Duplication of the Primary Encapsidation and Dimer Linkage Region of Human Immunodeficiency Virus Type 1 RNA Results in the Appearance of Monomeric RNA in Virions

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The dimerization initiation site (DIS) and the dimer linkage sequences (DLS) of human immunodeficiency virus type 1 have been shown to mediate in vitro dimerization of genomic RNA. However, the precise role of the DIS-DLS region in virion assembly and RNA dimerization in virus particles has not been fully elucidated, since deletion or mutation of the DIS-DLS region also abolishes the packaging ability of genomic RNA. To characterize the DIS-DLS region without altering packaging ability, we generated mutant constructs carrying a duplication of approximately 1,000 bases including the encapsidation signal and DIS-DLS (E/DLS) region. We found that duplication of the E/DLS region resulted in the appearance of monomeric RNA in virus particles. No monomers were observed in virions of mutants carrying the E/DLS region only at ectopic positions. Monomers were not observed when pol or env regions were duplicated, indicating an absolute need for two intact E/DLS regions on the same RNA for generating particles with monomeric RNA. These monomeric RNAs were most likely generated by intramolecular interaction between two E/DLS regions on one genome. Moreover, incomplete genome dimerization did not affect RNA packaging and virion formation. Examination of intramolecular interaction between E/DLS regions could be a convenient tool for characterizing the E/DLS region in virion assembly and RNA dimerization within virus particles.

Retrovirus RNAs packaged into virions are dimeric. The association between the two RNA molecules is noncovalent because the dimeric RNA dissociable into monomers under mild denaturing conditions, such as incubation at high temperature (~70°C) or treatment with denaturing reagents (for a review, see references 14 and 24). Electron microscopic analysis of genomic RNA dimers obtained from several species of retroviruses reveals a symmetrical form with a contact point between the RNA situated in a region near the 5′ end (4, 5, 19, 27, 31, 32, 37, 48, 57). It is likely that the presence of two genomes in single virus particles is advantageous for virus survival, facilitating recovery from physical damage to the RNA or providing genetic variety to the virus progeny (15, 28).

Synthetic RNA fragments derived from the 5′ region of retrovirus RNA can spontaneously dimerize in vitro upon incubation in appropriate buffer without protein factors (3, 6, 9, 11, 13, 16–18, 25, 26, 29, 33, 39, 51, 53, 56, 58). The contact point between the two monomers that constitute in vitro-synthesized dimers is referred to as the dimer linkage sequences (DLS). In human immunodeficiency virus type 1 (HIV-1), the 5′ untranslated region just downstream of the splicing donor (s.d.) was first reported to be a DLS, because the RNA fragments harboring deletions in this region have formed dimers in vitro at significantly reduced efficiency (3, 39, 58). Recently, several groups reported that another site within the 5′ untranslated region is also important for RNA dimerization in vitro. This site is located upstream of the 5′ s.d. and designated the dimer initiation site (DIS) (33, 47, 51, 56). The DIS consists of a stem-loop structure with a conserved palindromic sequence at the top of the loop. In their proposed model, the palindromic sequences on two RNA molecules first contact each other, forming a “kissing hairpin” interaction when dimer formation is initiated (33, 47, 51, 56). Mutation within this region also abolished in vitro RNA dimerization (13, 46).

In contrast to these in vitro data, several lines of evidence indicate that dimer formation in vivo is not as simple. The viral nucleocapsid protein (NC) appears to act as a molecular chaperon to refold viral RNA so that it has the appreciate secondary structure (16, 17, 20). We and other groups reported that mutations introduced in and around the encapsidation signal and DIS-DLS (E/DLS) region did not affect the stability of dimers in virus particles (7, 12, 54). We also found that it is likely that the regions located far from the primary E/DLS region affect the stability of the RNA dimer (54). Furthermore, electron microscopic observation indicates that the dimeric form of HIV-1 RNA contains more than one contact point in the primary E/DLS (27).

