations they carried. The pHIVgptSVPA plasmid contains a derivative of the HIV-1 HXB2 clone carrying the SV40 promoter-*gpt* cassette and the SV40 polyadenylation signal, which replaced the *env* coding sequences and the 3' LTR, respectively. The HIVgptSVPA construct produces VLPs from transfected cells (44, 48).

FIG. 4. Zinc fingers with site-directed lysine substitutions demonstrate broader RNA binding affinities. (A) Zinc finger mutations. The locations of the lysine substitutions were decided following an alignment of the two zinc fingers. These substitutions are demonstrated above the sequence, and the mutation numbering and random mutations are demonstrated below the sequence. (B) The lysine substitution mutants (indicated at the top of the panel) were tested in the yeast three-hybrid assay, and their binding to HIVΨ, HaMSVΨ, or IRE RNA was determined. The activation of *lacZ* was determined by measurement of the β-galactosidase activity (as described in the legend for Fig. 2A).

FIG. 5. Expression of and VLP production with HIV-1 Gag mutants. The figure shows the results of a Western blot analysis of Gag proteins in cell lysates (top panels) and virion pellets (bottom panels). 293T cells were transfected with pHIVgptSVPA encoding wild-type Gag or the listed NC mutations. Two days following transfection, the cells were harvested and lysed, the medium was collected, and the virions were purified from it by centrifugation through a 25% sucrose cushion. The different panels represent different experiments and thus show the wild-type activity for that specific experiment. The proteins were electrophoresed in a 10% acrylamide gel and transferred to a polyvinylidene difluoride membrane for Western analysis with an anti-CA antibody.

To test the mutants for Gag expression and for the ability to assemble and release VLPs, we transfected 293T cells with wild-type or mutant proviral DNA. In addition to the single point mutants mentioned above, we also analyzed the protein expression of double mutants (N17K/E42K and N17K/M46K) that we created (see details below) and of the E42G/D48N mutant. At 2 days posttransfection, VLPs were purified from culture supernatants by centrifugation through a 25% sucrose cushion. In addition, cell lysates of the transfected cells were prepared. Gag protein levels were assayed in cell lysates and in VLP pellets by Western blot analysis (Fig. 5). Gag protein expression in cell lysates was similar for all of the mutants and for the wild type (Fig. 5, top panels), except for the E42G/D48N mutant, which appeared to produce unstable cytoplasmic Gag proteins and failed to produce virions (data not shown). Gag processing in the cell lysates was similar for the wild type and the mutants. An analysis of the cognate VLPs revealed that all of the mutants had similar levels and processing of Gag in the particles to those of wild-type VLPs (Fig. 5, bottom panels). The mutants also incorporated similar levels of RT into the VLPs, as demonstrated by an analysis of the VLP samples with anti-RT antibodies (data not shown). These results indicate that the NC mutations in Gag, except for E42G/D48N, did not hamper virion assembly.

Gag proteins with single point mutations in the zinc fingers have improved vector transduction efficiencies. The HIVgpt SVPA clone can package its own genomic RNA, but this genome cannot undergo full reverse transcription and integration due to the absence of the 3' LTR (44). To quantify the effect of mutations in the zinc finger motifs on virus infectivity, we measured the transduction efficiencies of a GFP-containing retroviral vector encapsidated by particles made of the wild-type or mutated Gag proteins. 293T cells were transfected with wild-type or mutated pHIVgptSVPA clones, together with

plasmids expressing a GFP retroviral vector (pHR'-CMV-GFP) and the vesicular stomatitis virus envelope G protein (pMD.G). Pseudotyped particles in culture supernatants were then used to infect naïve 293T cells, and the transduction efficiencies in a single-cycle infection assay were calculated (see Materials and Methods). All mutants tested were able to transduce the vector. The M46K and D48K mutants had similar levels of transduction as wild-type Gag (Fig. 6). This analysis, however, revealed a small but reproducible enhancement (approximately twofold relative to the wild-type Gag protein) in the transduction efficiency of the retroviral vector by several other mutants, including the A25K, E42K, and M46V mutants (Fig. 6). As an exception to the other mutants, the N17K mutant showed an even higher transduction efficiency which was approximately sevenfold higher than that of the wild type.

