JOURNAL OF VIROLOGY, Nov. 2002, p. 11757–11762 Vol. 76, No. 22
0022-538X/02/$04.00+0 DOI: 10.1128/JVI.76.22.11757–11762.2002

Reversible Binding of Recombinant Human Immunodeficiency Virus Type 1 Gag Protein to Nucleic Acids in Virus-Like Particle Assembly In Vitro

Ya-Xiong Feng, Tong Li,† Stephen Campbell,‡ and Alan Rein*

HIV Drug Resistance Program, National Cancer Institute—Frederick, Frederick, Maryland 21702-1201

Received 5 March 2002/Accepted 25 July 2002

Recombinant human immunodeficiency virus type 1 (HIV-1) Gag protein can assemble into virus-like particles (VLPs) in suitable buffer conditions with nucleic acid. We have explored the role of nucleic acid in this assembly process. HIV-1 nucleocapsid protein, a domain of Gag, can bind to oligodeoxynucleotides with the sequence $d(TG)_n$ with more salt resistance than to $d(A)_n$ oligonucleotides. We found that assembly of VLPs on $d(TG)_n$ oligonucleotides was more salt resistant than assembly on $d(A)_n$; thus, the oligonucleotides do not simply neutralize basic residues in Gag but provide a binding surface upon which Gag molecules assemble into VLPs. We also found that Gag molecules could be "trapped" on internal $d(TG)_n$ sequences within 40-base oligonucleotides, rendering them unable to take part in assembly. Thus, assembly on oligonucleotides requires that Gag proteins bind near the ends of the nucleic acid, and binding of Gag to internal $d(TG)_n$ sequences is apparently cooperative. Finally, we showed that nucleic acids in VLPs can exchange with nucleic acids in solution; there is a hierarchy of preferences in these exchange reactions. The results are consistent with an equilibrium model of in vitro assembly and may help to explain how Gag molecules in vivo select genomic RNA despite the presence in the cell of a vast excess of cellular mRNA molecules.

A single protein, termed Gag, is the major structural component in retrovirus particles. Expression of Gag is sufficient for assembly of retrovirus-like particles in mammalian cells. After the particle is released, Gag is cleaved by the viral protease (PR) into the Gag-derived proteins of the mature particle. The cleavages, collectively termed "maturation" of the particle, are essential for infectivity. The cleavage products always include at least three proteins, i.e., from the N terminus to the C terminus, matrix (MA), capsid (CA), and nucleocapsid (NC) (reviewed in reference 19).

Sequences present in the MA, CA, and NC domains of Gag are all essential for correct particle assembly. Of the three cleavage products, only NC is known to interact with nucleic acids. This protein is quite basic and also contains one or two CCHC zinc fingers; it is complexed with the genomic RNA in the interior of a mature retrovirus particle. NC engages in diverse interactions with nucleic acids in vitro, involving both the basic residues and the zinc fingers (for reviews, see references 8 and 18).

At some point in the assembly process, the Gag protein interacts with a packaging signal, termed "ψ," on the genomic RNA. This interaction leads to the incorporation of the RNA into the nascent particle (for a review, see reference 4). However, this RNA is completely unnecessary for efficient particle assembly in mammalian cells. Thus, if the cellular pool of ψ+ RNA is depleted by treatment of virus-producing cells with actinomycin D (14) or if ψ is simply deleted from the viral RNA (16), virus-like particles (VLPs) continue to be formed at a rate similar or identical to that in control cells.

The fact that the genomic RNA is unnecessary for particle assembly might suggest that assembly is driven simply by protein-protein interactions between Gag molecules. However, there are several lines of evidence indicating that RNA is required for assembly but that other RNAs from the cell can perform this function in the absence of genomic RNA. Thus, some viral mutants with alterations in NC impairing its interactions with RNA (I-domain mutants) fail to assemble efficiently and frequently form aberrant, low-density virus particles (3). Further, virus particles produced in cells lacking ψ+ RNA contain cellular mRNA molecules in place of the genomic RNA (2, 12, 17). Finally, digestion of detergent-lysed, immature murine leukemia virus (MuLV) particles (containing either ψ+ RNA or cellular mRNA) with RNase causes the disruption of the viral capsid and the solubilization of the Gag protein molecules (17). This observation strongly suggests that Gag-RNA interactions, as well as Gag-Gag interactions, are essential in maintaining the structure of the particle.

These findings raise at least two further questions concerning retrovirus assembly. One question is simply the nature of the role of RNA. That is, precisely how does it facilitate the proper association of Gag molecules? Second, as noted above, Gag can use cellular mRNA molecules in place of ψ+ RNA in constructing the virion. Why, then, does it not use cellular mRNA efficiently when ψ+ RNA is present in the cell? What is the nature of the packaging advantage that ψ confers upon an RNA molecule?

