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Retroviral Nucleocapsid Domains Mediate the Specific Recognition of Genomic Viral RNAs by Chimeric Gag Polyproteins during RNA Packaging In Vivo

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The retroviral nucleocapsid (NC) protein is necessary for the specific encapsidation of the viral genomic RNA by the assembling virion. However, it is unclear whether NC contains the determinants for the specific recognition of the viral RNA or instead contributes nonspecific RNA contacts to strengthen a specific contact made elsewhere in the Gag polyprotein. To discriminate between these two possibilities, we have swapped the NC domains of the human immunodeficiency virus type 1 (HIV-1) and Moloney murine leukemia virus (M-MuLV), generating an HIV-1 mutant containing the M-MuLV NC domain and an M-MuLV mutant containing the HIV-1 NC domain. These mutants, as well as several others, were characterized for their abilities to encapsidate HIV-1, M-MuLV, and nonviral RNAs and to preferentially package genomic viral RNAs over spliced viral RNAs. We found that the M-MuLV NC domain mediates the specific packaging of RNAs containing the M-MuLV Ψ packaging element, while the HIV-1 NC domain confers an ability to package the unspliced HIV-1 RNA over spliced HIV-1 RNAs. In addition, we found that the HIV-1 mutant containing the M-MuLV NC domain exhibited a 20-fold greater ability than wild-type HIV-1 to package a nonviral RNA. These results help confirm the notion that the NC domain specifically recognizes the retroviral genomic RNA during RNA encapsidation.

Retroviral RNA encapsidation is the process by which two copies of the viral genomic RNA are packaged into an assembling virion inside an infected cell. The process is selective, in that the viral genomic RNA constitutes less than 1% of the RNA in the infected cell's cytoplasm yet constitutes most of the RNA inside the virion. The mechanism by which retroviruses preferentially encapsidate their own genomic RNA over cellular RNAs and subgenomic viral RNAs presumably involves a specific association between the genomic RNA and a component of the assembling virion. Since viral particles generated in the absence of the *pol* and *env* genes encapsidate normal levels of viral genomic RNA (43, 45), the Gag polyprotein is apparently sufficient for the process of RNA packaging.

Mutational analysis of *gag* has demonstrated that the NC domain of the polyprotein is necessary for RNA packaging. The NC domain is a small (49 to 95 residues) domain lying at or near the C terminus of the polyprotein and contains two conserved structural features. First, in all retroviruses except spumaviruses, NC contains one or two Cys-His boxes, a 14-residue zinc finger-like structure in which the tetrahelical coordination of zinc by three invariantly positioned cysteines and one histidine causes the region to adopt a compact, stable conformation (42, 47). Second, NC contains many basic residues, often in contiguous doublets or triplets flanking the Cys-His box(es). Both of these features have been shown to be required for RNA packaging. Mutations in the Cys-His box can cause drastic impairments in RNA packaging, especially if the mutations affect the ability of the box to coordinate zinc or fold properly (1, 8, 14, 15, 21–23, 39–41, 44). Simultaneous mutation of several basic residues near the Cys-His box can also impair packaging, with the severity of the defect increasing as the number of mutations or the proximity to the Cys-His box increases (18, 26, 44). In one instance, it was shown that only the basic nature of the residues, and not their identities per se, was required for packaging (26).

Is NC sufficient for specific viral genomic RNA encapsidation? Though RNA packaging-disruptive mutations have rarely been encountered outside of the NC domain, this question is difficult to address experimentally, since some regions of the Gag polyprotein are required for the assembly of virus particles and therefore cannot be analyzed for RNA packaging effects by mutational analysis. It is formally possible that these other regions play a direct role in RNA packaging as well as in virus assembly. Since NC is known to possess a nonspecific RNA-binding activity (12, 29, 31, 35), it is possible that the specific encapsidation of the viral genomic RNA requires a nonspecific interaction with the NC domain and a specific interaction with a region of Gag outside of NC. Several studies have analyzed the packaging specificity of retroviruses containing chimeric Gag proteins (16, 45).

In this report we describe efforts to determine whether NC contains the determinants of the Gag polyprotein that specifically interact with the viral genomic RNA during packaging. We constructed chimeric *gag* genes in which all or part of the NC domain was replaced by all or part of an NC domain from a different retrovirus and then characterized which RNAs could be packaged by the mutants. We found considerable evidence that NC mediated the specific encapsidation of the viral genomic RNA.

MATERIALS AND METHODS

Construction of plasmids. Numbering of viral sequences begins at the 5′ end of the 5′ long terminal repeat. A plasmid containing a simian virus 40 origin of replication and a modified human immunodeficiency virus type 1 (HIV-1) Hxb2 provirus lacking the 3′ long terminal repeat, described previously (36) and

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TABLE 1. Oligonucleotide primers used in PCRs during the construction of the chimeric Gag mutants[a]

Primer	Mutant	Sequence
A	HIV-Mobox	5′-TTGTTAAGTGTGCCTACTGCAAAG-3′
	HIV-MoNC	5′-TACCATAATGGCCACTGTCGTTAGTGG-3′
	HIV-MoCh	5′-TTGTTAAGGCCACTGTCGTTAGTGG-3′
	Mo-HIVNC	5′-AGCTATTGATGCAGAGAGGCAATTTTAGG-3′
B	HIV-Mobox	5′-TCTCTCAGTACAATCTTTAGCCCAG-3′
	HIV-MoNC	5′-GGCCAGATCTTCCCTAAAAACAGGAGGGAGGTCTGG-3′
	HIV-MoCh	Same as HIV-MoNC
	Mo-HIVNC	5′-ATCTAGGGTATTAGCCTGTCTCTCAG-3′
C	All 3 HIV mutants	5′-GATGGATAATCCTGGG-3′
	Mo-HIVNC	5′-TTGGCAGTCTGCCC-3′
D	HIV-Mobox	5′-TAGGCACACTTAACAATCTTTCTTTGG-3′
	HIV-MoNC	5′-ACAGTGGCCATTATGGTAGCTGAATTTG-3′
	HIV-MoCh	5′-ACAGTGGCCTTAACAATCTTTCTTTGG-3′
	Mo-HIVNC	5′-CTCTGCATCAATAGCTTGCTCATC-3′
E	HIV-Mobox	5′-GATTGTACTGAGAGACAGGCT-3′
	Mo-HIVNC	5′-AGGCTAATACCCTAGATGAGTAGG-3′
F	HIV-Mobox	5′-GGCCAGATCTTCCCTAAAAAATTAGCCTGTCTCTCAG-3′
	Mo-HIVNC	5′-ACTTTGCGATCCGTGG-3′