To evaluate if this enhancement could be further improved by creating additional mutations in zinc finger 2, we generated double mutants based on the N17K mutation in combination with mutations in zinc finger 2, namely, M46K and E42K. The resulting double mutants, N17K/M46K and N17K/E42K, were capable of virion assembly (Fig. 5), resembling wild-type Gag. When tested for their transduction efficiencies, these mutants did not show any additive effect in transduction efficiency. In fact, these double mutants were similar to the wild type, demonstrating a reduction in transduction efficiency compared to the N17K single mutant. Thus, the enhanced transduction efficiency shown by the N17K mutant appears to depend on the presence of wild-type sequences of the second zinc finger.

The N17K mutant packages a higher level of RNA. One possible explanation for the enhanced transduction efficiency of the N17K mutant is that this mutant is able to package more vector RNA in its particles. To test this hypothesis, we determined the level of packaged HIV-1 vector RNA and compared it to measurements of the virion protein content to approxi-

FIG. 6. HIV-1 NC mutants have different transduction efficiencies. 293T cells were cotransfected with pHR'-CMV-GFP, pMD.G, and DNAs encoding the listed NC mutations in the context of pHIVgpt SVPA. Two days following transfection, the cells were analyzed by fluorescence-activated cell sorting to quantify GFP expression. The culture medium from the cells was collected and used to infect naïve cells in the presence of 8 μ g/ml of Polybrene (hexadimethrine bromide). After 48 h, the infected cells were analyzed by fluorescence-activated cell sorting to detect GFP. The data are presented as infection indexes normalized to transfection efficiencies, with the samples standardized against the wild type as described before (48). The data shown represent a minimum of three independent experiments (means $\pm$ standard deviations).

mate the RNA encapsidation efficiency. Wild-type and N17K clones were coexpressed together with the GFP-expressing retroviral vector and the vesicular stomatitis virus G envelope. The virions were used to infect naïve cells, and the increase in infectivity by the N17K mutant approximated the mean of previous data (ninefold). Purified particles were normalized by an exogenous RT assay and tested for their vector RNA content by slot blot hybridization for GFP. This analysis showed that the N17K mutant had an estimated 13-fold increase in the amount of packaged HIV-1 vector RNA compared to the wild-type Gag protein (Fig. 7A). This increase in packaged vector RNA correlated approximately with the increase in infectivity of the N17K mutant. The HIVgptSVPA clone retains an intact packaging signal and is able to package its own defective RNA genome (44). Thus, both vector and helper RNA molecules are expected to coexist in the virions. Therefore, we also measured the contents of the RNAs encoding helper functions in these virions. Using the same procedure and RNA samples described above, we observed an increase in the amount of helper RNA packaged by the N17K mutant compared to the wild type (Fig. 7A), but this increase was only 5-fold, in contrast to the 13-fold increase in vector RNA. These results indicate that the N17K mutant encapsidates higher levels of vector RNA, in addition to an increased amount of the HIVgptSVPA RNA, than does wild-type Gag. The increase in the amount of vector RNA might explain the enhanced transduction demonstrated by this mutant.

The N17K Gag mutant interacted strongly with MoMLV Ψ in the yeast three-hybrid system (Fig. 2B). To investigate whether this mutant can also package RNA harboring the MoMLV Ψ sequence, we coexpressed the N17K mutant with a MoMLV-based retroviral vector harboring the GFP sequence

FIG. 7. Increased RNA encapsidation in virions of the N17K mutant. (A) For quantification of the vector and helper RNAs packaged in virions of the NC N17K mutant, 293T cells were transfected with pHR'-CMV-GFP, pMD.G, and pHIVgptSVPA expressing the N17K mutant or wild-type Gag. At 2 days posttransfection, RNAs were extracted from virions purified through a 25% sucrose cushion and analyzed by slot blot hybridization with either a GFP (top panel) or a GPT (bottom panel) riboprobe to detect vector and helper RNAs, respectively. To compare the encapsidation of foreign RNAs by wild-type and N17K virions, we transfected 293T cells with pQCXIP-gfp-C1 and pHIVgptSVPA expressing the N17K mutant or wild-type Gag. At 2 days posttransfection, total RNAs were extracted from the cells and from virions normalized by exogenous RT. The RNAs were reverse transcribed and the indicated dilutions of cDNA were amplified by PCR with primers specific to GFP. This semiquantitative analysis was performed with virion (B) and cellular (C) samples. Undiluted samples of virion and cellular RNA prior to reverse transcription (no RT) were used to verify the absence of contaminating plasmid DNA (D). PCR samples were electrophoresed in a 2% agarose gel.