Retroviral particle assembly can be studied in a fully defined system in vitro. As first shown by Campbell and Vogt (7), recombinant Gag proteins will assemble into VLPs in a buffer of neutral pH and moderate ionic strength in the presence of nucleic acids. (Portions of Gag molecules have also been

* Corresponding author. Mailing address: National Cancer Institute-Frederick, P.O. Box B, Frederick, MD 21702-1201. Phone: (301) 846-1361. Fax: (301) 846-7146. E-mail: rein@ncifcrf.gov.

† Present address: GlobalNet Services, Inc., Rockville, MD 20852.

‡ Present address: Cancer Protein Expression Laboratory, Cornell University, Ithaca, NY 14853.

shown to assemble in vitro in the absence of nucleic acids, but only at protein and salt concentrations far higher than those used here [13, 20].) Using HIV-1 Gag protein, we previously found that even simple oligodeoxynucleotides as short as 15 to 20 bases can support assembly (6). In the present work, we have used the in vitro assembly system to further explore the role of nucleic acid in particle assembly. Our results suggest that the nucleic acid provides a surface to which the NC domains of the Gag molecules bind. Further, we show that nucleic acid incorporated in assembled particles is still exchangeable with nucleic acid in solution and that some nucleic acids are released from assembled particles far more readily than others in these exchange experiments. The implications of these findings for assembly and RNA encapsidation in vivo are discussed.

Differential salt sensitivity of VLPs assembled on different oligonucleotides. One possible role for nucleic acids in retrovirus assembly might be to neutralize charges in Gag proteins, perhaps specifically the many positive charges in the NC domain of Gag. This hypothesis is supported by the ability of heparin to replace nucleic acids in VLP assembly by recombinant avian retroviral Gag proteins (22). Alternatively, nucleic acid molecules might provide a surface or "scaffold" to which Gag molecules must bind in order to participate in assembly. To attempt to distinguish between these possibilities, we measured the salt sensitivity of VLPs assembled from recombinant HIV-1 Gag protein on two simple 30-base oligodeoxynucleotides, i.e., $d(TG)_{30}$ and $d(A)_{30}$. [The subscript "30" indicates the length of the oligonucleotide; $d(TG)_{30}$ contains 15 repeats of the sequence TG.] These two oligonucleotides were chosen because HIV-1 NC protein, a cleavage product of Gag, binds with far higher affinity to $d(TG)_n$ than to $d(A)_n$ (10) (R. J. Fisher et al., manuscript in preparation). The interaction of NC with $d(TG)_n$ involves a major hydrophobic component, in addition to the electrostatic interaction between the basic protein and the nucleic acid backbone, so that binding to $d(TG)_n$ is far more salt resistant than binding to $d(A)_n$.

The assembly experiments were performed exactly as described previously (6), except that in some experiments the assembly reaction was scaled up to contain twice the amounts of all constituents in twice the volume. The human immunodeficiency virus type 1 (HIV-1) Gag protein used in all experiments was produced in *Escherichia coli* and lacks the p6 domain present in authentic HIV-1 Gag protein. It also differs from authentic viral Gag protein in that it lacks the myristate moiety at its N terminus. A 0.1-mg quantity of Gag protein was mixed with nucleic acid (Life Technologies, Inc., Gaithersburg, Md.) and diluted to 0.1 M NaCl in the presence of 20 mM Tris (pH 8.0)–10 mM dithiothreitol (DTT). After incubation for 1 to 2 h at room temperature, the mixture was fractionated by centrifugation at 21,000 × *g* at 4°C for 2 h. Supernatants and pellets from this centrifugation were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue. We calculate that structures as small as ~30S (containing roughly 20 to 25 Gag molecules) will be pelleted by this centrifugation.

Our previous work showed that the two oligonucleotides $d(TG)_{30}$ and $d(A)_{30}$ both support assembly under the standard conditions at an NaCl concentration of 0.1 M (6). Figure 1 shows the effects of increased NaCl concentrations upon assembly. As can be seen in the A30 panel, the extent of assembly on $d(A)_{30}$ was considerably reduced in 0.15 M NaCl (lanes 4 to 6), and only background levels of Gag protein were pelleted in 0.2 M NaCl (lanes 7 to 9). In contrast, when $d(TG)_{30}$ was used in the assembly reaction, the great majority of the Gag protein was pelleted in 0.2 M NaCl (lanes 4 to 6). A further increase to 0.3 M NaCl almost completely eliminated assembly (lanes 7 to 9). These results show that assembly on a $d(TG)_n$ substrate is significantly more resistant to increases in the ionic strength of the medium than assembly on a $d(A)_n$ substrate.

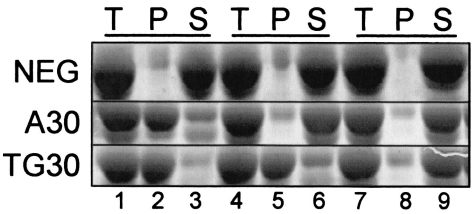

FIG. 1. Assembly on $d(A)_{30}$ is more salt sensitive than on $d(TG)_{30}$. No nucleic acid (NEG) was added to one panel. In all other lanes, the assembly mixture contained 8 μg of nucleic acid and 200 μg of Δp6. In $d(A)_{30}$ (A30), lanes 1 to 3 contain 0.1 M NaCl, lanes 4 to 6 contain 0.15 M NaCl, and lanes 7 to 9 contain 0.2 M NaCl. In NEG and $d(TG)_{30}$ (TG30), lanes 1 to 3 contain 0.1 M NaCl, lanes 4 to 6 contain 0.2 M NaCl, and lanes 7 to 9 contain 0.3 M NaCl. All reaction mixtures also contained 20 mM Tris (pH 8.0) and 10 mM DTT. T, total; P, pellet; S, supernatant.