[a] For explanations of the use of these primers, see Materials and Methods.

termed here pHIV, was used for the production of HIV-1 virus particles. A nontranslatable variant of this plasmid, also previously described (36) and termed here pHIV-Nde, was used as an HIV-1 RNA. Moloney murine leukemia virus (M-MuLV) particles were generated from a plasmid, originally termed pNCA (10) but termed here pMo, containing the M-MuLV provirus and a simian virus origin (4). To generate a nontranslatable M-MuLV RNA, the sequences between the *Pst*I restriction endonuclease sites at nucleotides (nt) 1016 and 1192 were deleted by standard techniques, generating plasmid pMo-dPst.

Mutant plasmids pHIV-Mobox, pHIV-MoNC, pHIV-MoCh, and pMo-HIVNC were each constructed by a multiple-step PCR protocol using plasmids pHIV and pMo. First, a PCR fragment containing the NC domain flanked by the proper sequences was generated with 5′ primer A and 3′ primer B (Table 1). At the same time, a PCR fragment lying immediately upstream of the NC fragment was generated with 5′ primer C and 3′ primer D, and a PCR fragment lying immediately downstream of the NC fragment was generated with 5′ primer E and 3′ primer F. Primers A and D contained overlapping sequences, as did primers B and E, such that the NC PCR fragment contained overlapping sequences with both flanking PCR fragments. The NC fragment was then connected to the upstream fragment by mixing the two fragments and performing PCR with primers C and B; the resulting fragment was then connected to the downstream fragment by mixing the two fragments and performing PCR with primers C and F. The final PCR product was digested with either *Acc*I and *Bgl*II (HIV-1 mutants) or *Bgl*II and *Dra*III (M-MuLV mutant) and subcloned into the *Acc*I (nt 1676)-*Bgl*II (nt 2093) sites of pHIV-1 or the *Bgl*II (nt 2355)-*Dra*III (nt 2785) sites of plasmid pMo by standard techniques. Note that mutants HIV-MoNC and HIV-MoCh did not require the downstream PCR fragment since the 3′ *Bgl*II site was located in the flanking sequences of the NC fragment. Mutant plasmid pHIV-d1 was constructed by subcloning the *Acc*I-*Bgl*II fragment of plasmid pGST-NCd1 (6) into plasmid pHIV. The four HIV-1 mutations were subcloned into the pHIV-Nde construct via the *Spe*I (nt 1504)-*Sal*I (nt 5783) fragment. All constructs were subjected to DNA sequence analysis to confirm that the mutations were as expected.

The M-MuLV Ψ fragment, from *Msc*I (nt 664) to *Pst*I (nt 1016), was used to replace 19 nt of the leader of wild-type HIV-1 and each of the four HIV-1 mutants in three steps. First, the large fragment of *Ssp*I-digested plasmid pHIV-SVPA-ΔP1 containing the 19-nt deletion (36) was circularized to generate the HIV-1 shuttle vector pdSsp. Then, the M-MuLV Ψ fragment was blunt end inserted into the 19-nt mutation-derived *Nae*I site of plasmid pdSsp. Then, the *Bss*HII (nt 708)-*Pst*I (nt 1416) fragment including the insertion was subcloned into each of the plasmids pHIV, pHIV-d1, pHIV-Mobox, pHIV-MoNC, and pHIV-MoCh to generate plasmids pHIV-MΨ, pHIV-d1MΨ, pHIV-MoboxΨ, pHIV-MoNCΨ, and pHIV-MoChΨ, respectively. DNA sequence analysis of these plasmids revealed that two had the expected sequence; however, three (pHIV-d1MΨ, pHIV-MoNCΨ, and pHIV-MoChΨ) had a deletion of 4 bp at the 5′ side of the insertion site.

The nonviral construct pCTM1 was constructed by the sequential insertion of segments of the cytomegalovirus major immediate-early gene promoter and first exon, the human triosephosphate isomerase cDNA, the Mason-Pfizer monkey virus genome, and the simian virus 40 origin of replication into the polylinker of the general utility plasmid pSP72 (Promega Corp., Madison, Wis.). Specifically, the cytomegalovirus segment was composed of the sequences between the *Msc*I site in the enhancer and the *Eag*I site 77 nt downstream of the transcription initiation site (provided by Mark Stinski, University of Iowa). The triosephosphate isomerase segment spanned nt 616 to 1518 (numbering consistent with GenBank accession no. M10036; provided by Lynne Maquat, Roswell Park Cancer Institute, Buffalo, N.Y.). The Mason-Pfizer monkey virus segment consisted of the 3′ 550 nt of the genome, including the *cis* transport element and the poly(A) signal (provided by E. Hunter, University of Alabama, Birmingham). The simian virus 40 segment was composed of the sequences between the *Hin*dIII and *Pvu*II sites.

