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# Interactions between HIV-1 Gag and Viral RNA Genome Enhance Virion Assembly

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**ABSTRACT** Most HIV-1 virions contain two copies of full-length viral RNA, indicating that genome packaging is efficient and tightly regulated. However, the structural protein Gag is the only component required for the assembly of noninfectious virus-like particles, and the viral RNA is dispensable in this process. The mechanism that allows HIV-1 to achieve such high efficiency of genome packaging when a packageable viral RNA is not required for virus assembly is currently unknown. In this report, we examined the role of HIV-1 RNA in virus assembly and found that packageable HIV-1 RNA enhances particle production when Gag is expressed at levels similar to those in cells containing one provirus. However, such enhancement is diminished when Gag is overexpressed, suggesting that the effects of viral RNA can be replaced by increased Gag concentration in cells. We also showed that the specific interactions between Gag and viral RNA are required for the enhancement of particle production. Taken together, these studies are consistent with our previous hypothesis that specific dimeric viral RNA-Gag interactions are the nucleation event of infectious virion assembly, ensuring that one RNA dimer is packaged into each nascent virion. These studies shed light on the mechanism by which HIV-1 achieves efficient genome packaging during virus assembly.

**IMPORTANCE** Retrovirus assembly is a well-choreographed event, during which many viral and cellular components come together to generate infectious virions. The viral RNA genome carries the genetic information to new host cells, providing instructions to generate new virions, and therefore is essential for virion infectivity. In this report, we show that the specific interaction of the viral RNA genome with the structural protein Gag facilitates virion assembly and particle production. These findings resolve the conundrum that HIV-1 RNA is selectively packaged into virions with high efficiency despite being dispensable for virion assembly. Understanding the mechanism used by HIV-1 to ensure genome packaging provides significant insights into viral assembly and replication.

**KEYWORDS** HIV-1, RNA genome, genome packaging, virus assembly

Gag proteins drive retrovirus assembly. In the absence of other viral components, the expression of many orthoretroviral Gag proteins in cell culture is sufficient to generate virus-like particles (1–3). Additionally, under appropriate conditions, purified Gag proteins can generate particles *in vitro* (4–8). Although Gag alone can multimerize to form particles, it must interact with multiple viral and host components during the assembly process to generate infectious virions. The viral RNA genomes and other viral proteins, such as Gag-Pol, as well as cellular factors must be recruited to facilitate the release of infectious particles from the host cells (reviewed in references 2, 9, and 10).

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Like all retroviral Gags, HIV-1 Gag contains three conserved domains: the matrix (MA), the capsid (CA), and the nucleocapsid (NC). Additionally, HIV-1 Gag also contains p6, the spacer peptide 1 (SP1) between CA and NC, and the spacer peptide 2 (SP2) between NC and p6. HIV-1 Gag is expressed as a polyprotein and processed at the domain junctions during or soon after virus assembly/budding (2). To drive assembly, HIV-1 Gag participates in Gag-Gag, Gag-membrane, and Gag-RNA interactions. The Gag-Gag interactions rely heavily on the CA domain of Gag proteins. The Gag-membrane interaction is mediated by the N-terminal MA domain of Gag, which targets Gag to the inner leaflet of the plasma membrane, which is the major site of Gag multimerization and particle assembly. The Gag-RNA interaction is mediated through a specific interaction between the NC domain of Gag and sequences in the viral genome, including those in the 5′ untranslated region (UTR) (2, 9, 10).

To better understand the regulation of HIV-1 RNA genome packaging, we have previously developed an imaging-based assay termed single-virion analysis (11). Briefly, we engineered HIV-1 genomes to harbor stem-loop sequences embedded in the *pol* gene recognized by RNA-binding proteins so that only full-length, but not spliced, viral RNAs contain such sequences. When coexpressed with RNA-binding proteins tagged with fluorescent proteins such as yellow fluorescent protein (YFP) or mCherry, HIV-1 RNA can be specifically labeled and visualized. We also tagged HIV-1 Gag with cerulean fluorescent protein (CeFP); when Gag-CeFP is coexpressed with untagged Gag, particles that are morphologically indistinguishable from immature HIV-1 particles can be generated (11, 12). In this system, viral particles can be imaged using fluorescence microscopy: the Gag-CeFP signal is used to identify HIV-1 particles, and other fluorescence signals, such as YFP or mCherry, can be used to identify the presence of viral RNA. Using this method, we were able to determine that most HIV-1 particles contain the viral RNA genome; furthermore, two copies of the genome are packaged (11). We further examined the mechanism that regulates HIV-1 RNA genome packaging and found that two copies of viral RNAs are packaged regardless of whether the RNAs were 17 kb or 3 kb, indicating that HIV-1 RNA packaging is not regulated by the mass of the RNA genome (12). To examine whether HIV-1 RNA packaging is regulated by the recognition of two copies of RNA or one dimeric RNA, we engineered the HIV-1 genome to contain a second dimerization signal as previously described (13, 14). The two dimerization sites in the same RNA molecule can interact to form a "self-dimer"; we found that in this case, only one copy of the RNA, or one "self-dimer," is packaged. These studies indicate that one dimeric RNA, not two monomers, is the packageable unit that is encapsidated into the viral particles (12).

