Adaptation of HIV-1/HIV-2 Chimeras with Defects in Genome Packaging and Viral Replication

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ABSTRACT Frequent recombination is a hallmark of retrovirus replication. In rare cases, recombination occurs between distantly related retroviruses, generating novel viruses that may significantly impact viral evolution and public health. These recombinants may initially have substantial replication defects due to impaired interactions between proteins and/or nucleic acids from the two parental viruses. However, given the high mutation rates of retroviruses, these recombinants may be able to evolve improved compatibility of these viral elements. To test this hypothesis, we examined the adaptation of chimeras between two distantly related human pathogens: HIV-1 and HIV-2. We constructed HIV-1-based chimeras containing the HIV-2 nucleocapsid (NC) domain of Gag or the two zinc fingers of HIV-2 NC, which are critical for specific recognition of viral RNA. These chimeras exhibited significant defects in RNA genome packaging and replication kinetics in T cells. However, in some experiments, the chimeric viruses replicated with faster kinetics when repassaged, indicating that viral adaptation had occurred. Sequence analysis revealed the acquisition of a single amino acid substitution, S18L, in the first zinc finger of HIV-2 NC. This substitution, which represents a switch from a conserved HIV-2 residue to a conserved HIV-1 residue at this position, partially rescued RNA packaging and replication kinetics. Further analysis revealed that the combination of two substitutions in HIV-2 NC, W10F and S18L, almost completely restored RNA packaging and replication kinetics. Our study demonstrates that chimeras of distantly related retroviruses can adapt and significantly enhance their replication by acquiring a single substitution.

IMPORTANCE Novel retroviruses can emerge from recombination between distantly related retroviruses. Most notably, HIV-1 originated from zoonotic transmission of a novel recombinant (SIVcpz) into humans. Newly generated recombinants may initially have significant replication defects due to impaired interactions between viral proteins and/or nucleic acids, such as between cis- and trans-acting elements from the two parental viruses. However, provided that the recombinants retain some ability to replicate, they may be able to adapt and repair the defective interactions. Here, we used HIV-1 and HIV-2 Gag chimeras as a model system for studying the adaptation of recombinant viruses. We found that only two substitutions in the HIV-2 NC domain, W10F and S18L, were required to almost fully restore RNA genome packaging and replication kinetics. These results illustrate the extremely flexible nature of retroviruses and highlight the possible emergence of novel recombinants in the future that could pose a significant threat to public health.

KEYWORDS Gag, HIV-1, HIV-2, RNA packaging, adaptation, chimeric virus, evolution, lentivirus, nucleocapsid, recombination, replication, zinc finger

Retroviruses package two copies of their full-length RNA into viral particles as genomes (1). Upon infecting a new cell, the viral enzyme reverse transcriptase (RT) uses the RNA as a template to synthesize DNA, which integrates into a host chromosome and becomes
a provirus (2–4). During DNA synthesis, RT can switch between copackaged genomes, resulting in a chimeric DNA copy that contains portions derived from each RNA (5–8). This process, called recombination, usually occurs between retroviruses with very similar sequences. For example, frequent recombination in human immunodeficiency virus type-1 (HIV-1) has been shown to quickly reassort mutations to generate variants that are resistant to antivirals or able to evade the host immune response (9, 10). However, on occasion, recombination occurs between distantly related retroviruses, resulting in formation of novel viruses with unique properties. The generation of simian immunodeficiency virus in chimpanzees (SIVcpz), the progenitor of HIV-1, is the best-known example of this process (11, 12). SIVcpz is a recombinant of at least two primate lentiviruses, SIVs from red-capped mangabeys (SIVrcm) and Cercopithecus monkeys (SIVmus/mon/gsn) (11, 13). This recombination event generated a virus with novel features that promoted its replication in chimpanzees. Phylogenetic analysis suggests that there have been at least 12 other instances of recombination between distinct SIV lineages (13). Thus, although rare, these recombination events can significantly impact viral evolution and public health.

For a novel retrovirus to arise through recombination, multiple steps must occur. First, the parental viruses must coinfect the same cell. Second, the parental RNA genomes must be copackaged into the same viral particle (6, 14). Third, during infection of a new cell, recombination must occur during reverse transcription to generate a hybrid DNA copy, which must be able to complete reverse transcription and integration. Lastly, the recombinant must be able to replicate. Lentiviruses have extremely compact genomes, with many overlapping reading frames and regulatory elements. Furthermore, there are complex interactions between cis- and trans-acting elements that are critical during multiple stages of viral replication. Thus, when recombinants are generated from distantly related viruses, proteins and/or nucleic acids from the two parental viruses may not function well together, leading to significant defects in replication fitness. In support of this idea, we previously demonstrated that viral fitness is a significant bottleneck to the generation of replication-competent intersubtype HIV-1 recombinants (15).

Based on our prior observations, we hypothesized that most recombinants of distantly related primate lentiviruses would have significant replication defects due to impaired interactions between elements from the two different parental viruses. However, given the high mutation rates of lentiviruses (16, 17), we hypothesized that, if they are able to replicate, these recombinants would adapt to enhance the compatibility of viral elements, resulting in improved replication kinetics. To test this hypothesis, we examined the replication and adaptation of chimeras between two distantly related human pathogens: HIV-1 and HIV-2. HIV-1 originated from SIVs in chimpanzees or gorillas (SIVcpz and SIVgor), whereas HIV-2 originated from SIV in sooty mangabey monkeys (SIVsmm), which belongs to a distinct lineage of SIV (12, 18, 19).