Inhibition of assembly by the presence of internal d(TG) sequences in longer oligonucleotides. Using our standard assembly conditions (i.e., 0.1 M NaCl), we previously measured the ability of $d(TG)_n$ and $d(A)_n$ oligonucleotides to support assembly as a function of both the length and the concentration of the oligonucleotide (6). We found that, for oligonucleotides of ≥ 20 bases in length, 2 to 4% nucleic acid/protein (wt/wt) was sufficient for assembly of all of the Gag protein into pelleted structures. However, at mass ratios of >4%, the degree of assembly decreased for $d(TG)_n$ oligonucleotides ≥25 bases long, but not for $d(A)_n$ oligonucleotides of the same length. This inhibition of assembly by supraoptimal concentrations of $d(TG)_n$ oligonucleotides ≥25 bases long was not explained.

We have now examined the properties of chimeric oligodeoxynucleotides, composed partly of d(TG) and partly of d(A) sequences, in an effort to localize the bases in these longer oligonucleotides responsible for the inhibition of assembly at high concentrations. The sequences of these two 40-base chimeric oligonucleotides were $(TG)_{10}(A)_{20}(TG)_{10}$ (hereafter called TG/A/TG) and $(A)_{10}(TG)_{20}(A)_{10}$ (hereafter called A/TG/A). Figure 2 shows the results of assembly experiments with these two oligonucleotides, as well as $d(A)_{40}$ and $d(TG)_{40}$, at concentrations relative to Gag protein of 2, 4, 8, and 16%. As can be seen, at the high concentrations (i.e., 8 and 16%), the A/TG/A oligonucleotide inhibited assembly even more than did $d(TG)_{40}$ (lanes 7 to 12). No inhibition was observed with the TG/A/TG oligonucleotide. Thus, with these chimeric oligonucleotides, inhibition of assembly is associated with the presence of TG sequences in the middle, not the ends, of the nucleic acid. These results suggest that TG sequences in the central portion of longer $d(TG)_n$ oligonucleotides are responsible for inhibition of assembly when the oligonucleotide is present in excess.

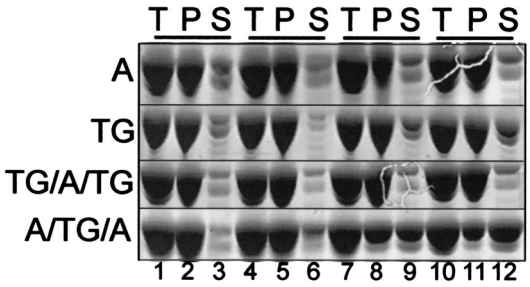

FIG. 2. Assembly on increasing concentrations of oligonucleotides. Assembly was performed on $d(A)_{40}$, $d(TG)_{40}$, $d(TG)_{10}(A)_{20}(TG)_{10}$ (TG/A/TG), and $d(A)_{10}(TG)_{20}(A)_{10}$ (A/TG/A). Lanes 1 to 3, 2% nucleic acid/protein; lanes 4 to 6, 4% nucleic acid/protein; lanes 7 to 9, 8% nucleic acid/protein; lanes 10 to 12, 16% nucleic acid/protein. T, total; P, pellet; S, supernatant.

Exchange between nucleic acids in VLPs and nucleic acids in solution. The VLPs assembled under our experimental conditions are large, organized structures (6). Since nucleic acid molecules are an essential element in these structures, we might expect these molecules to be in continuous, tight contact with Gag molecules in the VLPs. On the other hand, the VLPs are apparently not completely closed structures, as RNA in these particles is still accessible to RNase (6). As another way of probing the state of nucleic acids in VLPs, we tested the possibility that the DNA or RNA in VLPs could be exchanged for DNA or RNA molecules in solution. In our initial experiments, VLPs were first assembled using a panel of different nucleic acids. These nucleic acids included the following: oligonucleotides 20 or 40 nucleotides long, either with the sequence $d(A)_n$ or with the alternating sequence $d(TG)_n$; yeast tRNA (Life Technologies); and *E. coli* rRNA (Life Technologies). One microgram of nucleic acid was mixed with 25 μg of Gag in 20 μl of buffer A (20 mM Tris HCl [pH 8.0], 0.1 M NaCl, 10 mM DTT). The mixture was incubated at room temperature for 30 min, and assembled particles were then isolated by centrifugation at 19,300 × *g* for 60 min. The pellets were resuspended in 20 μl of buffer A and reincubated for 30 min with 0.1 μg of the same nucleic acid. Twenty nanograms of ^{32}P-labeled HIV-1 ψ+ RNA was then added to the mixture; after 60 min at room temperature, the mixtures were centrifuged again, and the amounts of ^{32}P incorporated into the pellet and remaining in the supernatant were determined by scintillation counting. Control experiments (not shown) demonstrated that virtually all of the Gag protein in these experiments was present in the final pellet. We estimate that structures containing ≥~65 Gag molecules will be pelleted by this centrifugation.