Although incorporation of viral RNA is not necessary for particle assembly in a cell, it is essential to generate an infectious particle. Given the specificity of Gag for its cognate RNA genome, it is remarkable that a virus particle composed of a few thousand Gag molecules, each with an NC domain that binds RNA, would contain one, but not more than one, RNA dimer. We hypothesized that the specific interaction between Gag and the viral RNA genome is the nucleation point of the virus assembly; once this interaction takes place, the dynamics or kinetics of the virus assembly makes it less likely to incorporate another RNA dimer (12).

RNA plays an intriguing role in the assembly of several retroviruses. It has been shown that RNA is an integral part of murine leukemia virus (MLV) virion structure (15). However, in the absence of MLV RNA, host cellular mRNAs can replace this function (15–17). Similarly, the HIV-1 RNA genome is not required for virus assembly *in vitro* or in cell culture systems, as viral particles can be generated in the absence of the viral RNA genome (7, 16). The fact that the HIV-1 assembly does not require the viral RNA genome seems to be contradictory to our hypothesis that the Gag-RNA genome interaction serves as the nucleation point of virus assembly. It is very likely that although not absolutely required for assembly, the Gag-RNA interaction enhances virus assembly and provides the Gag-viral RNA complex with an advantage for assembly.

In this study, we aimed to define the role of HIV-1 RNA in virus assembly in the cell. We show that the HIV-1 RNA genome provided in *trans* enhances the production of

HIV-1 particles and this enhancement requires viral RNA specifically recognized and packaged by Gag. We also show that the RNA-dependent enhancement is diminished at high Gag expression levels but is observed at Gag expression levels similar to those in cells containing one integrated provirus. These studies indicate that HIV-1 RNA plays a role in facilitating virus assembly.