Plasmids used as templates for the production of riboprobes were constructed as follows. Plasmid pHRCS, used for most HIV-1 constructs, has been described previously (36). Plasmids pChR1 and pChR2, used for the HIV-1 constructs containing the M-MuLV Ψ insertion, were constructed by the blunt-end insertion of the *Sca*I (nt 315 in the HIV-1 promoter)-*Eag*I (nt 795 in the M-MuLV insert) fragment of plasmid pHIV-MΨ (pChR1) or plasmid pHIV-d1MΨ (pChR2) into the *Eco*RV site of plasmid pSP72. Plasmid pChr1 was used for the HIV-MΨ and HIV-MoboxΨ constructs, while pChR2 was used for the HIV-d1MΨ, HIV-MoNCΨ, and HIV-MoChΨ constructs because of the 4-bp deletion in these three plasmids. Plasmid pMoR was used for the M-MuLV constructs and was constructed by ligating the *Xba*I (nt 298 in the promoter)-*Eag*I (nt 795 in the leader) fragment of plasmid pMo to the *Eag*I-*Xba*I fragment of plasmid pBluescript II KS− (Stratagene Cloning Systems, La Jolla, Calif.). Plasmid pCTR was used for the nonviral construct pCTM1 and was constructed by the blunt-end insertion of the *Aat*II (in the cytomegalovirus promoter)-*Pfl*MI (nt 718 in triosephosphate isomerase) fragment of plasmid pCTM1 into the *Eco*RV site of plasmid pSP72.

Production of virus, analysis of viral proteins, and RT assay. Cos cells were maintained in Dulbecco's modified Eagle medium containing 9% fetal bovine serum and were transfected by a calcium phosphate protocol (Speciality Media Inc., Lavallette, N.J.). Forty-eight hours posttransfection, culture media were removed from the Cos cells, filtered, and subjected to centrifugation over a 25% sucrose cushion to pellet the virus particles. The Cos cells were lysed in 50 mM Tris (pH 8.0)–100 mM sodium chloride–5 mM magnesium chloride–0.5% Nonidet P-40, and the nuclei were removed by centrifugation. Virions were lysed in TNE (10 mM Tris [pH 7.5], 100 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid [EDTA]) containing either 0.1% Triton X-100 (HIV-1 virions) or 0.06% Nonidet P-40 (M-MuLV virions). Reverse transcriptase (RT) assays were performed on the M-MuLV virion lysates as described previously (20). The HIV-1 virion lysates were assayed for RT activity as follows: an aliquot of the lysate was mixed with an equal volume of 90 mM Tris (pH 7.5)–2 mM dithiothreitol–500 mM sodium chloride–40% glycerol–0.5% Triton X-100. Ten microliters of the mixture was mixed with 10 μl of 5× RT buffer (200 mM Tris [pH 7.5], 37.5 mM magnesium chloride, 250 mM potassium chloride, 0.25% Triton X-100), 5 μl of 50-μg/ml oligo(dT)–50-μg/ml dTTP–100-μg/ml poly(rA), 1.2 μl of 200 mM dithiothreitol, 0.5 μl of 800-Ci/ml [^{32}P]dTTP, and 23.3 μl of water.