The results of such an exchange experiment are shown in Fig. 3. We found that if particles were initially assembled on $d(A)_n$ oligonucleotides, then virtually all of the ^{32}P-labeled HIV-1 ψ+ RNA was incorporated into the final pellet. This observation suggests that Gag molecules in VLPs might still be capable of interacting with nucleic acids in solution and taking them into VLPs. Figure 3 also shows that when particles had been assembled on $d(TG)_n$ oligonucleotides, the level of ^{32}P-labeled RNA incorporated was somewhat less than in the case of $d(A)_n$ and was also somewhat dependent upon the length of the oligonucleotide. Thus, more ^{32}P was incorporated if the

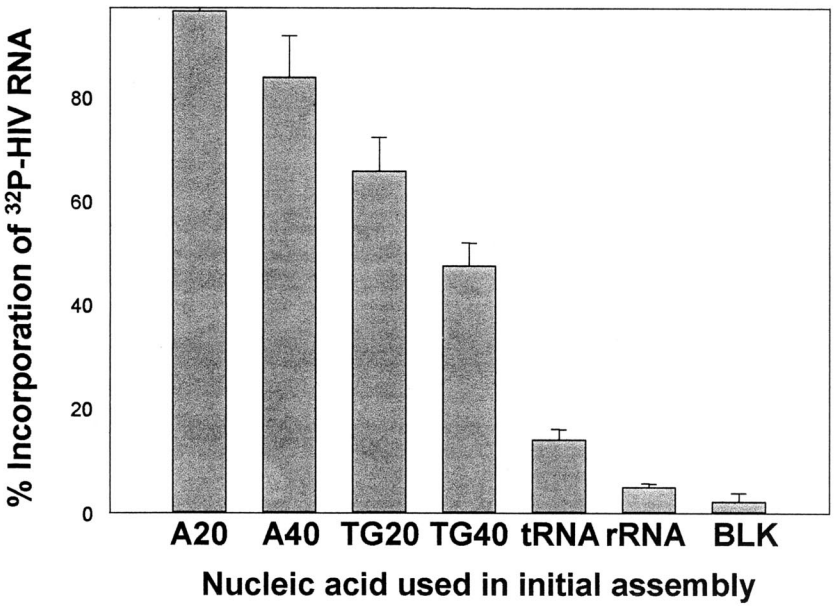

FIG. 3. Incorporation of ^{32}P-labeled HIV-1 ψ+ RNA following assembly on other nucleic acids. VLPs were assembled on the indicated nucleic acids. After the VLPs were pelleted, they were resuspended and incubated with 0.1 μg of the same nucleic acid for an additional hour. They were then incubated with 20 ng of ^{32}P-labeled HIV-1 ψ+ RNA (containing ~10,000 cpm) and recentrifuged. The fraction of total radioactivity in the reaction (i.e., supernatant plus pellet) that was present in the final pellet was determined. In the first four columns, the numeral indicates the length of the oligonucleotide. BLK, fraction of ^{32}P-HIV-1 RNA pelleted in the absence of Gag protein. Mean values and error bars (standard deviations) were obtained by pooling data from separate experiments; the small standard deviations indicate the reproducibility of the results.