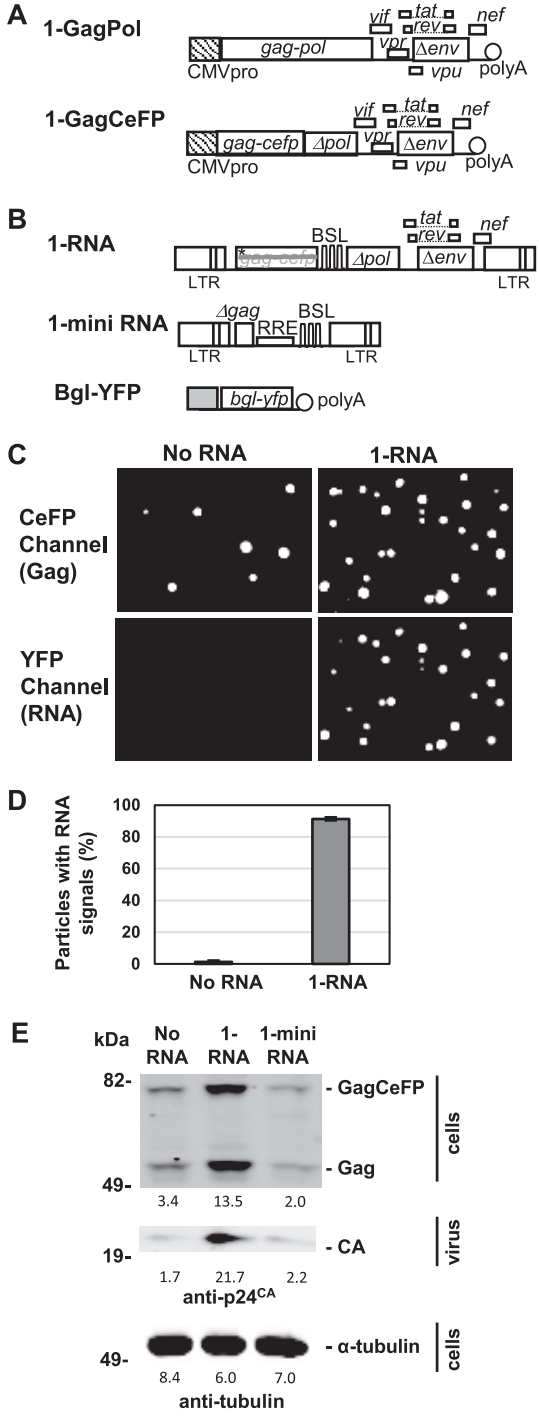
## RESULTS

**HIV-1 viral genomic RNA stimulates virus particle production.** Full-length HIV-1 RNA serves both as the mRNA template for Gag/GagPol translation and as the viral genome that is encapsidated into assembling virions. To examine the effect of viral RNA on assembly, we uncoupled these two functions of HIV-1 RNA by using separate plasmids to express HIV-1 Gag and packageable viral RNA. Specifically, Gag polyproteins were expressed from cytomegalovirus (CMV) promoter-driven helper constructs, 1-GagPol and 1-GagCeFP, that lack the HIV-1 packaging signal so that their RNAs are not efficiently packaged into viral particles (Fig. 1A). RNA that can be packaged by HIV-1 Gag was expressed from a construct, 1-RNA, that encodes a nearly full-length HIV-1 genome containing a frameshift mutation in *gag* and stem-loops (BSL) in the *pol* gene that are recognized by the *Escherichia coli* BglG protein (Fig. 1B). To examine virus production and 1-RNA incorporation, we performed single-virion analyses (11). Briefly, we transfected equal amounts of 1-GagPol and 1-GagCeFP plasmids along with a plasmid expressing Bgl-YFP, harvested viruses, and captured the images using fluorescence microscopy. Bgl-YFP is a truncated BglG and YFP fusion protein that specifically binds to BSL (Fig. 1B). Viral particles were detected by signals in the CeFP channel, whereas the 1-RNA signals were detected by the YFP channel; representative images of particles generated with 1-RNA and without 1-RNA ("no RNA") are shown in Fig. 1C. We consistently found more CeFP particles when Gag proteins were coexpressed with the 1-RNA construct (Fig. 1C). Furthermore, 1-RNA was efficiently packaged into the Gag particles because most of the CeFP particles from the samples cotransfected with 1-RNA contained YFP signals (Fig. 1C); the results of RNA incorporation into HIV-1 particles from three sets of independent experiments are summarized in Fig. 1D.

Plasmids 1-GagPol, 1-GagCeFP, and 1-RNA were all derived from HIV-1 molecular clone NL4-3, and RNAs expressed from these constructs contain largely HIV-1 sequences. However, the 1-GagPol and 1-GagCeFP contain deletions in the 5′ UTR, including elements important for RNA packaging (18), whereas 1-RNA contains the intact 5′ UTR and its RNA can be efficiently packaged. Therefore, these results suggest the possibility that the presence of packageable RNA enhances HIV-1 particle production.

The particle counts determined using single-virion analyses do not address the expression of Gag in the producer cells. To determine whether the presence of HIV-1 RNA can enhance particle production, we examined the amounts of Gag and CA in the producer cells and released particles in the supernatant by Western blotting and quantified these results (Fig. 1E). The efficiency of particle production was determined as the amount of CA in supernatant divided by the total amount of CA and Gag, which is calculated by adding the amount of CA in the supernatant with the amount of CA and Gag in the cell lysate. We then set the efficiency of particle production from the "no RNA" sample, in which only 1-GagPol and 1-GagCeFP plasmids were transfected, to 1; when normalized to the "no RNA" control, virus production was increased ~2.5-fold in samples cotransfected with the 1-RNA construct ($P = 0.005$, *t* test) (Fig. 1F).

Although the 1-RNA construct does not express Gag, it does express both Tat and Rev. To address the possibility that the increase in particle production was the result of additional Tat and Rev expression contributed by the 1-RNA plasmid, we used a previously described minimal RNA construct (12), termed 1-mini RNA. The 1-mini RNA construct expresses an ~3-kb HIV-1-based RNA that contains the native 5′ UTR, ~350 nucleotides (nt) of *gag* sequence, Rev response element (RRE), BSL, and 3′ UTR but does not express Gag, Tat, or Rev (Fig. 1B). Cotransfection of 1-GagPol, 1-GagCeFP, and the 1-mini RNA construct resulted in a slight reduction in Gag and GagCeFP expression in



cells, possibly due to competition for Tat and Rev (Fig. 1E). However, the efficiency of particle production is increased compared to the no-RNA control ($P = 0.01$, *t* test) (Fig. 1F). These results indicate that packageable HIV-1 RNAs enhance HIV-1 particle production and this activity is independent of the expression of Tat and Rev but is due to the presence of packageable RNA.

**RNA genome-mediated enhancement of particle production is dependent on Gag expression levels.** HIV-1 particle assembly is dependent upon the level of Gag expression in the cells (19). To determine whether the Gag expression levels affect the RNA genome-mediated enhancement of particle production, we transfected increasing amounts of 1-GagPol and 1-GagCeFP DNA into 293T cells and compared particle productions in these samples using Western blot analyses. A set of representative Western blots are shown in Fig. 2A. Results from three sets of independent experiments are summarized in Fig. 2B; in each pair of samples, the virus production level of the helper plasmid transfection without HIV-1 RNA (no RNA) was set as 1. Although HIV-1 RNA enhanced particle production when 0.2 $\mu$g of the Gag-expressing plasmids was transfected, this enhancement became less apparent when more Gag-expressing plasmids were used, indicating that the benefit of the RNA genome can be replaced by large amounts of Gag expression. For example, HIV-1 RNA had little effect on particle production when 0.8 $\mu$g of the Gag-expressing plasmids was transfected ($P = 0.06$, *t* test) (Fig. 2B). This observation is consistent with the hypothesis that the RNA genome can act as a nucleation point for virus assembly and suggests that the need of the viral RNA-dependent nucleation can be overcome in the presence of high levels of Gag.