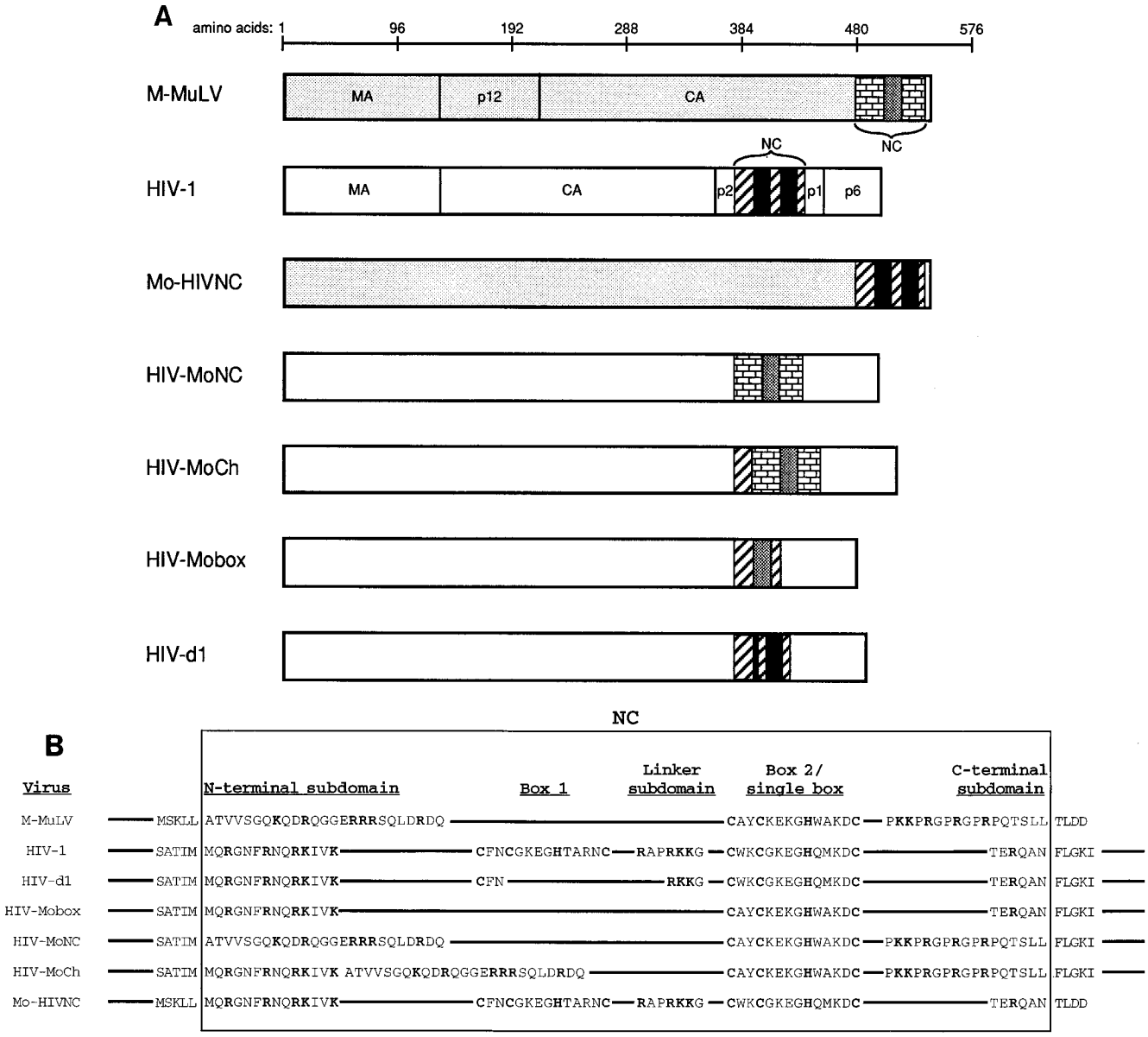

FIG. 1. Gag mutants and packaging constructs used in this study. (A) Schematic of wild-type M-MuLV, wild type HIV-1, and chimeric Gag precursor polyproteins. The M-MuLV Gag polyprotein is lightly shaded, the NC domain is depicted with a brick pattern, and the Cys-His box is darkly shaded. The HIV-1 NC domain is hatched, and the HIV-1 Cys-His boxes are in black. For descriptions of each of these mutants, see the text. (B) Amino acid residues of the constructs' NC domains. The NC residues are boxed, with the immediately flanking N-terminal residues to the left and the immediately flanking C-terminal residues to the right of the box. The horizontal lines are used to help divide the region into the different subdomains and do not indicate regions of deletions. Zinc-coordinating residues in the Cys-His boxes and basic residues outside the Cys-His boxes are indicated in boldface. (C) M-MuLV and HIV-1 packaging constructs and the nonviral expression construct CTM1. The *gag* start codon is deleted in the M-MuLV construct and is point mutated in the HIV-1 construct to render these genes untranslatable. For a description of the CTM1 construct, see Materials and Methods. SV, simian virus; CMV, cytomegalovirus; TPI, triosephosphate isomerase; MPMV, Mason-Pfizer monkey virus.

The reaction mixtures were incubated at 37°C for 15 min, and 10 μl was spotted onto DE-81 filter paper (Whatman); the filter was rinsed extensively with 2× SSC (300 mM sodium chloride, 30 mM sodium citrate, pH 7.4) and dried. Results of both HIV-1 and M-MuLV RT assays were quantified with a Betascope model 603 blot analyzer (Betagen Corp., Waltham, Mass.).

For the analysis of viral proteins, the cell and virion lysates were denatured and serial dilutions were subjected to electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and then transferred to a nitrocellulose filter by standard techniques. The filter was probed with polyclonal antisera to either HIV-1 capsid (AIDS repository no. 384), HIV-1 RT (AIDS repository no. 716), M-MuLV capsid (National Cancer Institute no. 79S-107), or M-MuLV RT anti-30-2 (7) by an enhanced chemiluminescence protocol (Amersham Corp., Arlington Heights, Ill.).

Analysis of encapsidated RNAs. RNA was isolated from the virion and cell lysates as follows. The cell lysates (minus nuclei) were supplemented with SDS and proteinase K to final concentrations of 0.2% and 125 μg/ml, respectively,