(pQCXIP-gfp-C1). Initial attempts to detect the MoMLV vector RNA by a hybridization procedure failed, probably due to the relatively small amounts of MoMLV vector RNA in the virion pellets. Thus, we analyzed the RNA contents of the virions with a more sensitive RT-PCR method. RNAs were extracted from equal amounts of virions (normalized by an exogenous RT assay) and reverse transcribed, and decreasing amounts of cDNA were amplified by PCR with GFP-specific

primers. This semiquantitative analysis revealed that the N17K mutant packaged the MoMLV vector about threefold more than wild-type Gag (Fig. 7B). However, we also analyzed the cellular RNA for the presence of vector sequences and estimated that in cells expressing wild-type Gag, there was a 10-fold higher level of synthesis than in cells expressing the N17K mutant (Fig. 7C). This suggests that the difference in packaged MoMLV vector RNA may be even higher for the N17K mutant than was estimated. The detected RT-PCR products were due to authentic cDNA generation and not to contamination with plasmid DNA, as shown by the controls presented in Fig. 7D. Thus, the enhanced binding of this HIV-1 Gag mutant to the MoMLV Ψ sequence correlated with the enhanced packaging of non-HIV RNA molecules containing this signal.

DISCUSSION

In this work, we have described several HIV-1 Gag proteins with altered RNA binding activities. These proteins were able to interact with the HIV-1 packaging signal but, in addition, showed affinities for other RNA structures that were higher than those of the wild-type Gag protein. Thus, a more relaxed RNA binding specificity enabling an expanded RNA recognition rather than a shift in target recognition characterizes these proteins.

Gag mutants that were selected to bind HaMSV Ψ also bound MoMLV Ψ , probably because of the sequence similarity of these two elements. However, their ability to also bind HIV-1 Ψ , an element with no clear primary sequence similarity to the other RNAs, but not another structured RNA (the IRE RNA), indicates that these packaging signals may fold into similar spatial structures that are recognized by the Gag proteins. This may require the formation of exposed guanosines, which are important for both HIV and MoMLV NC- Ψ interactions (23).

The mutations that altered the RNA binding specificity of the Gag protein and that were selected from pools of random mutations throughout the *gag* sequence were all localized to the NC domain, but not to other Gag domains. This implies that the NC domain, and the zinc knuckles in particular, is indeed the main determinant of the selective RNA recognition of Gag. However, we cannot rule out the possibility that mutations in other parts of Gag will also affect RNA binding, since the screens that we conducted were not saturated, as they did not detect the mutations that were generated by site-directed mutagenesis and that also caused altered RNA binding specificities. Indeed, second-site mutations that restored encapsidation signal binding to defective Gag mutants have recently been identified in Rous sarcoma virus Gag by use of the yeast three-hybrid system, and these mutations were localized to the p10 and CA domains (37). In addition, four mutations, in the MA, CA, SP1, and NC domains of HIV-1 Gag, were found to restore the infectivity of a virus with deletions in SL1 of the packaging signal (40). Similarly, other mutations in the SP1 and NC domains rescued the replication of HIV-1 with a deletion in SL3 of the packaging signal (54).