A problem that has hampered in vivo analysis of the DIS-DLS is that deletion or mutation of the dimerization site also abolishes RNA packaging, since the putative dimerization site largely overlaps the packaging signal. To try to obviate this problem, we generated mutant viral RNA carrying additional dimerization sites to see whether two dimerization sites within the same RNA molecule might interact with each other and interfere with normal intermolecular dimer formation. If such
intramolecular interaction negatively affects intramolecular inter-
teration, it might be possible to modify one dimerization site 
without affecting packaging efficiency and thereby functionally 
segregate the encapsidation and DIS-DLS regions. We report 
here that the duplication of a packaging-dimerization site on the 
same RNA molecule indeed caused the appearance of 
monomeric RNA in virions. Such monomers were not ob-
served when the original packaging-dimerization site was 
deleted, suggesting that the duplicated region actually mediates 
RNA–RNA interaction in the virion. This system could be used 
for defining and examining the exact location of dimer linkage 
sites within virus particles.

MATeRIALS AND METHODS

Plasmids and viral expression constructs. The replication-competent HIV-1 
proviral clone pNL4-3 (1) and its defective derivative pMSMBA (40), which had 
about a 900-bp deletion in the env gene, were used as the progenitors for all the 
mutants listed below. The nucleotide designations refer to the DNA sequence of 
plNL3-3 starting from the beginning of U3. To construct a series of mutants 
containing two copies of the dimer linkage site, we first constructed pGEM-MM, 
which contains the 5′ leader region (nucleotide positions 454 to 1523) of 
plNL3-3 with mutations in both the s.d. and the polyadenylation signal. First, 
two overlapping fragments were PCR amplified from pMSMBA with primers 
containing mutations in the s.d. Sense primer 438–464 BamHI (5′-GCTTTTTT 
CCGGATCCGGGTCTCTCTG-3′) and antisense primer 1535–1512 SpeI (5′-CATCCTATTGGATCCTGAAGGGT 
-3′) were used to generate fragment 1. Fragments 2 and 3 were digested with 
BclI, ligated, digested with BstII and SpeI, and cloned into pMSMBA that 
had been cut with BstII and SpeI to construct pGEM-MD (43). Next, two over- 
lapping fragments were PCR amplified from pMSMBA s.d. (43) with primers 
containing mutations in the polyadenylation signal. Sense primer 438–464 BamHI 
and antisense primer 543–520 PvuI (5′-TCAAGGCAACCATGTTGAGGC 
-3′) were used to generate fragment 2. Fragments 4 and 2 were digested with 
BclI, ligated, digested with BstII and SpeI, and cloned into pMSMBA that 
had been cut with BstII and SpeI to construct pGEM-MM (43). The amplified region of 
plNL4-3 which had been similarly blunt ended with T4 DNA polyme-
erase to construct pDDN. pssS was constructed by ligating fragment 5 into 
the plasmid DNA polymerase- 

Northern blotting. At 48 to 72 h after transfection, the medium and cytoplasmic 
RNA were concurrently collected as described elsewhere (40). Pelleted 
RNA was resuspended in T-buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1% 
sodium dodecyl sulfate [SDS], 100 mM NaCl, and 10% formamide), and the 
thermostability of dimeric viral RNA was determined by incubating RNA ali-
quotes for 10 min at the temperatures indicated in the relevant figures (54). Viral 
RNA was electrophoresed at room temperature in nondenaturing 0.75% native 
gel. Sense primer 438–464 BamHI and antisense primer 543–520 PvuI (5′-TCAAGGCAACCATGTTGAGGC 
-3′) were used to generate fragment 2. Fragments 4 and 2 were digested with 
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RESULTS

Duplication of the E/DLS region of HIV-1 RNA results in the accumulation of monomeric RNA in virions. To see whether 
duplication of the packaging-dimerization region (E/DLS) of HIV-1 affects encapsidation of genomic viral RNA, we 
constructed a variety of mutants carrying two copies of the 
capsulation and dimer linkage region. The duplicated region 
was approximately 1,000 bp in length and included 

Xhol, and a 2.7-kb env region fragment (6208 to 8888) was isolated. The env 
fragment was then inserted into the Xhol and T4 DNA polymerase-treated MhI 
sites of p5′ssglob to construct p5′ssEnvEX. pGEMbasic (Promega) was digested 
with Xhel, blunt ended with T4 DNA polymerase, and digested with SalI, and the 
270-bp fragment containing the simian virus 40 polyadenylation signal was iso-
lated. This fragment was ligated into the Xhol and T4 DNA polymerase-
treated Xhol sites, located in the nef gene, of p5′ssEnvEX to construct p5′ssEnvEXSV.