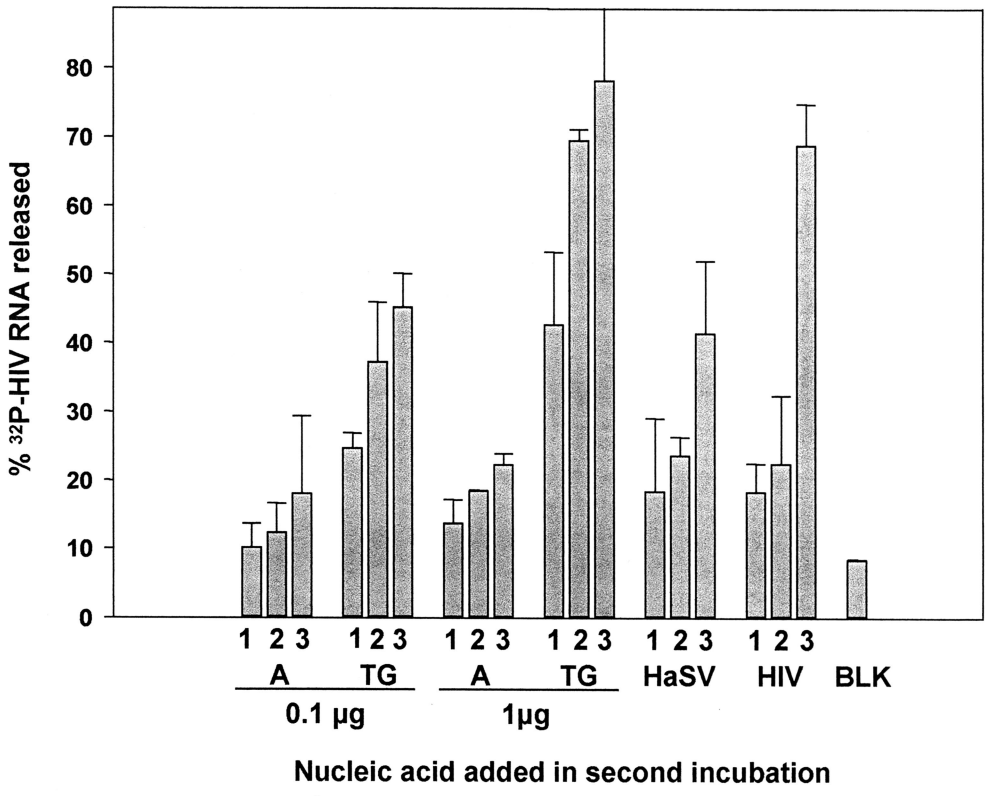

FIG. 4. Release of ^{32}P-labeled HIV-1 ψ+ RNA upon incubation of VLPs with other nucleic acids. As described in the text, VLPs were assembled on ^{32}P-labeled HIV-1 ψ+ RNA and were incubated with the indicated nucleic acid and then reisolated by centrifugation. The fraction of the total (supernatant plus pellet of the final centrifugation) radioactivity in the VLPs that was present in the supernatant in this final centrifugation is shown. In the case of the oligonucleotides (A and TG), lanes 1, 2, and 3 represent 20-, 40-, and 80-base oligonucleotides, respectively. In the case of HaSV and HIV, lanes 1, 2, and 3 represent 1, 10, and 100 ng of these RNAs, respectively. BLK, fraction of ^{32}P-labeled HIV-1RNA released when no nucleic acid was present in the second incubation.

particles had been initially assembled on $d(TG)_{20}$ than if the first nucleic acid was $d(TG)_{40}$. When tRNA or rRNA was used in the initial assembly, the levels of ^{32}P-labeled HIV-1 ψ+ RNA incorporated were only slightly above background levels. These results show that the ability of a VLP to take up ^{32}P-labeled HIV-1 ψ+ RNA from the solution is critically dependent upon the identity of the nucleic acid already present in the VLP: it is high for VLPs containing $d(A)_n$, very low for VLPs containing tRNA or rRNA, and intermediate for VLPs with $d(TG)_n$. In the latter case, the length as well as the sequence of the nucleic acid is important.

Figure 3 shows that VLPs assembled on oligonucleotides can still incorporate ^{32}P-labeled HIV-1 RNA. This incorporation might reflect a special affinity for this RNA species; perhaps Gag molecules bound to oligonucleotides or tRNA in a VLP can simultaneously interact with ψ+ HIV-1 RNA. Alternatively, the incorporation might represent one side of an exchange reaction, in which the uptake of the added nucleic acid molecule is accompanied by the release of the nucleic acid upon which the VLP was first assembled. To test this possibility, we performed an experiment reciprocal to that shown in Fig. 3. VLPs were first assembled on ^{32}P-labeled HIV-1 ψ+ RNA and were then incubated with a panel of different nucleic acids; the fraction of ^{32}P released from the VLPs was then determined. Twenty-five micrograms of Gag protein was incubated with 1 μg of ^{32}P-labeled HIV-1 ψ+ RNA (containing 5,000 to 10,000 cpm) in 20 μl of buffer A, incubated at room temperature for 30 min, and then centrifuged as described in the legend to Fig. 3. The pellets were suspended in 20 μl of buffer A, and a second (unlabeled) nucleic acid was added. The mixture was incubated at room temperature for 60 min and then fractionated again by centrifugation; radioactivity in the pellet and supernatant was determined. As can be seen in Fig. 4, there was very little ^{32}P released when particles were incubated in either 0.1 or 1.0 μg of $d(A)_n$ oligonucleotide (or in the control BLK reaction, in which no nucleic acid was added in the second incubation). Oligonucleotide lengths of 20, 40, and 80 bases all gave similar results. In contrast, when the oligonucleotide was composed of the alternating sequence d(TG), a substantial fraction of the ^{32}P was released from the preassembled VLPs. The amounts released were higher with longer oligonucleotides than with shorter oligonucleotides and greater with 1 μg of $d(TG)_n$ than with 0.1 μg of $d(TG)_n$. These results show that the nucleic acid in a VLP can be released by exchange with nucleic acid in solution. In addition, the data show that there is a distinct hierarchy in the ability of nucleic acids to compete with HIV-1 ψ+ RNA for inclusion in VLPs. We also incubated the VLPs with a concentration series of unlabeled HIV-1 ψ+ RNA and with another retroviral RNA fragment, consisting of nucleotides 34 to 378 of Harvey sar-

coma virus (HaSV) (transcribed as described previously [9]). Significant amounts (40 to 70%) of the ^{32}P were released when the VLPs were incubated with 0.1 μg of these RNAs (columns 3 in Fig. 4); as shown in Fig. 4, the HaSV transcript was nearly as effective as HIV-1 RNA in this experiment.