In both HIV-1 and HIV-2, RNA genome packaging is mediated by interactions between the structural protein Gag and the RNA packaging signal, which is located in the 5′ untranslated region (UTR) and the 5′ portion of the gag gene in unspliced, full-length RNA (20–23). Both HIV-1 and HIV-2 package their own viral genomes efficiently, with more than 90% of particles containing viral RNA (24, 25). Gag is translated as a polyprotein, which is cleaved into mature proteins by viral protease during or soon after virus assembly. HIV-1 and HIV-2 Gag polyproteins consist of matrix (MA), capsid (CA), nucleocapsid (NC), and p6 domains with two spacer peptides (SP); SP1 is located between CA and NC, whereas SP2 is located between NC and p6 (Fig. 1A) (26). Of these domains, NC is the most important for RNA genome packaging (27–30). Both HIV-1 and HIV-2 NC have two zinc fingers that contain a conserved C-X2-C-X4-H-X4-C motif and are important for specific recognition of genomic RNA (Fig. 1B). Intriguingly, HIV-1 and HIV-2 exhibit nonreciprocal cross-packaging of genomic RNA: HIV-1 Gag efficiently packages HIV-2 RNA, but HIV-2 Gag does not package HIV-1 RNA (31, 32). These findings indicate that HIV-2 NC has impaired interactions with the HIV-1 packaging signal. Thus, we reasoned that HIV-1-based chimeras containing HIV-2 NC or zinc fingers would result in substantial replication defects, providing an experimental system to study the adaptation of recombinants of distantly related primate lentiviruses.
To determine whether chimeric viruses can replicate and adapt, we generated HIV-1-based chimeras that contained the NC domain of HIV-2 Gag or the two zinc fingers of HIV-2 NC. These chimeras had significant defects in RNA genome packaging but were able to replicate in T cells, albeit with delayed kinetics. When the chimeric viruses were repasaged in T cells, they replicated with faster kinetics in some cases, indicative of viral adaptation. By analyzing the adapted viruses and performing additional mutational studies, we identified two key amino acid differences in the NC domain that affected RNA packaging. We found that switching these two amino acids from conserved HIV-2 residues to conserved HIV-1 residues nearly completely restored both RNA packaging and replication kinetics. These findings demonstrate that defective interactions between viral elements derived from distantly related viruses can be repaired through a small number of substitutions. These results highlight the flexible nature of lentiviruses and the potential for emergence of novel and unexpected recombinants in the future.

RESULTS

HIV-1 chimeras with HIV-2 NC or zinc fingers exhibit impaired RNA packaging. HIV-2 Gag cannot package HIV-1 genomic RNA (31, 32), indicating impaired interactions between HIV-2 NC and the HIV-1 RNA packaging signal. Thus, we reasoned that HIV-1 Gag chimeras with HIV-2 NC would exhibit impaired interactions with the HIV-1 packaging signal. We generated two HIV-1-based chimeras containing portions of HIV-2 NC (Fig. 2A) based on an NL4-3-derived HIV-1 vector (1-Gag in Fig. 2A). In 1-Gag 2NC, the HIV-1 NC domain was replaced with HIV-2 NC from the molecular clone ROD12. Several amino acids of HIV-1 NC, three at the N terminus and one at the C terminus, were not swapped to maintain the proteolytic cleavage sites. In 1-Gag 2ZF, the two zinc finger motifs, as well as the linker connecting the motifs, were replaced with those from ROD12. Relative to NL4-3 NC, the 1-Gag 2NC and 1-Gag 2ZF chimeras introduce 19 or 10 amino acid substitutions and deletions, respectively (see Fig. S1 in the supplemental material).

To examine RNA genome packaging, we used a previously established system called single-virion analysis, in which RNA genome packaging is measured in individual particles by fluorescence microscopy (24). This assay uses two structurally similar HIV-1 constructs that express Tat and Rev. Both constructs also contain 18 copies of the BglG stem-loop (BSL) sequence, which is specifically recognized by the *Escherichia coli* protein BglG. Because the stem-loops are located in the pol gene, only full-length RNA contains this sequence.
Additionally, one construct expresses untagged Gag and the other expresses Gag fused to cerulean fluorescent protein (CeFP). For simplicity, only the Gag-CeFP version is shown in Fig. 2 (1-Gag), although constructs expressing tagged and untagged Gag were used in all single-virion analysis experiments. When equal amounts of constructs expressing Gag and Gag-CeFP are cotransfected with Bgl-YFP, a plasmid that expresses BglG fused to yellow fluorescent protein (YFP), HIV-1 particles with normal immature morphology are generated. These particles can be visualized by performing fluorescence microscopy to detect Gag-CeFP. In addition, the full-length HIV-1 RNAs in the particles can be visualized by detecting the Bgl-YFP signal. The data represent averages and standard deviations (SD) from 11 independent experiments. ***, $P < 0.001$ (relative to 1-Gag); one-way ANOVA with Bonferroni’s posttest.

**FIG 2** HIV-1 Gag chimeras with HIV-2 NC or zinc fingers exhibit impaired packaging of HIV-1 RNA. (A) Constructs for single-virion analysis and Western blotting. HIV-1-based Gag chimeras containing HIV-2 NC (1-Gag 2NC) or zinc fingers (1-Gag 2ZF) were constructed in the context of 1-Gag-BSL (not shown), which expresses untagged Gag, and 1-GagCeFP-BSL (1-Gag), which expresses Gag fused to cerulean fluorescent protein (CeFP). These constructs express RNA containing the HIV-1 packaging signal and BglG stem-loops (BSL), which bind to the fusion protein Bgl-YFP. (B) Gag expression and particle production was determined by transfecting 293T cells with the constructs that express untagged Gag and performing Western blotting with an anti-p24 antibody. GAPDH was used as a loading control. (C) HIV-1 RNA packaging. The percentage of virus particles containing HIV-1 RNA was determined by cotransflecting constructs into 293T cells, centrifuging virus particles onto glass slides, and performing fluorescence microscopy. Viral particles were detected by Gag-CeFP signal, whereas viral RNA was detected by Bgl-YFP signal. The data represent averages and standard deviations (SD) from 11 independent experiments. ***, $P < 0.001$ (relative to 1-Gag); one-way ANOVA with Bonferroni’s posttest.
To determine RNA packaging efficiencies, we performed single-virion analysis. Particles were collected and imaged by fluorescence microscopy, and RNA packaging efficiencies were determined by measuring the proportion of CeFP\textsuperscript{+} particles with colocalized YFP signals. For 1-Gag, we found that 94% of particles contained HIV-1 RNA (Fig. 2C). For the 1-Gag 2NC and 1-Gag 2ZF chimeras, 61% and 49% of particles, respectively, contained HIV-1 RNA. Thus, both chimeras had significant defects in HIV-1 RNA packaging (\( P < 0.001 \) for both chimeras; one-way analysis of variance [ANOVA]). These results indicate that HIV-1 Gag chimeras with HIV-2 NC or zinc fingers have an impaired ability to recognize the HIV-1 packaging signal.