Transfection. Approximately 7 × 10⁶ 293 cells (23) or 293tat cells (49) were 
seeded on 150-mm-diameter plates the day before transfection and transfected with 20 μg of plasmid DNA using the calcium phosphate precipitation method 
(2). The day after transfection, the supernatant was discarded and replaced with 
fresh medium.
TAR, R/U5, U5/L, SL1, SL2, SL3, and SL4 stem-loops and the 5' half of the gag gene. These stem-loops are located in the 5' region of the HIV-1 genome and play important roles in viral genome packaging and dimerization. The polyadenylation signals and the s.d. in the ectopic fragment were deleted to obviate undesired polyadenylation and ectopic splicing. We initially generated four mutants (pDDS, pDDB, pDDN, and pDDX) that contain an additional E/DLS region at various locations in the HIV-1 genome (Fig. 1). These mutants and the wild-type plasmid (pMSMBA) were transfected in parallel into 293tat cells along with pCMV259Δ21 (40). Plasmid pCMV259Δ21, which was used as a helper plasmid, efficiently produces all the viral structural proteins except Env but does not produce packageable RNA. Cytoplasmic RNA was isolated from the transfected cells, and the virion RNA was isolated from the culture supernatant (40). The amount of RNA within virus particles was then quantitated by an RNase protection assay (40) using a riboprobe which detects both wild-type and mutant RNAs. Although slight variations were observed in packaging efficiency among the various mutants, each of the mutant RNAs was packaged with an efficiency similar to that of the wild type (Fig. 2). This indicates that the presence of an additional E/DLS region at several alternative positions in the viral genome had little effect on RNA packaging.

FIG. 1. Diagram of pMSMBA and mutants containing two copies of viral sequence. Open and solid boxes indicate open reading frames and the long terminal repeat (LTR), respectively. The polyadenylation signal (polyA) and a major s.d. site on the E/DLS fragment were mutated as described in Materials and Methods. Nucleotide positions of restriction endonuclease recognition sites used for constructing these mutants are also shown.
ond E/DLS affects RNA dimer formation by analyzing virion-
yments. Error bars indicate standard errors.
points represent the means of three or more independent measure-
RNA to p24. The RNA/p24 ratio for the wild type was set at 1. Data
efficiency was determined by calculating the ratio of virus-associated
were measured using a p24 antigen capture assay. The packaging
was quantified by phosphorimage analysis, and physical virus particles
to be measured from virus particles was dimeric and exhibited a dena-
tration profile with conversion to single-stranded RNA with
increasing temperature (Fig. 3). However, the RNA from the
particles of the mutants with two E/DLS regions contained a
readily detectible amount of monomeric RNA (25 to 40% of
total signal) even under nondenaturing conditions (Fig. 3A).
This indicates that duplication of the 5’ region of HIV-1 RNA
causes the appearance of monomeric RNA in virions. These
data also indicate that the duplication that resulted in this
effect was position independent, since each of these mutants
harbored monomeric RNA within virus particles.

To determine whether duplication of a region outside of the
primary dimer linkage region could result in an increase in
monomeric RNA in virus particles, we constructed two mu-
tants that had a duplication of a segment of the pol or env gene
(PDN and EDN, respectively) (Fig. 1) and compared dimer
formation of these mutants with that of the wild type and
pDDN. DDN particles, which contained viral RNAs with two
dimer linkage regions, contained detectable monomeric RNA,
as expected. However, the other two mutants, which contained
an additional copy of either pol or env, exhibited an RNA
profile similar to that of the wild type (Fig. 4).