We have recently found that the addition of inositol pentakisphosphate (IP5) to assembly reaction mixtures dramatically alters the assembly of HIV-1 Gag protein into VLPs (5). Thus, the VLPs formed in the presence of IP5 or related compounds are ~100 nm in diameter, rather than 25 to 30 nm in diameter, and are resistant to treatment with high concentrations of salt or RNase. It seemed possible that IP5 alters the exchange of nucleic acids by VLPs. However, when this hypothesis was tested, we found that the labeled RNA in VLPs assembled in HIV-1 RNA with IP5 was only slightly more resistant to displacement by $d(TG)_n$ than VLPs assembled in HIV-1RNA alone; similarly, $d(A)_n$ was unable to displace significant levels of HIV-1 RNA, whether or not IP5 had been present during assembly (data not shown).

RNA is a structural element in retrovirus particles (17), but its role in the structure of the particle is not yet clear. We also do not understand how ψ+ genomic RNA is normally incorporated into retrovirus particles with high selectivity, despite the fact that MuLV particles can assemble efficiently in the absence of this RNA, incorporating cellular mRNA molecules in place of genomic RNA (17). In the experiments reported here, we have tried to explore the role of nucleic acid and the mechanisms involved in specific packaging of ψ+ RNA by looking for experimental situations in which recombinant HIV-1 Gag protein discriminates between different nucleic acids in in vitro assembly. The in vitro system used here is artificial in many ways, and results obtained in this system cannot necessarily be applied to assembly in vivo. However, the extreme simplicity of this fully defined in vitro system makes it possible to explore questions experimentally that are not easily approached in vivo. (In fact, the structural role of RNA in retrovirus particles was first revealed in studies on VLPs assembled in vitro [7].)

Briefly, the results can be summarized as follows. First, assembly on d(TG) oligonucleotides is more salt resistant than assembly on d(A) oligonucleotides (Fig. 1). Second, an excess of long oligonucleotides containing centrally placed d(TG) inhibits assembly (Fig. 2). Third, exchange can occur between nucleic acids in assembled VLPs and nucleic acids in solution; there is a clear hierarchy of preferences observed in these exchange reactions (Fig. 3 and 4).

Previous work has shown that heparin can replace nucleic acid in the assembly of VLPs in vitro from avian retroviral Gag protein (22). This observation suggested the possibility that nucleic acid functions simply as a polyanion in assembly: its role might be to neutralize positive charges in Gag, since another polyanion can also perform this function. However, the preference for d(TG) over d(A) at increased salt concentrations (Fig. 1) would appear to argue against this hypothesis. HIV-1 NC protein, which of course is present as a domain of the Gag protein used here, binds to d(TG) sequences with a far higher affinity than it binds to d(A) sequences (10). This binding, involving hydrophobic interactions in addition to the electrostatic attraction between the basic protein and the phosphate backbone, is more salt resistant than the binding to d(A) (10). Thus, the present results strongly suggest that assembly on nucleic acids involves the binding of Gag to the nucleic acid, rather than the simple neutralization of basic charges by the nucleic acid backbone. The binding can evidently occur by a combination of electrostatic and hydrophobic forces. The salt-dependent discrimination between d(TG) and d(A) is also evidence that the binding of Gag molecules to nucleic acid molecules in in vitro assembly is mediated by their NC domains.

The fact that high concentrations of an oligonucleotide with centrally, but not terminally, placed d(TG) sequences inhibit assembly (Fig. 2) implies that this sequence acts as a "sink" for Gag molecules, sequestering them in the middle of the nucleic acid where they cannot take part in VLP assembly. In turn, this result implies that the Gag-Gag interactions in assembly are weaker than the binding of Gag to this DNA sequence. It also shows that Gag molecules bound to the interior of an oligonucleotide cannot assemble by side-to-side interactions; rather, they must be placed near a nucleic acid end (i.e., within ~10 nucleotides) in order for assembly to occur on a short nucleic acid molecule.

This model implies that assembly on short nucleic acids involves a series of Gag-Gag interactions across these gaps. This series would ultimately result in the formation of a long, linear chain of Gag molecules bound to oligonucleotides. In turn, this chain might wind up into a distorted helix: as pointed out by Campbell and Vogt (7), theoretically, a spherical particle could be formed from such a chain.