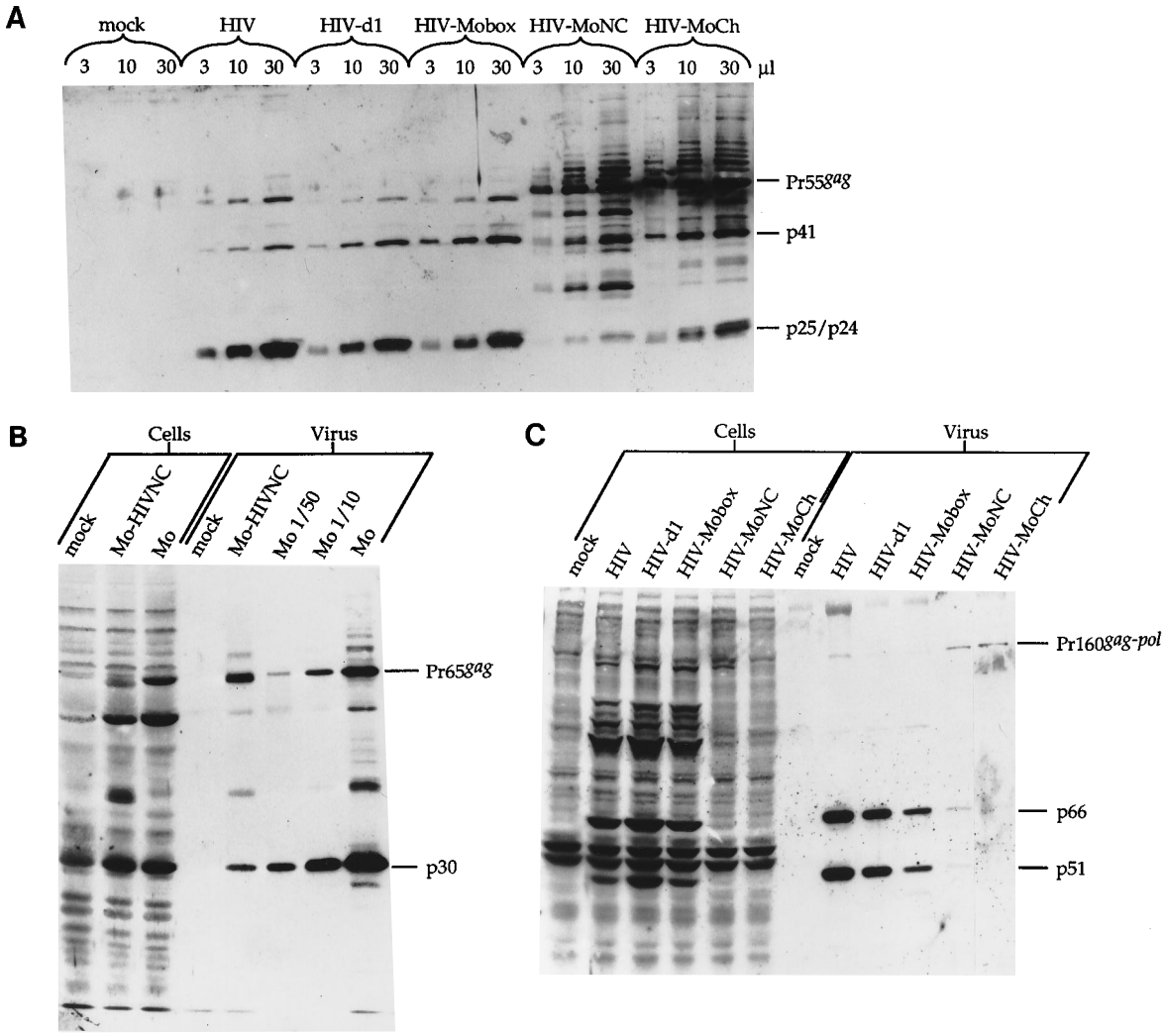

FIG. 2. Viral protein content of mutant virions. Virus particles collected from the media of Cos cells transfected 48 h earlier with the indicated provirus were lysed and subjected to Western blotting using specific antisera. (A) HIV-1 mutants probed with antiserum to p24 capsid. For each mutant, a range of volumes of the virus lysate was analyzed. (B) M-MuLV mutant probed with antiserum to p30 capsid. For the wild-type (Mo) virus lysate, several dilutions were analyzed; in addition, the Cos cell lysates were included. (C) HIV-1 mutants probed with antiserum to p66 RT. Virus lysates were first normalized by p24 Western blotting as described for panel A. Cell lysates were included to discriminate frameshifting or stability versus incorporation phenotypes. Mock, mock-transfected Cos cells; Mo, wild-type M-MuLV.

incubated at 37°C for 15 min, and extracted with phenol-chloroform, and nucleic acids were precipitated with ethanol. The virion lysates were adjusted to 50 mM Tris (pH 7.5)–100 mM sodium chloride–10 mM EDTA–1% SDS–100 μg of proteinase K per ml–10 μg of tRNA per ml, incubated at 37°C for 15 min, extracted with phenol-chloroform, and precipitated with ethanol. RNAs were suspended in water and assayed for the presence of discrete species by an RNase protection assay (Ambion Inc., Austin, Tex.) using antisense, radiolabelled RNA probes synthesized as described previously (6). In a typical assay, approximately 1/10 of the RNA isolated from a 10-cm-diameter dish of transfected Cos cells and 1/2 of the RNA isolated from the virus collected from the dish were each annealed to approximately 375 pg (2.5×10^5 dpm) of the riboprobe; under these conditions, the probe is in excess, guaranteeing linearity of signal. The protected fragments of the probe were subjected to denaturing polyacrylamide gel electrophoresis and autoradiography; the amount of radioactivity in the fragments was quantitated directly with a Betascope model 603 blot analyzer (Betagen Corp.).

Electron microscopy. Suspensions of transfected Cos cells and collected virus particles were fixed in fresh 2.5% glutaraldehyde in phosphate-buffered saline (PBS) at pH 7.0 and postfixed with 1% osmium tetroxide. After thorough rinsing in PBS and agar block enclosure, the fixed cells and virus particles were dehydrated and embedded in Epon. Thin sections were made approximately 60 to 80 nm thick and were poststained with 1% uranyl acetate and 1% lead nitrate. Specimens were analyzed with a Zeiss CEM 902 electron microscope, equipped with a spectrometer, at an accelerating voltage of 80 kV.

RESULTS

Construction of mutants. To probe whether the NC domain of the Gag polyprotein was sufficient to determine the specificity of the RNAs encapsidated by retroviruses, the NC domains of HIV-1 and M-MuLV were exchanged and the specificity of RNA packaging was determined for each resultant chimeric mutant. The 55-residue NC domain of the Hxb2 strain of HIV-1 was replaced with the 56-residue M-MuLV NC domain, generating the HIV-1 mutant HIV-MoNC, while the reverse substitution generated the M-MuLV mutant Mo-HIVNC (Fig. 1). In addition, two other HIV-1 mutants containing portions of M-MuLV NC were constructed. In mutant HIV-MoCh, the C-terminal 41 residues of the HIV-1 NC domain were replaced by the entire M-MuLV NC domain, generating a chimeric NC (hence "Ch" for chimera); this mutant is similar to HIV-MoNC but retains the normal NC N-terminal 14 residues. Mutant HIV-MoCh was constructed because all previous HIV-1 mutants lacking both Cys-His boxes were not competent for virion particle assembly except for one contain-

TABLE 2. RT activities of the HIV-1 mutants

Expt	RT activity (cpm) in cells					
	Mock transfected	Transfected with[a]:				
		HIV-1	HIV-d1	HIV-Mobox	HIV-MoNC	HIV-MoCh
1	2	8,057	ND	1,503	52	ND
2	7	3,972	ND	667	140	17
3	<1	3,965	ND	426	182	18
4	6	13,968	6,352	1,103	312	148

[a] Forty-eight hours after Cos cells were transfected with each mutant, particle-associated RT activity was assayed as described in Materials and Methods. ND, not determined. In the M-MuLV RT assay, the activity in mock-, M-MuLV-, and Mo-HIVNC-transfected cells was <1, 647, and <1 cpm, respectively.

ing the N-terminal 14 residues of NC (3, 17, 19, 28). In mutant HIV-Mobox, 35 residues of the HIV-1 NC interior, including both Cys-His boxes and the residues in between, were replaced with the sole M-MuLV Cys-His box. We constructed a single-box HIV-1 mutant lacking M-MuLV sequences as a control; 14 residues, including almost all of the N-terminal Cys-His box, were deleted from the NC domain of HIV-1, generating mutant HIV-d1.