A single ectopic E/DLS can mediate efficient encapsidation
and RNA dimerization. Since the mutants containing RNAs
with two E/DLS regions were encapsidated efficiently, we
wanted to determine whether the ectopic E/DLS sequences
located in different positions throughout the genome were
sufficient for packaging. The HIV-1 construct p5’ssglob, which has a large deletion in the primary encapsidation (E/psi)
region and nearby cis sequences, exhibits severely reduced
RNA packaging efficiency compared to the wild type (41).
However, p5’ssglob is able to express viral genes via spliced
mRNAs, since this construct contains the splice donor from
the first intron of the human β-globin gene. The leader region
of p5’ssglob was introduced in place of the primary E/DLS
region of DD series plasmids to create pssS, pssB, pssN, and
pssX (Fig. 5). 293tat cells were transfected with these plasmids,
and the amounts of cellular and virus particle-associated p24
and virus-specific RNA contents were measured. As shown in
Fig. 6A, the efficiency with which the various mutants were
packaged was within 40 to 70% of the wild-type value, whereas
that of pCMV259A21 and p5’ssglob was only 10 to 20% of
wild-type levels. These results indicate that the ectopic E/psi
region can at least partially function as a packaging signal in
the absence of the authentic E/psi site. They also indicate that
E/psi can function at several different locations in the genome.

We next analyzed the conformation of RNA within virus
particles by native agarose gel electrophoresis followed by
Northern blot hybridization. No apparent monomer RNAs
were observed in virus particles from the mutants containing
a single ectopic E/DLS (Fig. 6B and C). These results are con-
sistent with a requirement for two intact E/DLS regions for
the generation of monomeric RNA. In addition, most of the mu-
tant RNA dimers exhibited thermostability similar to that of
the wild-type virus. It is noteworthy that the mutant ssS dimer
RNA showed slightly higher stability than the wild-type and

![FIG. 2. Relative encapsidation efficiency of viral RNAs containing
two E regions. The relative amount of RNA from the virus particles
was quantified by phosphorimage analysis, and physical virus particles
were measured using a p24 antigen capture assay. The packaging
efficiency was determined by calculating the ratio of virus-associated
RNA to p24. The RNA/p24 ratio for the wild type was set at 1. Data
points represent the means of three or more independent measure-
ments. Error bars indicate standard errors.](http://jvi.asm.org/)

![FIG. 3. Representative phosphorimage analysis of RNA detected
on March 26, 2021 by guest http://jvi.asm.org/](http://jvi.asm.org/)
To overcome this complication, we constructed two more mutants, pDDN and pssN. The infectivity of these mutant viruses was first examined by infection of the MAGI cell assay (30). We then compared the infectivity of those mutants with that of the wild-type, PDN, EDN, and 5’ssβGlob viruses. Since all these constructs carried a mutation in the env gene, HIV-1 Env proteins were supplied by cotransfection of env expression vector p5’ssEnvEXSV to produce infectious virions by complementation. Seventy-two hours after transfection, culture supernatants were assayed for the levels of viral p24 protein, and equivalent amounts of virus in p24 were used to infect MAGI cells (30). Forty-eight hours after infection, cells were fixed and stained, and the cells that were successfully infected, as evidenced by bacterial β-galactosidase expression, were enumerated. As shown in Table 1, there was a more than 100-fold reduction in infectivity of DDNΔPBS and ssNΔPBS compared to the wild-type virus, while the duplication of the pol and env regions had a very little or no effect on viral infectivity. Since ssNΔPBS carried only one intact E/DLS region at an ectopic position and formed dimeric RNA as efficiently as wild-type virus, it seemed unlikely that the presence of an ectopic E/DLS site affected infectivity of DDNΔPBS and ssNΔPBS was not due simply to the lack of dimeric RNA in virions. Instead, it seemed more likely that the presence of an ectopic E/DLS site affected one or more steps between virus penetration and gene expression. In particular, it seemed likely that some step in viral nucleic acid replication was arrested.