All of the isolated mutants with altered RNA binding activities had mutations confined to the NC zinc finger motifs. Several features were common among these mutations: all were single or double substitution mutations, and although all

mutations were located in the zinc finger motifs in the NC domain, none changed the zinc-coordinating cysteine and histidine residues. This is not surprising, since our screen for relaxed specificity required RNA binding, which in turn is dependent on zinc-mediated NC folding (21, 22, 28). In addition, the majority of these mutations were characterized by an increase in the positive charge of the Cys-His boxes. These included replacements of negatively charged residues by positively charged ones (E21K, E42K, D46K, and D48K), replacements of negatively charged residues by neutral residues (E42G/D48N), and replacements of neutral residues by positively charged ones (N17K, M46K, and A25K). An exception to this was the M46V mutation, which did not result in a gain of a positive charge. However, Gag proteins harboring the M46V mutation activated the *lacZ* reporter gene in the yeast three-hybrid assay to a lesser extent than did Gag proteins with the same methionine changed to a lysine. Similar observations were made when the E42G and D48K mutations were compared to the E42K and D48K mutations, respectively (data not shown). These results emphasize the strong effect of lysine substitutions on the RNA binding specificities of Gag mutants.

How may NC mutations change the RNA binding specificity of the Gag protein? Several explanations can be considered that are not mutually exclusive. It is conceivable that the addition of a positive charge to the zinc finger motif will result in a new and direct interaction between the positively charged residue and the negatively charged RNA molecule. Such an explanation has recently been hypothesized for the N21K mutant, which acts as a second-site suppressor of NC mutations (15). Alternatively, negatively charged amino acids might repel some RNA structures, thus preventing strong binding to sequences other than the genuine packaging signal. In this case, the removal of such residues will result in an overall reduction in the RNA binding specificity. Another possible explanation is that the lysine substitutions found in most of the Gag mutants resulted in alterations of the RNA binding activity because both the size and the charge of the lysine residue enforced structural changes in the NC domain. For example, in a nuclear magnetic resonance (NMR) structure that was obtained for a complex composed of the HIV-1 NC stem-loop 3 (SL3) of the packaging signal (21), the asparagine at position 17 is buried and is surrounded by an arginine at position 32 and two lysines at positions 33 and 34. Modeling the lysine of the N17K mutation into this structure results in a "clash" between the lysine side chain and the amine group of an adjacent adenine (the adenine in the tetraloop of SL3 [data not shown]). Moreover, asparagine 17 (in F1) is involved in several interactions, including hydrophobic contacts with the adenine of the tetraloop of SL3 and with tryptophan 37 of F2 and hydrogen bonding with cysteine 28, proline 31, and lysine 33 (21). Thus, the introduction of a larger positively charged residue by the N17K substitution does not necessarily form a new contact with the RNA but is likely to cause conformational changes in the NC that in turn change its RNA binding specificity. Since N17 is involved in a relatively large number of interactions, it is feasible that the N17K mutation has a larger effect on the NC structure than do the other mutations. This in turn may explain the ability of the N17K mutant, and not the other mutants, to interact with the IRE structure. The occurrence of the N17K mutation appears to depend on the presence of an intact zinc

finger 2, since combined mutations of N17K and mutations in zinc finger 2 abolished the enhanced infectivity of the N17K mutant. However, this does not preclude the possibility that other combinations of mutations would not demonstrate an additive effect.

In line with the idea that some of the mutations introduced conformational changes into the zinc knuckles, the salt bridge of lysine 14 and glutamic acid 21, which appears to stabilize the folding of the F1 domain, and that of lysine 33 and glutamic acid 42, which appears to stabilize F2 knuckle-linker interactions, were likely disrupted by the E21K and the E42K mutations, respectively. Likewise, hydrophobic clefts in F1 and F2 that are formed in part by alanine 25 and methionine 46, respectively, and that pack nucleotide bases G9 and G7 of SL3 are probably affected by the A25K and M46K mutations. It is important, however, that the above assumptions are based on an NMR structure that was obtained for a free NC molecule bound to one stem-loop structure (21) and that differences may exist in the RNA-protein interactions when full-length Gag precursors bind the four stem-loops of HIV Ψ or other RNA molecules such as the packaging signal of HaMSV. Importantly, it has been reported that mutations at some of these positions are tolerated and that the resulting mutant viruses are replication competent. Specifically, N17A, E21A, A25G, and N27A mutants were reported to retain significant levels of replication (22), and the E21K mutation has been reported to act as a secondary mutation that restores efficient replication of an HIV-1 NC mutant (15).