**HIV-1 Gag chimeras with HIV-2 NC or zinc fingers preferentially package HIV-2 RNA.** To further investigate the nature of the RNA packaging defect in the chimeras, we determined whether the chimeras have a preference for packaging HIV-1 or HIV-2 RNA. To accomplish this, we coexpressed the chimeras with a plasmid, 2-fsGag-MSL, that expresses HIV-2 RNA but not Gag protein (Fig. 3A). The 2-fsGag-MSL construct expresses RNA containing the MS2 stem loops (MSL). HIV-1 and HIV-2 RNAs were labeled with Bgl-mCherry and MS2-YFP, respectively. (B) HIV-1 versus HIV-2 RNA packaging. The percentages of virus particles containing only HIV-1 RNA (mCherry\textsuperscript{+} only), only HIV-2 RNA (YFP\textsuperscript{+} only), or both RNAs (mCherry\textsuperscript{+}/YFP\textsuperscript{+}) were determined by cotransfecting the indicated constructs into 293T cells, centrifuging virus particles onto glass slides, and performing fluorescence microscopy to detect Gag-CeFP, Bgl-mCherry, and MS2-YFP. (C) The ratios of particles containing HIV-1 RNA to HIV-2 RNA were determined. The data in panels B and C are averages and SD from 3 independent experiments. ***, \( P < 0.001 \); ns, not significant (relative to 1-Gag); two-way (B) or one-way (C) ANOVA with Bonferroni\'s posttest.
recognized by the MS2 bacteriophage coat protein. We cotransfected 293T cells with the chimeras (or 1-Gag as a control), 2-fsGag-MSL, Bgl-mCherry, and MS2-YFP. The Bgl-mCherry plasmid expresses BglG fused to the mCherry fluorescent protein, while the MS2-YFP plasmid expresses the MS2 coat protein fused to YFP. In this experiment, cotransfected cells can generate four types of CeFP\textsuperscript{1} particles: those without RNA, those with only HIV-1 RNA (mCherry\textsuperscript{1}), those with only HIV-2 RNA (YFP\textsuperscript{1}), and those with both HIV-1 and HIV-2 RNA (mCherry\textsuperscript{1}/YFP\textsuperscript{1}). For 1-Gag, we found that 42% of particles contained only HIV-1 RNA, 40% contained only HIV-2 RNA, and 12% contained both RNAs (Fig. 3B). The total ratio of particles containing HIV-1 RNA to particles containing HIV-2 RNA was 1.05 (Fig. 3C). Thus, consistent with our previous findings (32), 1-Gag efficiently packaged HIV-2 RNA and did not exhibit a significant preference for HIV-1 or HIV-2 RNA. For the 1-Gag 2NC chimera, 16% of particles contained only HIV-1 RNA, 52% contained only HIV-2 RNA, and 9% contained both RNAs. For the 1-Gag 2ZF chimera, we found that 7% of particles contained only HIV-1 RNA, 64% contained only HIV-2 RNA, and 5% contained both RNAs (Fig. 3B). Relative to 1-Gag, the chimeras exhibited a significantly lower proportion of particles with only HIV-1 RNA and a higher proportion of particles with only HIV-2 RNA (< 0.001; two-way ANOVA) (Fig. 3B). The proportion of particles containing both RNAs (HIV-1 and HIV-2) was not significantly different. The total ratio of HIV-1 RNA to HIV-2 RNA was 0.41 for 1-Gag 2NC and 0.17 for 1-Gag 2ZF, significantly lower than the ratio of 1.05 observed for 1-Gag (< 0.001 for both chimeras; one-way ANOVA) (Fig. 3C). Thus, the chimeras preferentially packaged HIV-2 RNA over HIV-1 RNA. These results further show that HIV-1 Gag chimeras with HIV-2 NC or zinc fingers have defective interactions with the HIV-1 packaging signal.

HIV-1 Gag chimeras with HIV-2 NC or zinc fingers have significant defects in particle infectivity. To investigate whether the Gag chimeras can support viral replication, we next cloned the two chimeras into the infectious molecular clone NL4-3 to generate NL4-3 2NC and NL4-3 2ZF. The HIV-1 Gag-Pol polyprotein is expressed using programmed ribosomal frameshifting facilitated by a set of cis-acting elements including a slippery site and a stem-loop located at the NC-SP2 junction and SP2-coding region, respectively. Because the last four residues of HIV-1 NC were unchanged to maintain the proteolytic cleavage site, both NL4-3 2NC and NL4-3 2ZF contain the HIV-1 slippery site, the intervening sequence, and the stem-loop sequence important for frameshifting and the synthesis of the Gag-Pol polyprotein.

To determine if the chimeras affect Gag expression, particle release, or processing, 293T cells were transfected with each chimera or NL4-3 as a control. Western blotting was performed on cell and viral lysates using an anti-HIV-1 p24 antibody. We found that the chimeras had no effect on Gag expression or particle release (Fig. 4A). However, there was a small, but statistically insignificant, increase in Gag processing intermediates (e.g., p41 and p25) for
the chimeras. To determine whether these minor processing defects affected particle morphology, we performed transmission electron microscopy on viral particles from transfected 293T cells (Fig. S2). We found that particles produced by the chimeras were similar to NL4-3 in terms of particle size, the fraction of immature versus mature particles, and particle/core morphology. Next, to determine the infectivity of the chimeras, we transfected 293T cells, harvested virus, measured the amount of virus produced by p24 enzyme-linked immunosorbent assay (ELISA), and infected TZM-bl indicator cells with normalized amounts of virus (1 ng p24). TZM-bl cells are HeLa cells engineered to express CD4 and CCR5. In addition, they harbor a Tat-inducible firefly luciferase reporter gene that is expressed upon HIV-1 infection. To prevent reinfection, the protease inhibitor lopinavir (500 nM) was added to TZM-bl cells 6 h after infection. Luciferase activity was measured 72 h after infection. Relative to NL4-3, NL4-3 2NC and NL4-3 2ZF had infectivities of 9% and 7%, respectively (Fig. 4B). Thus, the chimeras had significant defects in particle infectivity ($P < 0.001$ relative to NL4-3; one-way ANOVA).