We have also examined the viral RNA content of the viral particles generated by transfecting different amounts of Gag-encoding plasmids while maintaining the same amount of 1-RNA plasmid; the results from three sets of experiments are summarized in Fig. 2C. We found that the majority of the Gag particles in the 1-RNA group contain viral RNA signals under all experimental conditions; however, the proportion of particles without the viral RNA signals increased when a larger amount of Gag-encoding plasmids was used. These results suggest that under the experimental condition with higher Gag expression, more viral particles were generated without encapsidating viral RNA.

To determine whether the conditions in which we observe RNA-dependent enhancement of particle production were similar to those in an HIV-1-infected cell, we compared the Gag expression levels of our transfected cells to those of a cell line containing one integrated provirus. Western analyses using lysates from cells transfected with 0.2 $\mu$g of the helper plasmids and from T6-2, a cell line in which 60 to 80% of the cells express an HIV-1 provirus, were performed. T6-2 was generated by infecting 293T cells at a low multiplicity of infection (MOI), $<0.1$, with viruses derived from HIV-1 vector pON-T6 (20), which expresses Gag/Gag-Pol, Tat, Rev, and a mouse Thy1.2 protein; cells expressing the mouse Thy1.2 protein were enriched by repeated cell sorting. The Western blots were first probed with an antibody against HIV-1 CA (p24), followed by an antibody against $\alpha$-tubulin. Using the host protein $\alpha$-tubulin level as a

**FIG 1** Examining the effects of the packageable HIV-1 RNA on particle production. (A) General structures of HIV-1 helper constructs that express HIV-1 Gag/GagPol or HIV-1 Gag tagged with CeFP. (B) General structures of constructs that express packageable HIV-1 RNAs but not Gag proteins and fusion protein Bgl-YFP. (C) Representative images of HIV-1 particles generated in the absence (no RNA) and presence (1-RNA) of packageable HIV-1 RNA. Viral particles were detected using the CeFP channel, whereas 1-RNA signals were detected using the YFP channel. (D) Proportion of HIV-1 particles containing 1-RNA (YFP) signal. Results shown are averages from three experiments; error bars represent standard deviations. (E) Representative Western blots of cell lysates (top panel) and virus (middle panel) from 293T cells transfected with the helper constructs 1-GagPol and 1-GagCeFP without packageable HIV-1 RNA (No RNA), with 1-RNA, or 1-mini RNA and probed with anti-p24$^{CA}$ antibody. The bottom panel shows cell lysates immunoblotted for $\alpha$-tubulin as a loading control. Equal amounts of 1-GagPol and 1-GagCeFP were transfected. Quantitation of the Western blot data is shown below the panels. (F) Particle production measured by quantitation of the Western blot data from three independent experiments and expressed as the CA signal in the supernatant divided by the total CA/Gag signal in both supernatant and cell lysates and then normalized to the "No RNA" sample. Error bars represent standard deviations of the means from the three experiments.