**DISCUSSION**

We have found that duplication of the E/DLS region of HIV-1 RNA results in the appearance of monomers in virions without markedly affecting encapsidation efficiency. In contrast, no monomers were observed in virions of mutants that have only one E/DLS region at an ectopic position. Duplication of viral RNA per se does not interfere with dimerization, since monomers were not observed when a segment of the pol or env region was duplicated. We speculate that the presence of an additional E/DLS region at the ectopic position results in interaction between those two regions and competitively interferes with intermolecular dimer formation. Consistent with this notion, monomeric RNAs from mutant virions that had not been subjected to heat treatment exhibited less variation in mobility following electrophoresis through native gels than heated samples. This result is consistent with the idea that the mutant monomeric RNA forms a particular secondary or tertiary structure that results in relatively uniform migration, similar to that observed for dimeric RNA from the wild-type particles. In contrast, monomeric RNA profiles from protease-deficient particles were heterogeneous (21, 22, 50), suggesting a more disordered secondary or tertiary structure of the viral RNA in immature particles. Alternatively, it is possible that a higher proportion of monomers in the mutant virus than in the wild-type virus reflects the relative fragility of the dimers, since it is hard to exclude the possibility that the monomers observed in RNA extracted from virions came from dissociated dimers. In fact, some RNA is observed in the monomer position in all of the unheated RNA profiles (Fig. 4 and 6C). Although this

**FIG. 4.** Thermal dissociation kinetics of dimeric viral RNA. The relative amounts of monomeric and total RNA in each lane were quantitated with a PhosphorImager, and the percentage of monomer RNA was calculated for each RNA sample. Similar results were obtained in three separate experiments. (A) Comparison of wild-type (pMSMBA) and mutant viruses containing two E regions. (B) Comparison of pMSMBA and mutants containing two copies of various viral sequences.

other mutant RNAs. The ssS mutant has a tandemly repeated E/DLS. It is possible that four E/DLS sites positioned closely within two RNA molecules result in a more stable dimer.

**Presence of an ectopic E/DLS region affects the infectivity of virus.** To determine whether the duplication of the E/DLS region affects the viral life cycle, we analyzed the infectivity of mutant viruses using the MAGI cell assay (30). Since the presence of an ectopic E/DLS region would also result in a virus with an extra primer-binding site (PBS), it was highly unlikely that those mutants retained infectivity. To overcome this complication, we constructed two more mutants, pDDNΔPBS and pssNΔPBS, that contained only naturally occurring PBS. The profiles and packaging efficiency of these mutant RNAs were first examined by native agarose gel electrophoresis followed by Northern blot hybridization and an RNase protection assay. This showed that DDNΔPBS and ssNΔPBS were similar to their progenitors, DDN and ssN, respectively, in packaging efficiency and dimerization or lack of dimerization (data not shown). Western blot analysis showed that the proteins of both viruses were properly expressed and processed (Fig. 7). We then compared the infectivity of those mutants with that of the wild-type, PDN, EDN, and 5’ssβGlob viruses. Since all these constructs carried a mutation in the env gene, HIV-1 Env proteins were supplied by cotransfection of env expression vector p5’ssEnvEXSV to produce infectious virions by complementation. Seventy-two hours after transfection, culture supernatants were assayed for the levels of viral p24 protein, and equivalent amounts of virus in p24 were used to infect MAGI cells (30). Forty-eight hours after infection, cells were fixed and stained, and the cells that were successful-
possibility could not be excluded, it is still reasonable to con-
clude that duplication of the E/DLS site affected dimeriza-
tion of viral RNA and that the E/DLS site plays an important role
in dimer formation.

We and other groups have found that mutation of the DIS
loop does not affect dimer formation in vivo (7, 12, 54) and a
region other than E/DLS affected dimer formation of retrovi-
rus RNA (54, 59), also indicating a limited role for the DIS-
DLS region in dimer formation. There are probably multiple
sites on the virus genome that contribute to RNA dimerization.
It is possible that the dimer linkage site observed by electron
microscopy is the final dissociating point of RNA dimer under
denaturing conditions and that such a point might not coincide
with the primary contact point of RNA following virion assem-
bly.

Although the presence of two E/DLS sites results in the
presence of monomeric RNA in particles, it is still unclear
whether one or two monomeric RNAs are encapsidated in
each virion. Determination of the relative amounts of Gag and
RNA molecules in particles might help to resolve this question.
Recent studies with a Rous Sarcoma Virus MA mutant that
packages monomeric RNA suggests the presence of only one
RNA molecule for that mutant (52). However, it appears that
only 10% of HIV-1 particles contain virus RNA (M. S.
McBride, personal communication). Moreover, several reports
describe data indicating that significant quantities of cellular
RNA are incorporated into retrovirus particles (for a review,
see reference 8). Therefore, it is difficult to deduce whether
one or two monomeric RNA molecules are encapsidated in
individual mutant virions.