Assembly and release of mutant virus particles. The mutants were analyzed for virion production after transient expression of the viral proteins in Cos cells. Forty-eight hours after transfection of the cells, virions were collected from the culture media and lysed; serial dilutions of the virion proteins were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with polyclonal capsid antisera (Fig. 2A and B). By comparing the sum of the intensities of the CA (p24) and $Pr55^{gag}$ bands among the dilutions, virus particles can be normalized within a factor of two. All of the HIV-1 mutants produced wild-type levels of particles. In fact, comparison of virion-associated Gag protein levels with cell-associated Gag protein levels indicated that mutants HIV-MoNC and HIV-MoCh were fivefold more efficient in virion assembly or release than wild-type HIV-1 (data not shown). Mutant Mo-HIVNC was also able to produce virus particles but produced 20- to 40-fold fewer particles than wild-type M-MuLV, supporting prior results indicating a role for M-MuLV NC in virion assembly (24).

To characterize the mutant virions for *pol* gene products, the collected virus particles were assayed for RT activity. Mutant HIV-d1 exhibited RT levels twofold lower than wild-type, but in multiple experiments the rest of the mutants exhibited further reduced levels of RT activity (Table 2). The HIV-Mobox RT level was reduced 5- to 13-fold, the HIV-MoNC RT level was reduced 20- to 50-fold, and the HIV-MoCh RT level was reduced greater than 100-fold. Mutant Mo-HIVNC exhibited no detectable RT activity. These results suggest that some of the mutants have reduced levels of RT in spite of the normal virion-associated Gag levels.

Interestingly, a general correlation was observed between the amount of RT activity detected in the RT assay and the amount of protease-mediated Gag processing detected in the Western (immunoblot) analysis, indicating a general defect in *pol* function for some of the constructs. To determine the cause of the *pol* defect, equivalent numbers of transfected cells and virus particles (as determined by the capsid immunoblot) were assayed for RT protein by Western blotting, using RT antisera (Fig. 2C). Mutant HIV-Mobox contained normal levels of intracellular *pol* gene products but exhibited reduced levels of virion-associated RT protein, indicating that this mutant was partially defective in Gag-Pol polyprotein incorporation during virion assembly. Mutants HIV-MoNC and HIV-MoCh exhibited extremely reduced levels of intracellular and virion-associated RT protein, much of which still resided in the Gag-Pol polyprotein, indicating that the *pol* defect in these mutants lies in either Gag-Pol protein stability or, more likely, Gag-Pol protein synthesis. Both mutants contained guanine as the last nucleotide in the NC coding region; the current model for the frameshifting mechanism requires that this nucleotide be a thymidine, to allow the slippage of the phenylalanine tRNA containing the nascent polypeptide into the −1 reading frame (27). Mutant Mo-HIVNC produced too few virus particles to readily reveal virion-associated Pol protein levels.

Visualization of mutant virus particles. The mutant virions were collected after transient transfection of Cos cells and analyzed by electron microscopy for structural differences from the wild-type particles. In particular, changes in the size or shape of the virions and in the packaging of the core material within the virions were assessed. Previously, HIV-1 and M-MuLV NC domains have been implicated in regulating virion density, and mutations in the HIV-1 capsid domain have been found to alter the proper redistribution of the core material during virion maturation (13, 37).

The wild-type HIV-1 virions were observed to contain the characteristic cone-shaped core structure (25) (Fig. 3A). The HIV-Mobox particles were heterogeneous in appearance, exhibiting both immature particles and mature particles containing globular core structures (Fig. 3B); cone-shaped core structures were never observed. The HIV-MoNC particles were entirely immature, consistent with the lack of *pol* function in this mutant (Fig. 3C). The HIV-MoCh particles were predominantly immature and varied in size, ranging from 120 to 240 nm in diameter. Occasionally, particles containing a lateral body of core material adjacent to the envelope or a round dense central core structure were observed (Fig. 3D). The irregular packing of core material appears to parallel that of HIV-1 CA mutants (13, 37). The HIV-d1 particles were immature (Fig. 3E), even though the viral proteins were fully processed (Fig. 2A), raising the possibility that this mutant was impaired in core condensation. The Mo-HIVNC particles were also immature and varied in size, ranging from 90 to 170 nm (Fig. 3F). Altogether, the results suggest that in addition to regulating virus particle assembly and density, NC plays a role in the regulation of virion size and the mechanism of core condensation.

Encapsidation of mutant RNAs. The viral RNA content of the mutant virions was analyzed by an RNase protection assay to determine the effect of the mutations on the preferential encapsidation of the unspliced, genomic viral RNA over the spliced, subgenomic viral RNAs. Cos cells were transfected with each of the mutant proviruses, and virions were collected and normalized by the capsid immunoblot analysis as before. RNA was then extracted from the virions and the cytoplasm of the transfected cells. The virion-associated RNA and cytoplasmic RNA were each annealed to a radiolabelled antisense riboprobe RNA spanning the viral transcription initiation site and the major splice donor (Fig. 4). The mixtures were then digested with the single-strand-specific RNases A and T_1 and subjected to denaturing polyacrylamide gel electrophoresis. The resulting riboprobe fragments, each protected by a different viral RNA species, were visualized by autoradiography and quantified by a direct β-emission sensor (Fig. 5). Wild-type HIV-1 exhibited a 10-fold preference for packaging the genomic RNA over the spliced RNAs, since the spliced RNAs were twice as prevalent as the genomic RNA in the cytoplasm but the genomic RNA was 5-fold more prevalent than the