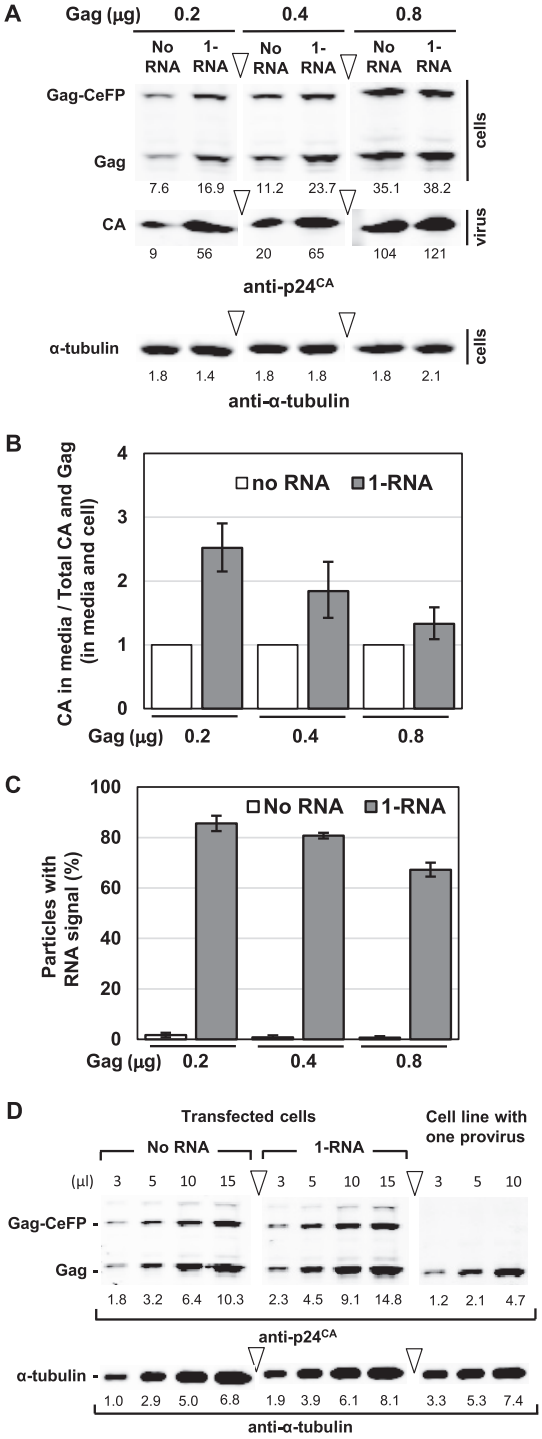



**FIG 2** Examining the effects of Gag concentration on viral RNA-dependent enhancement of particle production. (A) Representative Western blot analyses of cell lysate and viral particles generated from transfection of various amounts of Gag-encoding plasmids. (B) Particle production in the absence (white bars) or presence (gray bars) of 1-RNA over increasing amounts of transfected Gag helper DNA (1-GagPol and 1-GagCeFP). Particle production is expressed as the CA signal in the supernatant divided by the total CA/Gag signal in both supernatant and cell lysates and then normalized to the "No RNA" sample. Error bars represent the standard deviations from the means from three independent experiments. (C) Proportion of viral particles containing RNA signals. Results from three independent single-virion analyses experiments are shown; error bars show standard deviations. (D) Dilutions of cell lysates from cells transfected with 0.2 $\mu$g of Gag expression plasmids (left and center) or the T6-2 cell line in which ~60 to 80% of the cells harbor one provirus (right). Western blots of cell lysates were first probed with anti-p24$^{CA}$ antibody and then stripped and reprobed with anti-$\alpha$-tubulin antibody, which served as a loading control. Triangles indicate that all panels are from the same immunoblot but were cropped and repositioned for clarity. Quantitation of the Western blot is shown below the panels.

loading control and adjusting for the percentage of T6-2 cells that contain an HIV-1 provirus, we observed that the level of Gag expression in cells harboring one T6 provirus was close to that in our cells transfected with 0.2 $\mu$g of the helper plasmids (Fig. 2D). Furthermore, the Gag expression levels in the T6-2 cells did not reach the levels of those in the 0.8-$\mu$g plasmid transfection samples. These results imply that when Gag is expressed from one integrated provirus, the presence of viral RNA is likely to enhance HIV-1 particle assembly.

**Specific recognition between Gag and packageable RNA is required to enhance particle production.** If the viral RNA genome is a nucleation point of HIV-1 particle assembly, then specific recognition between Gag and RNA must play an important role for this enhancement. To test this hypothesis, we examined the effects of an HIV-1 RNA with deletion in the packaging signal on HIV-1 particle production and the effect of HIV-1 RNA on the particle production of MLV Gag, which does not efficiently package HIV-1 RNA (21, 22). Based on the aforementioned HIV-1 construct 1-RNA, we generated MΨ-RNA with the following two modifications: first, the 5′ long terminal repeat (5′ LTR) and most of the 5′ UTR were replaced with the CMV promoter, and second, an extended MLV packaging signal $\Psi^+$ was inserted in the *pol* gene (Fig. 3A). The previously defined MLV $\Psi^+$ has been shown to be modular and can function in positions other than the 5′ UTR in the viral RNA (23, 24). To confirm that the deletion of the 5′ UTR reduces RNA packaging by HIV-1 Gag, we performed single-virion analysis. Our results from four set of experiments are summarized in Fig. 3C and show that 1-RNA, but not MΨ-RNA, is efficiently packaged by HIV-1 Gag.

To test the function of the MLV packaging signal in MΨ-RNA, we modified the MLV GagPol expression construct pLGPS (25) by replacing the MLV U3 promoter with a CMV promoter to generate MLV-GagPol (Fig. 3B). We also generated a structurally similar MLV-Gag-mCherry construct that expresses MLV Gag-mCherry by inserting an mCherry gene at the end of Gag (Fig. 3B); although not translated, the *pol* gene sequence is maintained in this construct to facilitate Gag expression (26–28). Equal amount of MLV-GagPol and MLV-Gag-mCherry helper plasmids, neither of which contains the MLV packaging signal, were transfected into 293T cells alone ("no RNA" sample), with the 1-RNA, or with MΨ-RNA construct. The results from four sets of single-virion analyses experiments are summarized in Fig. 3C and show that the MΨ-RNA was packaged into MLV particles far more efficiently than the 1-RNA. Thus, the MLV packaging signal is functional in the context of MΨ-RNA.