We observed some dimeric RNA along with monomeric
RNA for mutants containing two E/DLS regions. The authen-
tic and ectopic E/DLS regions on individual RNAs may be
effectively located closer to each other than those on separate
RNA molecules. This might facilitate intramolecular contact
but not completely eliminate intermolecular interaction. Thus,
intermolecular interaction between two native DIS-DLS re-
gions might compete with the intramolecular E/DLS interac-
tion.

Mutants containing an E/DLS site at an ectopic position
but lacking the natural E/psi site were packaged efficiently
but not as efficiently as wild-type RNA. It is possible that the
ectopic E/psi site was fully functional but that the mutated
E/psi site at the original position had a negative effect on
packaging. It is also likely that the context of the E/psi region affects packaging efficiency. However, the ectopic E/DLS region appeared to be fully functional for dimer formation, since mutants containing a single E/DLS region at a single novel location formed RNA dimers with stability similar to that of the wild type. Mutants that had an ectopic E/DLS region were profoundly reduced in infectivity even though they fully retained a E/psi

FIG. 6. Analysis of mutants containing a single ectopic E region. (A) Encapsidation efficiency. The relative amount of RNA from virus particles was quantified by phosphorimage analysis, and encapsidation was quantitated as for Fig. 2. The value for the wild-type control was set at 1. pCMV259Δ21 was included as a negative control. The data are derived from at least three independent experiments. Error bars indicate standard errors. (B) Representative phosphorimage analysis of RNA detected by Northern blotting. Experiments were performed as in Fig. 3. Positions of dimeric (solid arrowheads) and monomeric (open arrowheads) viral RNAs are indicated. The temperatures (degrees Celsius) at which aliquots were incubated are indicated for each lane. (C) Thermal dissociation kinetics of RNA dimers. The thermal stability of RNA dimers and monomers was measured as in Fig. 4. Similar results were obtained in three separate experiments.
site, splice site, and PBS (Table 1). Surprisingly, duplication of the pol and env regions had only a small or slight effect on infectivity (Table 1). One explanation for this specific defect is that the 5′ region containing E/DLS may be more recombinogenic than the pol or env region. This may reflect a general higher efficiency of recombination associated with regions of the RNA normally located near the RNA termini. There may be an intrinsic feature of the termini that promotes transfer of minus-strand strong-stop DNA from the 5′ terminus of the viral RNA. In fact, a series of studies from Pedersen’s group (35, 36, 43–45) showed site-specific recombination within the viral RNA. In fact, a series of studies from Pedersen’s group (35, 36, 43–45) showed site-specific recombination within the viral RNA. In fact, a series of studies from Pedersen’s group (35, 36, 43–45) showed site-specific recombination within the viral RNA.

The generation of monomeric RNA due to the presence of a second E/DLS might provide a useful system for characterizing the requirements for in vivo dimer formation. Genetic analysis of one or both sites and the effect of mutations on these sites could be examined by assaying for the presence of monomers in virus particles.

**FIG. 7. Western blot analysis of virion proteins. Positions of the Gag precursor (Pr55) and gag products p24 and p17 are indicated.**

**TABLE 1. Infectivity of DD and ss mutants**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Relative titer</th>
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<tbody>
<tr>
<td>MSMB</td>
<td>++ +</td>
</tr>
<tr>
<td>DDN/PPS</td>
<td>++ +</td>
</tr>
<tr>
<td>ssNAPS</td>
<td>++ +</td>
</tr>
<tr>
<td>PDD</td>
<td>+ +</td>
</tr>
<tr>
<td>EDN</td>
<td>++ +</td>
</tr>
<tr>
<td>5′ssglob</td>
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*The infections were performed on CD4+ long terminal repeat (LTR)-galactosidase indicator cells as described. Titers: ++ +, >102; ++, 101 to 102; +, 100 to 101; −, <100 β-galactosidase-inducing units per ml.*