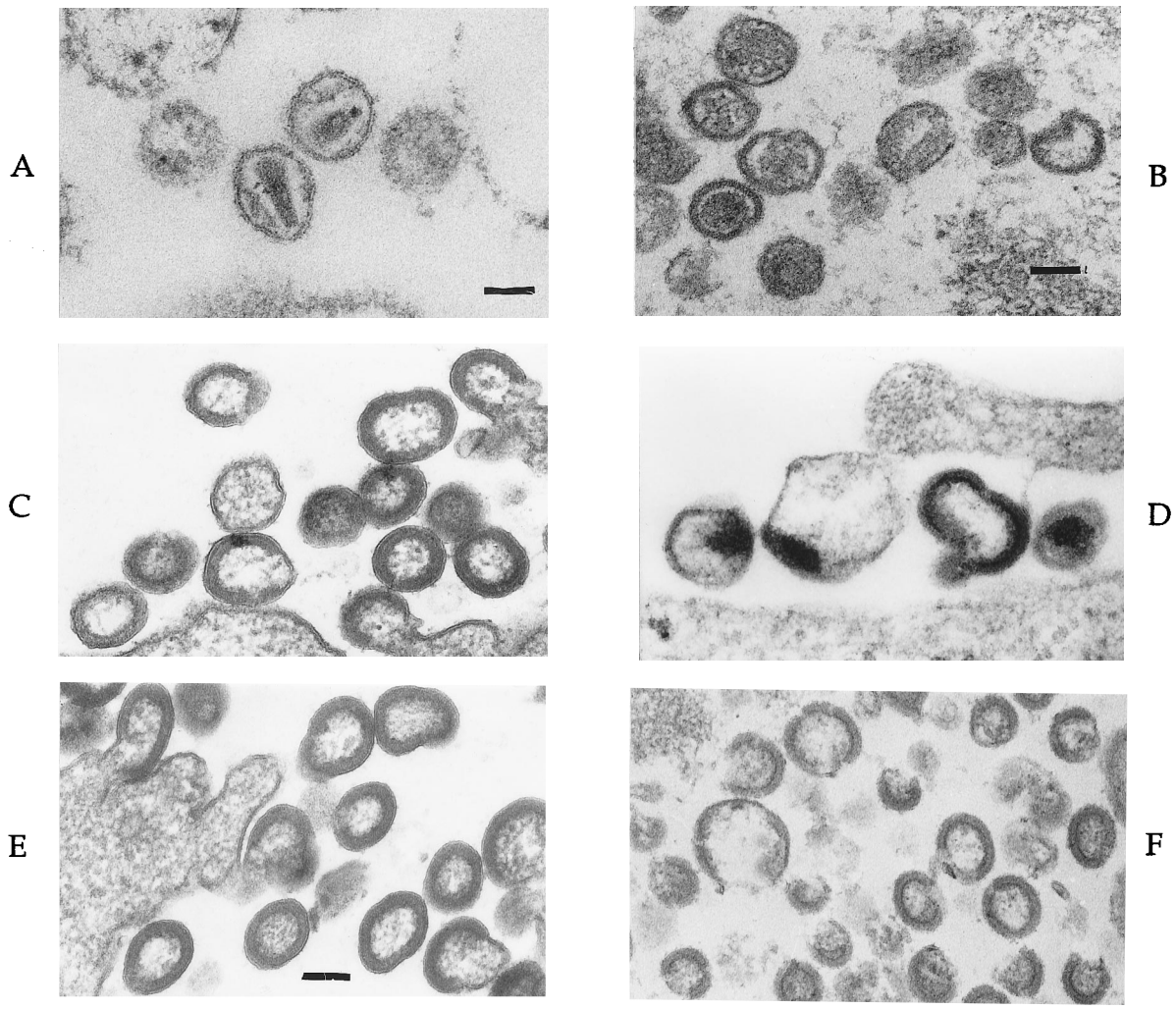

FIG. 3. Electron micrographs of transfected Cos cells and virus particles. Forty-eight hours after transfection, virus particles and cell debris in the culture media were collected by centrifugation and, along with the Cos cells, analyzed by electron microscopy as described in Materials and Methods. The magnification is ×70,000, and the bars are 100 nm in length. (A) Wild-type HIV-1, showing virions containing characteristic cone-shaped virion core structures; (B) mutant HIV-Mobox, showing virions containing aberrant core structures; (C) mutant HIV-MoNC, showing immature virions; (D) mutant HIV-MoCh, showing an immature virion (middle), a mature virion containing a centrally condensed core structure (right), and two mature virions containing laterally condensed core structures (left); (E) mutant HIV-d1, showing immature virions; (F) mutant Mo-HIVNC, showing immature virions varying in size.

spliced RNAs in the virions. Repeated trials, in this and all of the experiments in this study, yielded numbers for these ratios well within a factor of two. For the HIV-1 mutants, both HIV-MoNC and HIV-MoCh packaged roughly the same amount of unspliced viral RNA as wild-type HIV-1. However, these two mutants also packaged much spliced viral RNA, approximately 10-fold more than the wild type did; as a result, the ratio of spliced to unspliced RNA in the virus particles was the same as the ratio in the Cos cell cytoplasm. Thus, these two mutants have lost the ability to preferentially package the viral genomic RNA but have gained the ability to package more total viral RNA. Mutants HIV-Mobox and HIV-d1 also lost the preference for the genomic RNA but differed in the total amount of viral RNA encapsidated; HIV-Mobox packaged 40-fold less viral RNA than the wild type did, while mutant HIV-d1 packaged roughly the same amount of viral RNA as wild-type HIV-1. Mutant HIV-d1's phenotype was identical to that of a previously described mutant (HIV-SVPA-ΔP1) containing a 19-nt deletion just upstream of the *gag* start codon (36).

To address the possibility that these four mutants' packaging phenotypes were due to *cis* effects on the HIV-1 packaging signal in the viral RNA, the mutations were subcloned into a packageable, nontranslatable HIV-1 construct (Fig. 1C). The subclones were introduced into Cos cells along with a wild-type HIV-1 plasmid and assessed for their RNA's incorporation into the wild-type virus particles (data not shown). None of the four mutations affected the ability of the RNA to be packaged, confirming that the mutants' phenotypes resulted from alterations in the Gag polyprotein.

The ability of the M-MuLV mutant Mo-HIVNC to package its unspliced and spliced RNAs could not be determined (Fig. 5B), because of the low numbers of virus particles produced by the mutant and the low levels of M-MuLV RNA inside the Cos cell cytoplasm. In addition, M-MuLV particle-associated RNA appeared to be susceptible to nucleolytic degradation (see lane Mo*, containing 30-fold more wild-type virus particles).