These packaging experiments showed that 1-RNA can be efficiently packaged by HIV-1 Gag but not MLV Gag, whereas MΨ-RNA can be packaged more efficiently by MLV Gag than HIV-1 Gag. We then tested the ability of these RNAs to enhance particle assembly by cotransfecting cells with one set of Gag expression constructs and an RNA expression construct. Cell lysates and supernatant were harvested and analyzed in Western blots, which were probed with either an antibody against HIV-1 CA (p24) or an antibody against MLV CA (p30); the efficiency of particle production was quantitated and expressed as the ratio of CA signal in the supernatant to the total CA and Gag signals in the supernatant and in the cell lysate. One set of representative Western blot analyses is shown in Fig. 3D. The results from 5 sets of HIV-1 Gag experiments are shown in Fig. 3E; compared with the "no RNA" control, HIV-1 particle production is enhanced by 1-RNA ($P = 0.01$, *t* test) but not by MΨ-RNA ($P = 0.98$, *t* test). In contrast, the results from 3 sets of MLV Gag experiments showed that MLV particle production is enhanced by MΨ-RNA ($P = 0.002$, *t* test) but not by 1-RNA ($P = 0.9$, *t* test). Therefore, the enhancement of assembly is dependent upon the specific recognition between Gag and the viral RNA genome.

## DISCUSSION

During the assembly of an infectious virion, HIV-1 Gag needs to interact with viral components and host cell factors. Among these, the viral RNA genome needs to be packaged into the particle to allow the transfer of the viral genetic information to the new host cell. In this report, we investigated an apparent conundrum in HIV-1 assembly:

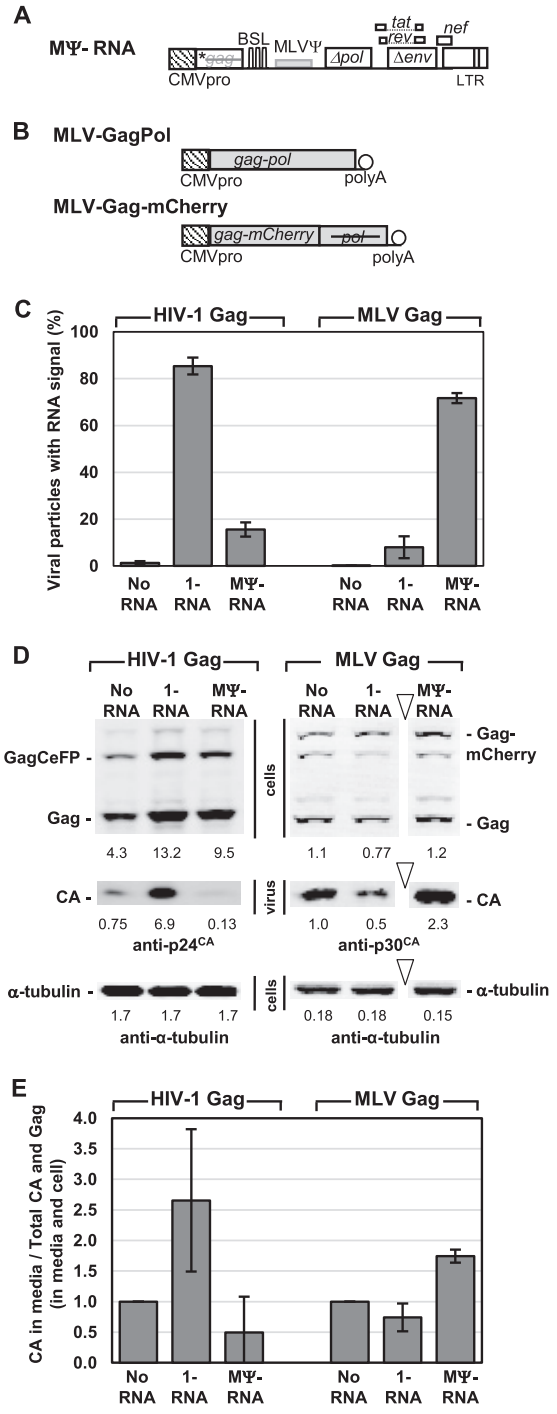



**FIG 3** Determining the specificity of retroviral RNA-dependent enhancement of particle production. (A) General structures of HIV-1-based constructs expressing RNA packageable by MLV Gag (MΨ-RNA). (B) General structures of MLV helper constructs MLV-GagPol and MLV-Gag-mCherry, which lack the 5′ leader sequences important for RNA packaging. (C) Proportion of viral particles containing RNA signals. Results shown are averages from four experiments. Error bars indicate standard deviations. The efficiencies of RNA packaging into HIV-1 particles are ~88% and ~16% for 1-RNA and MΨ-RNA, respectively. The efficiencies of RNA packaging into MLV particles are ~72% and ~8% for MΨ-RNA and 1-RNA, respectively. (D) Representative Western blot analyses of cellular lysates and viral lysates. Triangles indicate that all panels are from the same immunoblot but were cropped and repositioned for clarity. Quantitation of the Western blot is shown below the panels. (E) Particle production measured by quantitation of Western blot data from five (HIV-1 Gag) and three (MLV Gag) independent experiments. Particle production was measured as the CA signal in the medium divided by the total CA/Gag in both the medium and the cell lysate and then normalized to the "No RNA" sample. Error bars represent the standard deviations of the means.