Two studies (1, 23) have shown that the role of the NC domain in retroviral assembly can be filled by a leucine zipper domain. These leucine zippers are structures by which proteins dimerize; thus, it has been proposed that nucleic acids are scaffolds for the dimerization of Gag molecules and that the proteins become competent for assembly once they dimerize (15). However, our results suggest that dimerization of Gag is not sufficient to permit assembly. Thus, high concentrations of a 30-base oligonucleotide, i.e., $d(TG)_{30}$, were previously found to inhibit assembly (6). It seems likely that Gag molecules dimerize upon binding to $d(TG)_{30}$, since the site size for HIV-1 NC on d(TG) is only 5 bases (10) and since 25-base oligonucleotides are long enough to support efficient assembly under our experimental conditions (6). We would propose that the dimers that form on excess $d(TG)_{30,}$ like those on A/TG/A (Fig. 2), are unable to complete the assembly into VLPs because they are too far from the ends of the oligonucleotide. These results also suggest that binding of Gag to $d(TG)_n$ is cooperative and that the juxtaposition of two Gag molecules on a single oligonucleotide is energetically favored over Gag-Gag association across the gap between the ends of a pair of oligonucleotides.

The results presented in Fig. 3 and 4 show directly that the interaction between Gag and nucleic acid molecules leading to assembly in vitro is reversible and that Gag exhibits clear preferences in these interactions. In addition, we noted a preference (Fig. 3 and 4) for longer $d(TG)_n$ oligonucleotides over shorter $d(TG)_n$ oligonucleotides; this observation, like the inhibition of assembly by high concentrations of long $d(TG)_n$ oligonucleotides (6), suggests that binding of Gag to these nucleic acids is cooperative and that the energy gain for coop-

erative binding on a single oligonucleotide is greater than that for interactions across the ends of oligonucleotides.

We were careful in these experiments to ensure that all the Gag protein in our exchange experiments was already assembled in pelletable structures before it was exposed to the second nucleic acid. However, we have still not rigorously established that a Gag molecule in such a structure can interact with a second nucleic acid molecule. It is possible that the assembled VLPs normally undergo some level of spontaneous, transient dissociation or "breathing" (as would be expected if in vitro assembly were an equilibrium reaction) and that Gag molecules from such dissociated particles are capable of binding a new nucleic acid molecule as they reassemble. It should also be noted that the extent of the preferences we observed do not seem sufficient to account for the specificity of packaging in vivo. While we did not compare HIV-1 ψ+ RNA with HaSV ψ+ RNA in detail here, we observed only a limited preference for the former over the latter (Fig. 4). This appears to represent a clear difference between the admittedly artificial in vitro assembly system and virion assembly in living cells; it is not known how the intracellular environment (and/or the differences between the viral Gag protein and the protein purified from bacteria) contribute to specificity in RNA packaging.

As noted above, genomic RNA packaging in vivo (at least in the case of murine leukemia virus) involves the selective incorporation of viral RNA into the nascent virion despite the availability of a vast excess of cellular mRNA molecules that are also capable of supporting assembly (17). Thus, the presence of ψ on the viral RNA gives it a profound advantage in its competition with mRNAs for packaging into the particle. This advantage might be kinetic: in other words, Gag might interact with ψ+ RNA in the cell before it encounters other RNAs. For example, ψ might target viral RNA to a specific cellular location where it can interact with Gag molecules before assembly has begun. Alternatively, the advantage might be thermodynamic: the complex between Gag and ψ+ RNA might be more stable than complexes with other RNAs. According to this hypothesis, interactions between Gag molecules and nucleic acid molecules would be reversible, so that Gag can "sample" different nucleic acids before committing irreversibly to packaging one of them. Our results are consistent with the latter possibility.

Retrovirus particles are pleomorphic and lack perfect symmetry (11, 21). Because of these properties, we had previously suggested that Gag-Gag interactions might be too weak to drive particle formation and that Gag-nucleic acid interactions might contribute to assembly (21). The observations presented here are fully compatible with these proposals.

Taken together, our present results suggest that retrovirus particles are assembled through a series of relatively weak, reversible interactions. The Gag proteins apparently must bind to a scaffold in order to assemble; this binding presumably concentrates and aligns them properly to facilitate Gag-Gag interactions. The results are all consistent with an equilibrium model of in vitro assembly (24) involving cooperative binding of Gag molecules to nucleic acids.

Ya-Xiong Feng and Tong Li contributed equally to this work.

We thank Volker Vogt and members of his laboratory for many helpful discussions and particularly for pointing out that leucine zippers are dimerization rather than multimerization domains. We also thank Louis Henderson and Adam Zlotnick for illuminating comments and Judith Levin and Delphine Muriaux for thoughtful reviews of the manuscript.