HIV-1 Gag chimeras support viral replication, albeit with delayed kinetics in T cells, and adapt by acquiring an S18L substitution in the first zinc finger of HIV-2 NC. To determine if the Gag chimeras can support multiple cycles of viral replication, we infected two T cell lines, CEM-SS T cells and MT4 T cells, with 10 ng p24 of each virus and monitored virus replication by performing p24 ELISA on supernatants collected every 2 days. In CEM-SS T cells, NL4-3 replication peaked 9 to 11 days postinfection, but replication of the chimeras was not detected even after 60 days (data not shown). However, both the NL4-3 2NC and NL4-3 2ZF chimeras replicated in the highly permissive MT4 T cell line, but with delayed kinetics (Fig. 5A). When averaged across five independent experiments, NL4-3 replication
peaked 4 days postinfection, whereas NL4-3 2NC and NL4-3 2ZF replication peaked 9 and 16 days postinfection, respectively. Thus, both chimeras were able to replicate in MT4 cells, albeit with significantly delayed kinetics.

To determine whether adaptation occurred during replication of the chimeras, we collected viral supernatants from multiple cultures at the peak of replication based on p24 production. We then repassaged the viruses by infecting fresh MT4 cells. For these experiments, we initiated the infections with 0.1 ng p24 of each virus instead of 10 ng p24 to better reveal differences in replication kinetics. In multiple cases, NL4-3 2NC replicated significantly faster when repassaged (see Fig. 5B for one example). These results indicate that some of the repassaged viruses had successfully adapted. To identify the mutation(s) responsible for adaptation, we isolated genomic DNA from infected cells collected at the peak of infection during repassaging (e.g., NL4-3 2NC P2 in Fig. 5B). We then performed PCR and sequencing of the entire 5' UTR and gag region. We did not detect any mutations in the HIV-1 packaging signal in any of the cultures. However, in two independent cultures, we found a mutation that led to a single substitution, S18L, in the first zinc finger of HIV-2 NC. To test if this substitution was responsible for adaptation, we introduced the S18L substitution into both the NL4-3 2NC and NL4-3 2ZF chimeras by site-directed mutagenesis. We found that the S18L substitution alone significantly improved the replication of both the NL4-3 2NC (Fig. 5B) and NL4-3 2ZF (Fig. 5C) chimeras in MT4 cells. However, even with the S18L substitution, the chimeras replicated more slowly than NL4-3.

We next tested whether the S18L substitution would enable the replication of the NL4-3 2NC chimera in the less permissive CEM-SS T cell line. Strikingly, adding the S18L substitution allowed NL4-3 2NC to successfully replicate in these cells. On average (over five independent experiments), NL4-3 replication peaked 9 days postinfection, NL4-3 2NC never replicated (within 60 days), and NL4-3 2NC S18L replication peaked 17 days postinfection (Fig. 5D). These results demonstrate that the S18L substitution alone dramatically accelerates replication of the NL4-3 2NC chimera in CEM-SS cells. Similarly, the S18L substitution also improved the replication of NL4-3 2ZF in CEM-SS cells, although the results were more variable (Fig. S3).

The S18L adaptive substitution in HIV-2 NC improves packaging of HIV-1 RNA. The S18L substitution is located immediately after the zinc-coordinating histidine residue in the first zinc finger of HIV-2 NC (Fig. 6A). The analogous HIV-1 residue in NL4-3 NC is isoleucine 24 (I24). To determine whether this amino acid difference is conserved, we analyzed Gag sequence alignments from the Los Alamos HIV sequence database, which contained 9,524 HIV-1 and 53 HIV-2 sequences. In HIV-1, we found that this residue is nearly always (~97% of the time) isoleucine or leucine and rarely other residues, such as valine or threonine (Fig. 6B). In HIV-2, this residue is invariably serine or threonine (Fig. 6B). These differences remained consistent across all HIV-1 and HIV-2 groups and subtypes (data not shown). Thus, HIV-1 NC usually has a large, hydrophobic residue (I/L) at this position, whereas HIV-2 always has a small, polar residue (S/T) at the corresponding position. Intriguingly, the S18L adaptive substitution represents a change from a conserved HIV-2 amino acid residue to a conserved HIV-1 residue at this position, indicating that a large, hydrophobic residue at this position may be important for efficient recognition of the HIV-1 packaging signal.

To determine the impact of the S18L substitution on RNA packaging, we performed single-virion analysis by introducing the S18L substitution into the 1-Gag 2ZF and 1-Gag 2NC constructs. We then transfected 293T cells, collected particles, and performed fluorescence microscopy. The S18L substitution significantly enhanced HIV-1 RNA packaging for both chimeras (Fig. 6C). For 1-Gag 2NC, S18L increased packaging from 61% to 76% (P < 0.001; one-way ANOVA). For 1-Gag 2ZF, S18L increased packaging from 49% to 72% (P < 0.001; one-way ANOVA). However, 1-Gag packaged RNA into 94% of particles. Thus, even with the S18L substitution, the chimeras packaged HIV-1 RNA into fewer particles than 1-Gag (P < 0.001 for both chimeras; one-way ANOVA).

To determine whether the S18L substitution affects the preference of the chimeras for packaging HIV-2 RNA over HIV-1 RNA, we also performed single-virion analysis experiments in which HIV-1 and HIV-2 RNAs were coexpressed. With the S18L substitution, the total ratio of HIV-1 to HIV-2 RNA in particles was 0.45 for the 1-Gag 2NC chimera and 0.46
for the 1-Gag 2ZF chimera (Fig. 6D). Thus, even with the S18L substitution, the chimeras preferentially packaged HIV-2 RNA over HIV-1 RNA. Collectively, these data indicate that the S18L substitution only partially rescues recognition of the HIV-1 packaging signal by the Gag chimeras. These results are in good agreement with the partial rescue of chimera replication kinetics by the S18L substitution (Fig. 5).