although viral RNA is not required to generate particles, HIV-1 is extremely efficient at packaging one dimeric RNA genome in most viruses. We now report that although Gag particles can be generated without the viral genome, the presence of the viral RNA enhances particle production. Additionally, the lack of viral RNA genome can be compensated by a high level of Gag expression. These results explain seemingly contradictory observations of HIV-1 RNA packaging and are consistent with our hypothesis that the specific Gag-RNA interaction serves as a nucleation point in virus assembly.

The dynamic interactions between HIV-1 Gag and RNA genome on plasma membrane have been described (29–31). We have previously studied the population dynamics of HIV-1 RNA on the plasma membrane and found that the presence of Gag protein significantly extends the time that HIV-1 RNA resides near the plasma membrane, indicating that most viral RNAs on the plasma membrane are in Gag-RNA complexes (31). We have also examined HIV-1 RNA dimerization using live-cell total internal reflection fluorescence microscopy and observed that HIV-1 RNA molecules interact with each other dynamically on the plasma membrane, often when RNAs are associated with Gag signals. Furthermore, a significant number of HIV-1 Gag molecules are required to stabilize the RNA dimer (30).

It has been suggested that the role of the RNA in assembly is to induce Gag multimerization (32). Indeed, short oligonucleotides of nonviral origin have been shown to facilitate HIV-1 Gag assembly *in vitro* (4). Interestingly, retroviral particles contain multiple RNA species; in addition to the viral genome, many small noncoding RNAs are also packaged in virions (33–35). Some of the small RNAs have known functions for viral replication, such as certain tRNAs, which serve as primers for the initiation of reverse transcription. However, the roles of other small RNAs, such as 7SL RNA, Y RNA, and U6 small nuclear RNA, in viral replication are unclear, including whether such RNAs affect virus assembly. Particles generated in the absence of the viral genome contain cellular mRNAs, suggesting that nonviral RNA may replace the RNA genome in the role of assembly. Furthermore, Gag binds both viral RNA and nonviral RNA in the cytoplasm (36, 37). These observations raised the question as to why viruses are produced more efficiently in the presence of the viral RNA genome. One possibility is that dimeric viral RNA offers numerous specific binding sites, or avidity, to assist the initiation of the assembly-competent complex. The CA-SP1 junction region has been shown to be capable of forming a helical structure to facilitate Gag assembly (38, 39). Interestingly, the coil-to-helical structure transformation is concentration dependent and requires dimerization/multimerization of the SP1 region. It is possible that viral RNA is superior to cellular mRNA in increasing the local concentration of Gag to initiate the cooperative binding (32).

Taken together, we propose the following hypothesis for virus assembly: Gag binds to both HIV-1 RNA and nonviral RNA in the cell and on the plasma membrane. During the initiation of HIV-1 RNA dimerization, the RNA-RNA interaction either exposes or increases the number of specific Gag binding sites, which allows binding of multiple Gag proteins, effectively increasing the local Gag concentration; this process facilitates a switch in Gag conformation, making key surfaces of Gag more accessible for Gag-Gag interactions leading to particle assembly. In contrast, although Gag binds to nonviral RNA, cellular mRNA lacks multiple specific binding sites, and it is less likely for the complex to achieve sufficient local Gag concentration to cross the threshold to initiate assembly. Therefore, although HIV-1 RNA is only a small fraction of the total RNA in a cell, the Gag-dimeric RNA complex has a significant advantage to initiate virus assembly, providing a mechanism to ensure high-efficiency RNA genome packaging by HIV-1.

## MATERIALS AND METHODS

**Plasmids.** Helper construct pCMVΔR8.2 has been previously described (18) and expresses all HIV-1 genes except for *env*; for simplicity, this construct is referred to as 1-GagPol in this report. Plasmid 1-GagCFP is derived from pCMVΔR8.2 by inserting a DNA fragment immediately before the *gag* stop codon that encodes a short linker and the cerulean fluorescent protein (CeFP) gene; the GagCeFP fusion protein is similar to that expressed from the previously described GagCeFP-MSSL (11).