REFERENCES

1. **Accola, M. A., B. Strack, and H. G. Gottlinger.** 2000. Efficient particle production by minimal Gag constructs which retain the carboxy-terminal domain of human immunodeficiency virus type 1 capsid-p2 and a late assembly domain. J. Virol. **74:**5395–5402.
2. **Anderson, D. J., J. Stone, R. Lum, and M. L. Linial.** 1995. The packaging phenotype of the SE21Q1b provirus is related to high proviral expression and not *trans*-acting factors. J. Virol. **69:**7319–7323.
3. **Bennett, R. P., T. D. Nelle, and J. W. Wills.** 1993. Functional chimeras of the Rous sarcoma virus and human immunodeficiency virus Gag proteins. J. Virol. **67:**6487–6498.
4. **Berkowitz, R., J. Fisher, and S. P. Goff.** 1996. RNA packaging. Curr. Top. Microbiol. Immunol. **214:**177–218.
5. **Campbell, S., R. J. Fisher, E. M. Towler, S. Fox, H. J. Issaq, T. Wolfe, L. R. Phillips, and A. Rein.** 2001. Modulation of HIV-like particle assembly in vitro by inositol phosphates. Proc. Natl. Acad. Sci. USA **98:**10875–10879.
6. **Campbell, S., and A. Rein.** 1999. In vitro assembly properties of human immunodeficiency virus type 1 Gag protein lacking the p6 domain. J. Virol. **73:**2270–2279.
7. **Campbell, S., and V. M. Vogt.** 1995. Self-assembly in vitro of purified CA-NC proteins from Rous sarcoma virus and human immunodeficiency virus type 1. J. Virol. **69:**6487–6497.
8. **Darlix, J. L., M. Lapadat-Tapolsky, H. de Rocquigny, and B. P. Roques.** 1995. First glimpses at structure-function relationships of the nucleocapsid protein of retroviruses. J. Mol. Biol. **254:**523–537.
9. **Feng, Y. X., W. Fu, A. J. Winter, J. G. Levin, and A. Rein.** 1995. Multiple regions of Harvey sarcoma virus RNA can dimerize in vitro. J. Virol. **69:**2486–2490.
10. **Fisher, R. J., A. Rein, M. Fivash, M. A. Urbaneja, J. R. Casas-Finet, M. Medaglia, and L. E. Henderson.** 1998. Sequence-specific binding of human immunodeficiency virus type 1 nucleocapsid protein to short oligonucleotides. J. Virol. **72:**1902–1909.
11. **Fuller, S. D., T. Wilk, B. E. Gowen, H. G. Krausslich, and V. M. Vogt.** 1997. Cryo-electron microscopy reveals ordered domains in the immature HIV-1 particle. Curr. Biol. **7:**729–738.
12. **Gallis, B., M. Linial, and R. Eisenman.** 1979. An avian oncovirus mutant deficient in genomic RNA: characterization of the packaged RNA as cellular messenger RNA. Virology **94:**146–161.
13. **Gross, I., H. Hohenberg, C. Huckhagel, and H. G. Krausslich.** 1998. N-terminal extension of human immunodeficiency virus capsid protein converts the in vitro assembly phenotype from tubular to spherical particles. J. Virol. **72:**4798–4810.
14. **Levin, J. G., P. M. Grimley, J. M. Ramseur, and I. K. Berezesky.** 1974. Deficiency of 60 to 70S RNA in murine leukemia virus particles assembled in cells treated with actinomycin D. J. Virol. **14:**152–161.
15. **Ma, Y. M., and V. M. Vogt.** 2002. Rous sarcoma virus Gag protein-oligonucleotide interaction suggests a critical role for protein dimer formation in assembly. J. Virol **76:**5452–5462.
16. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33:**153–159.
17. **Muriaux, D., J. Mirro, D. Harvin, and A. Rein.** 2001. RNA is a structural element in retrovirus particles. Proc. Natl. Acad. Sci. USA **98:**5246–5251.
18. **Rein, A., L. E. Henderson, and J. G. Levin.** 1998. Nucleic acid chaperone activity of retroviral nucleocapsid proteins: significance for viral replication. Trends Biochem. Sci. **23:**297–301.
19. **Swanstrom, R., and J. W. Wills.** 1997. Synthesis, assembly, and processing of viral proteins, p. 263–334. *In* J. M. Coffin, S. H. Hughes, and H. E. Varmus (ed.), Retroviruses. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
20. **von Schwedler, U. K., T. L. Stemmler, V. Y. Klishko, S. Li, K. H. Albertine, D. R. Davis, and W. I. Sundquist.** 1998. Proteolytic refolding of the HIV-1 capsid protein amino-terminus facilitates viral core assembly. EMBO J. **17:**1555–1568.
21. **Yeager, M., E. M. Wilson-Kubalek, S. G. Weiner, P. O. Brown, and A. Rein.** 1998. Supramolecular organization of immature and mature murine leukemia virus revealed by electron cryo-microscopy: implications for retroviral assembly mechanisms. Proc. Natl. Acad. Sci. USA **95:**7299–7304.
22. **Yu, F., S. M. Joshi, Y. M. Ma, R. L. Kingston, M. N. Simon, and V. M. Vogt.** 2001. Characterization of Rous sarcoma virus Gag particles assembled in vitro. J. Virol. **75:**2753–2764.
23. **Zhang, Y., H. Qian, Z. Love, and E. Barklis.** 1998. Analysis of the assembly function of the human immunodeficiency virus type 1 Gag protein nucleocapsid domain. J. Virol. **72:**1782–1789.
24. **Zlotnick, A.** 1994. To build a virus capsid. An equilibrium model of the self assembly of polyhedral protein complexes. J. Mol. Biol. **241:**59–67.