We demonstrated improved vector transduction by Gag mutants, which in the case of the N17K mutant, correlated with improved packaging. We observed that both vector RNA and RNA encoding helper functions (HIVgptSVPA RNA) were increased in N17K mutant virions compared to wild-type Gag virions. These combined data suggest that RNA packaging is increased overall in these virions, rather than a case whereby the vector RNA is packaged at the expense of the HIVgpt SVPA RNA. These data imply that "empty" particles that are devoid of RNA harboring a packaging signal exist in helper systems that are commonly used for gene transfer protocols and may reduce the efficiency of gene transduction. Hence, an immediate improvement to such systems would be the introduction of N17K-like mutations that appear to improve the efficiency of vector packaging. However, the fact that the N17K mutant showed enhanced packaging of foreign RNA, such as the MLV-based vector, suggests that caution has to be exercised when using this mutant for the design of more efficient packaging and delivery systems, since enhanced packaging of foreign RNAs, including RNAs that encode helper functions, into HIV-1 particles may result in unwanted recombination between these RNAs and the vector RNA. In this regard, it would be interesting to study the extent of cellular RNA co-packaging.

As mentioned above, in our assays the wild-type and mutated Gag proteins that were used to package the vector RNA were translated from the RNA genome of the HIVgptSVPA clone, which retains its own packaging signal (44). This is an important difference between the system used for this study and retroviral gene transfer systems, which are designed to package only vector RNA, due to the deletion of the packaging signal from the RNA that encodes the Gag-Pol "helper" pro-

teins. Although it is unlikely that the presence of the genomic RNA assisted the packaging of the vector RNA by the Gag protein mutants, it will be interesting to test the NC mutations in the context of classical helper Gag expression plasmids that are missing the packaging signal.

Although the Gag mutants with NC mutations were active in single-cycle infection assays and some had significantly higher transduction activities than that of wild-type Gag, it is possible that there is a selection against the *in vivo* replication of viruses harboring these mutations. Using BLAST searches, we have identified some of the mutations reported in this study in sequence files describing HIV samples from infected individuals. These include the E21K mutation (files AAD28900, AAD28903, AAN39585, and AAK77516), the N27K mutation (files AAQ86724, AAQ86700, AAQ86708, AAQ86660, AAQ86612, BAC02525, AAQ86740, AAQ8668A, and AAQ86676), the E42K mutation (files AAB83306, AAL07717, and AAF28598), and the M46V mutation (files AAP68999, AAB61122, and AAP69138). This suggests that viruses harboring these mutations can replicate to detectable levels *in vivo*, but it does not preclude the possibility that additional mutations at secondary unidentified sites may have facilitated this replication. The double mutant E42G/D48N, which showed defects in virion production, could not be found in BLAST searches, although files that describe each of the two mutations do exist (for example, file AABB3064 for E42G and file AAL78453 for D48N). Other mutations, including the N17K, A25K, M46K, and D48K substitutions, were not found in BLAST searches, suggesting that there is selection against these mutants. The mechanism of this negative selection is not clear, but the relaxed specificity in RNA binding may introduce a disadvantage to the virus over multiple infectious cycles. Alternatively, the NC mutants may change other NC functions that were overlooked in our transduction assays which are based on overexpression of the viral components. In addition, these mutations may be more immunogenic, and hence, a more aggressive immune selection can be mounted against them.

A hallmark of HIV replication *in vivo* is the generation of a high degree of genetic variation due to a large number of replication cycles combined with a high mutation rate (18). In addition, insertions of short cellular sequences into the HIV-1 genome were documented in the past (42, 58, 59), and such insertions into the envelope gene might contribute to immune system evasion (58). It was recently proposed that these insertions are the result of a discontinuous synthesis of the minus-strand DNA that results in homologous and nonhomologous crossovers between the genomic RNA and cellular RNA molecules that are packaged inside the virions (4). Thus, mutations like the ones described in this work, which alter the RNA binding specificity, may cause enhanced encapsidation of the host RNA into the assembled virions and therefore contribute to natural HIV variation.

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