Plasmid MLV-GagPol was derived from the MLV helper construct pLGPS (25) by replacing the MLV U3 promoter with the CMV promoter from pCMVΔR8.2. Plasmid MLV-Gag-mCherry is similar to MLV-GagPol except for a DNA fragment encoding a short linker fused to the mCherry gene, which was inserted immediately before the stop codon of *gag* to express a Gag-mCherry fusion protein. Although it does not express the *pol* gene product, MLV-Gag-mCherry still has the *pol* gene sequence, which has been shown to be important for efficient MLV Gag expression (26–28). The MLV-GagPol plasmid contains an E231G mutation at the C-terminal region of CA; a plasmid lacking the mutation generated results similar to those shown in Fig. 3 (data not shown). Plasmid 1-RNA was derived from NL4-3-based plasmid GagCeFP-BglSL (11) and generated by SphI digestion and Klenow-mediated blunting, followed by ligation, which introduced a frameshift mutation in the *gag* gene. This construct contains 18 copies of the stem-loop sequences (BSL) recognized by the *E. coli* BglG protein in the *pol* gene and does not express functional Gag or CeFP. For clarity, previously described plasmid Mini-Bgl (12) is referred to as 1-mini RNA in this report. Briefly, this construct expresses an ~3-kb packageable HIV-1 RNA that harbors the 5′ UTR, ~350 nucleotides of *gag*, RRE, BSL, and the 3′ UTR but does not express any viral proteins.

Plasmid MΨ-RNA is similar to 1-RNA except for the following modifications: the HIV-1 LTR and 5′ leader sequence were replaced with the CMV promoter from pCMVΔR8.2; as a result, most of the HIV-1 leader sequence is deleted and *gag* contains a frameshift mutation. Additionally, an ~0.9-kb DNA fragment containing MLV $\Psi^+$ was amplified by PCR from the plasmid pAMS (40) and inserted into the *pol* gene. The MLV $\Psi^+$ fragment included sequences from downstream of primer binding site through the first 429 nucleotides of *gag*; however, the splice donor site and ATG start codon of *gag* were mutated. Plasmid pBgl-YFP has been described previously (41).

**Cell culture, transfections, and virus production.** Human embryonic kidney 293T cells were grown in a humidified 37°C incubator with 5% $CO_2$ and maintained in Dulbecco's modified Eagle's medium supplemented with fetal calf serum (10%), penicillin (50 U/ml), and streptomycin (50 μg/ml). Transfections were performed using FuGeneHD (Promega) or TransIT-LT1 (Mirus) reagent according to the manufacturer's recommendations. To measure particle production, the total amount of transfected DNA was kept constant; "no RNA" control samples were supplemented with the pUC18 plasmid (Thermo Scientific). T6-2 is a 293T-based cell pool that was established through infecting cells at a low MOI (<0.1) using a previously described, nearly full-length HIV-1 construct, ON-T6, carrying the mouse thy 1.2 gene (*thy*), followed by multiple rounds of cell sorting to enrich the cells containing provirus expressing the *thy* marker gene (20). As a result, T6-2 consists of a pool of >100,000 infected cells and ~60 to 80% of the cells express the Thy marker.

**Single-virion analysis.** Virus particles were collected by clarifying supernatant through a 0.22-μm- or a 0.45-μm-pore-size filter, mixing with Polybrene, and plating on glass bottom dishes (MatTek Corp.) or μ-Slide ibiTreat 8-well slides (Ibidi) for imaging. Image acquisition was achieved using an inverted Nikon Eclipse Ti microscope, the X-Cite series 120 Q system for illumination, an Andor Technology iXon camera, and NIS Element AR software (Nikon). Custom Matlab (11) and/or Localize (42) software was used to identify, quantitate, and colocalize Gag (CeFP or mCherry) and RNA (YFP) signals.

**Western immunoblot assay.** Virus and cells were harvested 20 h posttransfection. Virus particles were collected by clarifying medium using a 0.45-μm-pore-size filter and pelleting virus by centrifugation at 17,000 × *g* for 3 h in a Sorvall SuperT21 centrifuge at 4°C. Immunoblots were probed for HIV-1 Gag with a mouse anti-$p24^{CA}$ antibody (43) (a kind gift from Michael H. Malim through the NIH AIDS Reagent Program, NIAID, NIH), followed by a secondary goat anti-mouse antibody (IRDye-680RD; LI-COR). MLV Gag was probed with a rabbit anti-p30 antibody (a kind gift from the AIDS and Cancer Virus Program, Leidos, Frederick, Maryland) followed by a secondary goat anti-rabbit antibody (IRDye-800CW; LI-COR). Cell lysate immunoblots were stripped (Restore PLUS Western blot Stripping Buffer; Thermo Scientific) and reprobed for tubulin using a mouse monoclonal anti-α-tubulin antibody (T9026; Sigma) followed by a secondary goat anti-mouse antibody (IRDye-680RD; LI-COR). In some experiments, a rabbit antibody against host protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used (ab128915; Abcam) followed by a secondary goat anti-rabbit antibody (IRDye-800CW; LI-COR). Western blots were imaged and quantitated using the Odyssey infrared imaging system (LI-COR). Particle production efficiency was calculated as the total CA signal in the virus pellet divided by the total CA and Gag signal in the virus pellet and cell lysate; the particle production efficiency of the "no RNA" control was set to 1.

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