The first zinc finger of HIV-2 nucleocapsid has a much greater effect on RNA packaging specificity than the second zinc finger. Considering that the S18L substitution only partially rescued replication kinetics and RNA packaging, other amino acid differences in the chimeras must contribute to the observed defects. Compared to 1-Gag, the 1-Gag 2NC and 1-Gag 2ZF chimeras introduce 19 or 10 amino acid substitutions or deletions, respectively (Fig. S1). Both chimeras have 4 substitutions in each zinc finger and 2 in the central linker. Additionally, 1-Gag 2NC has 7 amino acids altered in the leader and 2 in the tail. Upon analyzing Gag alignments from the Los Alamos HIV sequence database, we found that 7 of the 8 differences in the HIV-1 and HIV-2 zinc fingers are highly conserved. The single exception is R20 (HIV-2)/K26 (HIV-1) in the first zinc finger. This residue is usually arginine or lysine in both HIV-1 and HIV-2 NC, although arginine is more common in both viruses.

To identify other residues in the chimeras that contribute to impaired Gag-RNA interactions, we constructed two additional chimeras in which only the first or second zinc finger of HIV-1 NC was replaced with the corresponding sequence from HIV-2 (1-Gag 2ZF1 and 1-Gag 2ZF2, respectively, in Fig. 7A). Each of these chimeras has 4 amino acid substitutions relative to 1-Gag (Fig. S1). We then analyzed genome packaging by single-virion analysis. We found that switching the first zinc finger (1-Gag 2ZF1), but not the second zinc finger (1-Gag 2ZF2), resulted in a significant decrease in HIV-1 RNA packaging (94% versus 79%; \( P < 0.001 \); one-way ANOVA) (Fig. 7B). However, 1-Gag 2ZF1 was not as defective as 1-Gag 2NC in packaging of HIV-1 RNA (\( P < 0.001 \); one-way ANOVA). Strikingly, when HIV-1 and HIV-2 RNAs were coexpressed, 1-Gag 2ZF1 had a strong preference for packaging HIV-2 RNA, indistinguishable from 1-Gag 2NC (Fig. 7C). In contrast, 1-Gag 2ZF2, like 1-Gag, had no preference for packaging HIV-1 or HIV-2 RNA. These results demonstrate that the amino acid differences in the first
The first zinc finger of HIV-2 NC are primarily responsible for the altered RNA packaging specificity of the 1-Gag 2NC chimera.

**The combination of two substitutions in the first zinc finger of HIV-2 nucleocapsid, W10F and S18L, restores chimera RNA packaging and replication kinetics.** Based on the above findings, we hypothesized that the packaging and replication defects of the 1-Gag 2NC chimera could be further rescued by combining S18L with other substitutions in the first zinc finger of HIV-2 NC. There are three other residues that differ between HIV-1 and HIV-2 in this region: W10, R20, and Q21 in HIV-2 NC, which correspond to F16, K26, and N27 in HIV-1 NC (Fig. 8A). The R20/K26 difference is not conserved, as both HIV-1 and HIV-2 sequences can have arginine or lysine in this position. Thus, we turned our attention to the other two differences: W10 (HIV-2)/F16 (HIV-1) and Q21 (HIV-2)/N27 (HIV-1). We introduced the W10F and Q21N substitutions into the 1-Gag 2NC chimera, either alone or in combination with S18L, and performed single-virion analysis to examine RNA packaging. In contrast to S18L, neither the W10F nor the Q21N substitution alone significantly improved RNA packaging with the 1-Gag 2NC chimera (Fig. 8B). However, the combination of W10F, but not Q21N, with the S18L substitution restored RNA packaging with the 1-Gag 2NC chimera to 90%, which was not significantly different than the packaging efficiency of 94% observed for 1-Gag (P = 0.12; one-way ANOVA). We next coexpressed HIV-1 and HIV-2 RNA to determine the effect of these substitutions on RNA packaging preference. Similar to S18L, the W10F and Q21N substitutions alone had no effect on the preference of the 1-Gag 2NC chimera for packaging HIV-2 RNA (Fig. 8C). However, the combination of the W10F and S18L substitutions eliminated the preference of the 1-Gag 2NC chimera for packaging HIV-2 RNA. With the W10F/S18L double substitution, the 1-Gag 2NC chimera had a total ratio of HIV-1 to HIV-2 RNA of 0.93, which was not significantly different from the ratio of 1.05 observed for 1-Gag (P > 0.99; one-way ANOVA). Therefore, the combination of two substitutions in the first zinc finger of HIV-2 NC, W10F and S18L, completely corrected recognition of the HIV-1 packaging signal by the 1-Gag 2NC chimera.

To test the effects of the W10F substitution on replication kinetics, W10F was introduced into the NL4-3 2NC chimera, either alone or in combination with S18L. We first produced virus
stocks by transfecting 293T cells and performed Western blotting and single-cycle infectivity analysis. The W10F and S18L substitutions did not affect Gag expression, particle release, or processing, alone or in combination (Fig. 9A). In TZM-bl cells, the S18L substitution alone significantly improved the single-cycle infectivity of NL4-3 2NC ($P < 0.001$; one-way ANOVA) (Fig. 9B). Relative to NL4-3, NL4-3 2NC had an infectivity of 9%, while NL4-3 2NC S18L had an infectivity of 29%. In contrast, the W10F substitution alone did not improve the infectivity of NL4-3 2NC. However, the introduction of both substitutions (W10F/S18L) into the NL4-3 2NC chimera led to a single-cycle infectivity of 97%, which was not significantly different from that of NL4-3 ($P = 0.94$; one-way ANOVA). Thus, the combination of the W10F and S18L substitutions completely restored the infectivity of the NL4-3 2NC chimera.

Lastly, to examine replication kinetics, we infected MT4 and CEM-SS T cells with normalized amounts of each virus and monitored viral replication by p24 ELISA. In MT4 cells, NL4-3 2NC W10F replicated slightly faster than NL4-3 2NC but more slowly than NL4-3 2NC S18L (Fig. 9C). Thus, the S18L substitution had a greater effect on replication kinetics than W10F, in agreement with our RNA packaging and infectivity data. Importantly, NL4-3 2NC W10F/S18L replicated with kinetics similar to that of NL4-3 in MT4 cells (Fig. 9C). In CEM-SS cells, NL4-3 replication peaked 9 to 11 days postinfection, while NL4-3 2NC failed to replicate within 60 days (Fig. 9D). Both NL4-3 2NC W10F and NL4-3 2NC S18L replicated in CEM-SS cells, but NL4-3 2NC S18L replication peaked faster than that of NL4-3 2NC W10F. When averaged across three independent experiments, NL4-3 replication peaked 9 days postinfection, while NL4-3 2NC W10F/S18L replicated only slightly more slowly than NL4-3 in CEM-SS cells. On average, NL4-3 2NC W10F/S18L replication peaked 11 days postinfection, just 2 days later than for NL4-3. In sum, the combination of just two amino acid substitutions, W10F and S18L, in the first zinc finger of HIV-2 NC completely restored RNA genome packaging and nearly completely restored replication kinetics of the NL4-3 2NC chimera. Thus, these two residues are key determinants of Gag-RNA interactions in this chimera. These findings indicate that recombinants of distantly related lentiviruses may be able to repair defective interactions between cis- and trans-acting elements through a small number of mutations.

**DISCUSSION**

RNA viruses have evolved several mechanisms to exchange genetic information. RNA viruses with segmented genomes can reassort segments during virus replication to...
generate hybrid viruses (33, 34), whereas RNA viruses with nonsegmented genomes generate hybrid viruses using recombination, which occurs by template switching during viral nucleic acid synthesis (35). Recombination occurs frequently in many plus-strand RNA viruses but less frequently in minus-strand RNA viruses (36–38). In addition, retroviruses package two complete copies of their RNA genome into each particle, thereby facilitating frequent template switching during reverse transcription and enhancing recombination (1, 6).

Retroviruses from distantly related lineages can recombine to generate novel viruses. In the case of SIV, phylogenetic analysis suggests that these recombination events have happened at least 13 times in the past, including the generation of SIVcpz, the precursor to HIV-1 (11, 13). Lentiviruses have complex genome structures; additionally, many steps of the viral replication cycle rely on intricate interactions between cis- and trans-acting elements. In a chimeric virus, cis- and trans-acting elements derived from genetically distinct parental viruses may not function well together; we hypothesized that adaptation must occur to repair these defective interactions and allow efficient viral replication. In this study, we examined the replication and adaptation of HIV-1-based Gag chimeras containing portions of HIV-2 NC that exhibited impaired recognition of the HIV-1 packaging signal and defects in viral replication. We found that the NL4-3 2NC chimera acquired a key amino acid substitution (S18L) during viral replication, which led to improved genome packaging and replication kinetics. Further analyses revealed that the combination of S18L with one additional substitution, W10F, resulted in the complete recovery of RNA genome packaging and near complete recovery of replication kinetics. Of the two substitutions, S18L was more critical, as it had greater effects on genome packaging, infectivity, and replication kinetics than W10F. These studies demonstrate that chimeric lentiviruses can repair defective interactions.
between cis- and trans-acting elements through a surprisingly small number of mutations. Further, these findings support our hypothesis that adaptation is a critical step in the generation of interlineage recombinant lentiviruses that can efficiently replicate.

Previous studies of Gag chimeras with heterologous NC domains have led to various conclusions. In some reports, swapping NC domains between viruses altered RNA packaging specificity, whereas in others it did not (29, 31, 39–42). However, none of the previous studies examined viral replication kinetics, likely because the chimeras were not able to replicate. To our knowledge, our study is the first to examine viral replication kinetics and adaptation of viruses that express chimeric Gag proteins. In our study, we found that HIV-1/HIV-2 Gag chimeras had an intermediate phenotype distinct from that of either parent: the chimeras could package HIV-1 RNA, unlike HIV-2 Gag, but they preferentially packaged HIV-2 RNA, unlike HIV-1 Gag. These results indicate that HIV-2 NC recognizes the HIV-1 packaging signal, at least within the context of the chimeric Gag protein, but that Gag-RNA interactions are impaired due to the W10/S18 differences in the first zinc finger.

Intriguingly, it was previously shown that the first zinc finger was more important than the second zinc finger of HIV-1 NC in RNA packaging (43). Additionally, mutations in Gag can compensate for imperfect Gag-5’ UTR interactions to improve genome packaging (44–46). The W10F and S18L substitutions in HIV-2 NC represent changes from conserved HIV-2 residues to conserved HIV-1 residues at these positions. Specifically, S/T18 in HIV-2 is equivalent to I/L24 in HIV-1, whereas W10 in HIV-2 is equivalent to F16 in HIV-1. In previous structural studies, F16 and I24 in HIV-1 NC formed part of a hydrophobic cleft that bound to an unpaired guanosine in the tetraloop of stem-loop 2 (SL2) or SL3 (47, 48). SL2 and SL3 are stem-loop elements located in the HIV-1 RNA 5’ UTR that play important roles in RNA packaging.

To better understand how the W10F and S18L substitutions led to improved HIV-1 RNA packaging, we performed molecular modeling studies of HIV-2 ZF1 (with or without the W10F and/or S18L substitutions) to examine the influence of these residues on the binding interface between NC and HIV-1 SL3 RNA. HIV-1 ZF1 and HIV-2 ZF1 were found to have similar tertiary structures (Fig. 10A). However, the HIV-2 ZF1 W10 residue exhibited extensive steric clashes with the tetraloop bases of HIV-1 SL3 RNA that could not be alleviated by local energy minimization of the W10 residue (Fig. 10B to D). In the context of HIV-2 ZF1, the W10F substitution alleviated these intermolecular clashes (Fig. 10E and F). This finding suggests that steric interference prevents the bulky indole ring of W10 from interacting with the HIV-1 SL3 tetraloop (bases 209G to 212G). While the S18 residue did not exhibit steric clashes with SL3, the side chain lacked the correct orientation to participate in hydrogen bonding with guanosine 212 (212G). However, with the S18L substitution, the leucine Cβ atoms were positioned in an orientation that may lead to favorable stacking interactions with 212G (Fig. 10G and H), similar to the base stacking and hydrophobic space filling frequently found at RNA-protein interfaces (49). Cumulatively, these results indicate that the W10F and S18L substitutions in the HIV-1 2NC chimera may improve the recognition of HIV-1 RNA by relieving steric clashes (W10F) and creating base stacking interactions (S18L).

NC is involved in multiple steps of HIV-1 replication. In addition to genome packaging, NC also plays important roles during reverse transcription and integration (26, 30, 50–53). NL4-3 2NC and 2ZF had greater defects in single-cycle infectivity (Fig. 4B) than in genome packaging (Fig. 2C). These findings indicate that the disruption of NC-nucleic acid interactions also interferes with other steps during viral replication, such as reverse transcription and/or integration. Intriguingly, NL4-3 2NC S18L/W10F replicated almost as quickly as WT NL4-3 (Fig. 9C and D), indicating that these two substitutions largely repaired any additional defects. Structural studies will be required to fully elucidate the mechanistic basis for the effects of these substitutions on NC-nucleic acid interactions. Of the two chimeras, NL4-3 2NC replicated better than NL4-3 2ZF despite more residues being altered. We speculate that interaction/cooperation between the N-terminal leader and zinc fingers of HIV-2 NC may allow better replication of NL4-3
NC. Alternatively, the long N-terminal leader of HIV-1 NC may interfere with the HIV-2 zinc fingers, thereby causing further replication defects of NL4-3 2ZF.

In West Africa, HIV-1/HIV-2 dual infection has been documented in multiple reports (54–56). However, natural HIV-1/HIV-2 recombinants have not been identified, even though we showed previously that HIV-1 and HIV-2 can copackage and recombine (14) and in the current report that certain HIV-1/HIV-2 chimeras can replicate. We note several possible explanations for the lack of naturally occurring HIV-1/HIV-2 recombinants: First, in dually infected individuals, the proportion of cells infected with both HIV-1 and HIV-2 may be very low; HIV-1 and HIV-2 use the same receptor/coreceptors and thus are subject to superinfection interference (57). Second, genome copackaging requires expression of both RNAs in the same cell. HIV-2 can suppress HIV-1 gene expression, partly because HIV-2 cis-acting elements outcompete their HIV-1 counterparts for binding to Tat and Rev (58–60). Third, HIV-1 and HIV-2 recombination is rare due to limited sequence homology (61–63). Lastly, HIV-1/HIV-2 recombinants are likely often defective and unable to replicate. However, as shown in our study, even a crippled recombinant that replicates at a low level might be able to adapt relatively quickly. We also cannot rule out the possibility that HIV-1/HIV-2 recombinants have arisen naturally but have not been detected due to limited study. Collectively, our findings illustrate the remarkable adaptability of retroviruses and highlight the potential emergence of novel and unexpected recombinants in the future.

MATERIALS AND METHODS

**Plasmids.** The HIV-1 vectors for single-virion analysis, 1-Gag-BSL and 1-Gag-CeFP-BSL, have been previously described (24). These constructs encode functional Gag (or Gag-CeFP), Tat, Rev, and Nef but not GagPol, Vif, Vpr, Vpu, or Env. Additionally, these constructs contain 18 copies of the Bgl stem-loop (BSL) in the pol gene. Portions of HIV-2 NC were introduced into 1-Gag-BSL and 1-Gag-CeFP-BSL by

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**FIG 10** Molecular modeling of HIV-2 ZF1 interactions with HIV-1 stem-loop 3 (SL3) RNA. Purple residues, HIV-1 ZF1; orange residues, HIV-2 ZF1; blue bases, HIV-1 SL3 RNA; white sphere, zinc ion in HIV-1 ZF1; gray sphere, zinc ion in HIV-2 ZF1; red solid lines, steric clashes; green dotted lines, stacking interactions. (A) Overlay of HIV-1 ZF1 (purple) and HIV-2 ZF1 (orange). (B) Steric clashes between HIV-2 ZF1 W10 and HIV-1 SL3 RNA, based on alignment with the HIV-1 NC-SL3 structure. (C) 2D projection of SL3 bases (blue) which contact HIV-1 ZF1 residues (purple) at the protein-RNA interface and proximal SL3 bases (gray). (D) 2D projection of SL3 bases (blue) which contact HIV-2 ZF1 residues (orange) at the protein-RNA interface and proximal SL3 bases (gray). (E) Overlay of HIV-1 ZF1 and HIV-2 ZF1 with W10F and S18L substitutions. (F) Decreased steric clashes between HIV-2 ZF1 W10F and SL3. (G) Stacking interactions between HIV-2 ZF1 S18L and SL3 212G. (H) 2D projection of HIV-2 ZF1 with W10F and S18L substitutions and SL3 bases at the protein-RNA interface. The gray dotted line indicates the relative change in residue position with the W10F substitution.
inserting DNA fragments containing chimeric genes (Integrated DNA Technologies [IDT]) using Splh and Xmal sites. The previously described HIV-2 RNA expression construct, 2-fsGag-MSL, encodes YFP, Vpx, Tat, and Rev but not Gag, GagPol, Vpr, Env, or Nef (25). Additionally, 2-fsGag-MSL contains 24 copies of MS2 stem-loop (MSL) in the pol gene. The Bgl-YFP, Bgl-mCherry, and MS2-YFP constructs were described previously (24, 64).

To generate replication-competent vectors, DNA fragments containing HIV-2 NC or zinc fingers were transferred from single-virion analysis vectors into pNL4-3. Point mutations encoding amino acid substitutions (e.g., S18L) were introduced into viral vectors using PCR and HiFi DNA assembly master mix (New England Biolabs [NEB]). All new constructs were verified by restriction digest mapping and sequencing of the entire cloned region.

**Cell lines.** The 293T human embryonic kidney cells were obtained from the American Type Culture Collection (catalog no. CRL-3216). TZM-bl cells were maintained in the NIH AIDS Reagent Program (catalog no. B129), Division of AIDS, NIAID, NIH, from John Kappes and Xiaoyun Wu. TZM-bl cells were derived from HeLa cells that stably express CD4, CXCR4, and CCR5; these cells also express beta-galactosidase and firefly luciferase upon HIV-1 infection (65, 66). The 293T and TZM-bl cells were obtained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. MT4 human T cells were a kind gift from Eric Freed (67). CEM-SS human T cells were obtained through the NIH AIDS Reagent Program (catalog no. 776), Division of AIDS, NIAID, NIH, from Peter Nara (68). MT4 and CEM-SS cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

**Single-virion analysis.** To determine HIV-1 RNA packaging efficiencies, 293T cells were transfected with 50 ng Gag-CeFP construct, 50 ng Gag (untagged) construct, and 1 μg Bgl-YFP using TransIT-LT1 (Mirus). To investigate whether there was a preference for packaging HIV-1 or HIV-2 RNA, 293T cells were transfected with 50 ng Gag-CeFP construct, 50 ng Gag (untagged) construct, 200 ng 2-fsGag-MSL, 1 μg Bgl-mCherry, and 1 μg MS2-YFP using TransIT-LT1. Virus-like particles were collected 20 h posttransfection, clarified through 0.45-μm-pore-size filters, and plated onto glass slides (μ-Slide 8-well ibiTreat; Ibidi) with Polybrene (50 μg/mL final concentration). The slides were centrifuged at 1,200 × g for 1 h at room temperature and imaged using fluorescence microscopy as previously described (69, 70). Images were analyzed using custom MATLAB (MathWorks) programs to determine the percentage of CeFP⁺ particles that contained HIV-1 RNA and/or HIV-2 RNA. At least 1,000 Gag-CeFP⁺ particles were analyzed per sample for each biological replicate, with at least three replicates performed for each construct.

**Replication kinetics.** Virus stocks were prepared by transfecting 293T cells with 10 μg plasmid (WT NL4-3 or chimeras) per 10-cm plate using TransIT-LT1. Virus supernatants were collected 48 h posttransfection, clarified through 0.45-μm-pore-size filters, and quantified by p24 ELISA (XpressBio). MT4 or CEM-SS cells were infected with 0.1 or 10 ng p24 of each virus, using 1 million cells and 3 mL medium per infection. Infections were performed in duplicate using the same virus stock (i.e., technical replicates). In addition, infections were performed at least three independent times (i.e., biological replicates) to ensure that differences in replication kinetics were reproducible. After the infections were initiated, cells and virus supernatants were collected every other day beginning 3 days after infection. Briefly, 2 mL of each culture were removed and 1 mL of the removed culture was centrifuged at 16,000 × g for 10 min. Supernatants and cell pellets were then separated and frozen at −80°C. The residual ~1 mL of removed culture was discarded. Lastly, 2 mL of fresh medium was added to the remaining 1 mL of culture to continue propagating the cells. The amount of virus in each supernatant was determined by p24 ELISA. To test for virus adaptation, the same protocol was followed, but virus stocks were from a previous spreading infection experiment rather than from 293T transfection. Putative adaptive mutations were identified by purifying genomic DNA from cell pellets (High Pure PCR template preparation kit; Roche), performing PCR of the 5′ UTR/gag region (using Phusion Hot Start II high-fidelity polymerase; Thermo Scientific), gel purifying the PCR product, and sequencing the entire amplicon.

**Single-cycle infectivity assay.** Virus stocks were produced and quantified by p24 ELISA as described above and used to infect TZM-bl indicator cells (65, 66). TZM-bl cells were plated in white 96-well plates using 4,000 cells per 0.1 mL medium per well. The medium was removed 24 h later and replaced with virus and medium to a final volume of 0.2 mL per well. Cells were infected with 1 ng p24 of virus per well. Each infection was performed in triplicate (technical replicates), and uninfected cells were included as controls. Six hours after infection, the medium was removed and fresh medium containing 0.5 μM of the protease inhibitor lopinavir (Sigma) was added to prevent reinfection. Luciferase activity was measured 72 h postinfection using a 96-well luminometer (LUMIstar Galaxy; BMG LABTECH, Cary, NC, USA). Data were analyzed by first subtracting the average luminescence from uninfected cells from that observed with infected cells. Next, luciferase activity was normalized to that of NL4-3, which was set to 100%.

**Western blotting.** To examine Gag expression, release, and processing, 293T cells were transfected with viral constructs, washed with phosphate-buffered saline (PBS), and lysed using Celllytic solution (Sigma) containing cComplete EDTA-free protease inhibitor cocktail tablets (Roche). Cell lysates were centrifuged at 16,000 × g for 10 min at 4°C, and supernatants were transferred to new tubes. Virus lysates were prepared by centrifuging 1 mL of filtered virus stock with a 0.2 mL 20% sucrose cushion at 16,000 × g for 2 h at 4°C. Concentrated virus was resuspended in 0.1 mL PBS. Lastly, for both cell and viral lysates, an appropriate volume of 4× Laemmlli sample buffer (Bio-Rad) containing 10% beta-mercaptoethanol was added, and the samples were incubated at 99°C for 5 min. SDS-PAGE was performed using 4% to 20% Criterion TGX precast gels (Bio-Rad). After transfer to polyvinylidene difluoride (PVDF) membranes, blots were probed with mouse anti-p24 antibody (at 1:5,000 dilution), obtained from the NIH AIDS Reagent Program (catalog no. 6458), Division of AIDS, NIAID, NIH, from Michael Malim, followed by goat anti-mouse IgG secondary antibody (IRDye 800CW; LI-COR) at 1:10,000 dilution. The blots were also probed with rabbit anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody.
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