Encapsidation of HIV-1 mutant RNAs containing the M-MuLV Ψ element. The HIV-1 constructs were assessed for their ability to package RNAs containing the M-MuLV Ψ

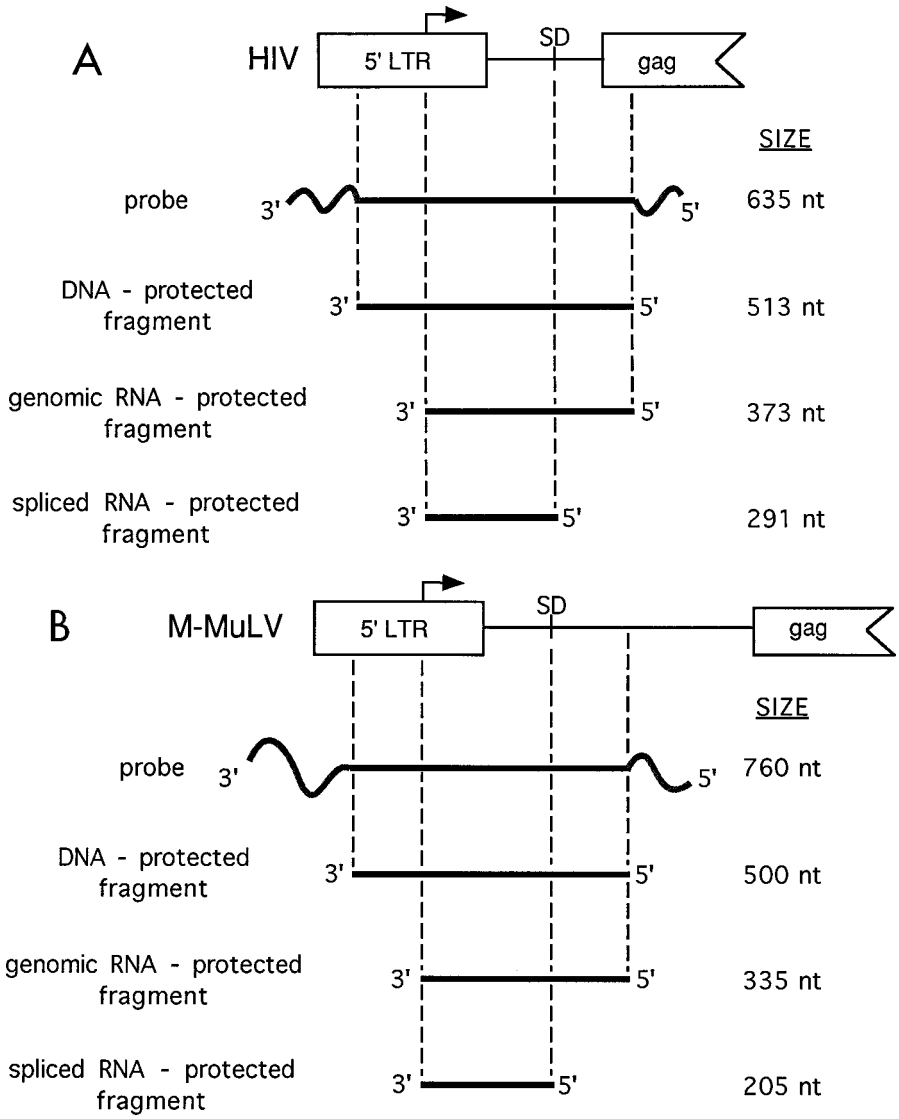

FIG. 4. Schematic of probes used in RNase protection assays and sizes of different protection fragments of the probe. (A) HIV-1 probe and fragments; (B) M-MuLV probe and fragments. (Top of each panel) Provirus schematic, with 5′ long terminal repeat (LTR) and *gag* genes boxed, splice donor indicated by "SD," and transcription initiation site indicated with an arrow; (middle of each panel) RNA probe containing antisense viral segment, aligned with the provirus to depict regions of complementarity and flanked by nonviral sequences; (bottom of each panel) three possible probe fragments, produced by protection of the probe by the proviral DNA, unspliced genomic viral RNA, or spliced viral RNA.

packaging signal preferentially over RNAs lacking the signal. The 351-nt Ψ region was inserted into the constructs' leaders, in between the splice donor and the *gag* start codon; as a result, each genomic RNA contained the Ψ insertion but the corresponding spliced RNAs did not. Cos cells were transfected with each of these constructs, virions were collected and normalized as before, and the RNase protection assay was utilized to determine the levels of virion-associated genomic and spliced viral RNAs (Fig. 6). The wild-type HIV-1 Gag protein was unable to package its M-MuLV Ψ-containing genomic RNA with preference over its spliced RNAs, indicating that the Ψ insertion interfered with the genomic RNA's preferential ability to be packaged. The HIV-d1 mutant also packaged its Ψ-containing genomic RNA with a similar lack of preference over its spliced RNAs. However, the HIV-MoNC and HIV-MoCh Gag proteins each exhibited a significant preference for packaging their Ψ-containing genomic RNA over their spliced RNAs; the selectivity exhibited by the two mutants was sixfold higher than that of wild-type HIV-1 Gag or mutant HIV-d1 Gag. For reasons unknown, the HIV-MoNC and HIV-MoCh mutants produced unusually high levels of cytoplasmic and virion-associated viral RNAs. The requirement of both M-MuLV Ψ and M-MuLV NC sequences for the restoration of the packaging preference supports the notion that these regions interact with each other in a specific fashion during M-MuLV genomic RNA encapsidation. Mutant HIV-Mobox was not able to package the Ψ-containing genomic RNA, indicating that the M-MuLV Cys-His box requires the proper flanking residues.

Encapsidation of heterologous retroviral RNAs. The HIV-1 mutants were also assessed for their abilities to encapsidate an M-MuLV RNA lacking HIV-1 sequences. Cos cells were cotransfected with each mutant DNA and a DNA encoding a nontranslatable M-MuLV RNA (Fig. 1C); the HIV-1 particles

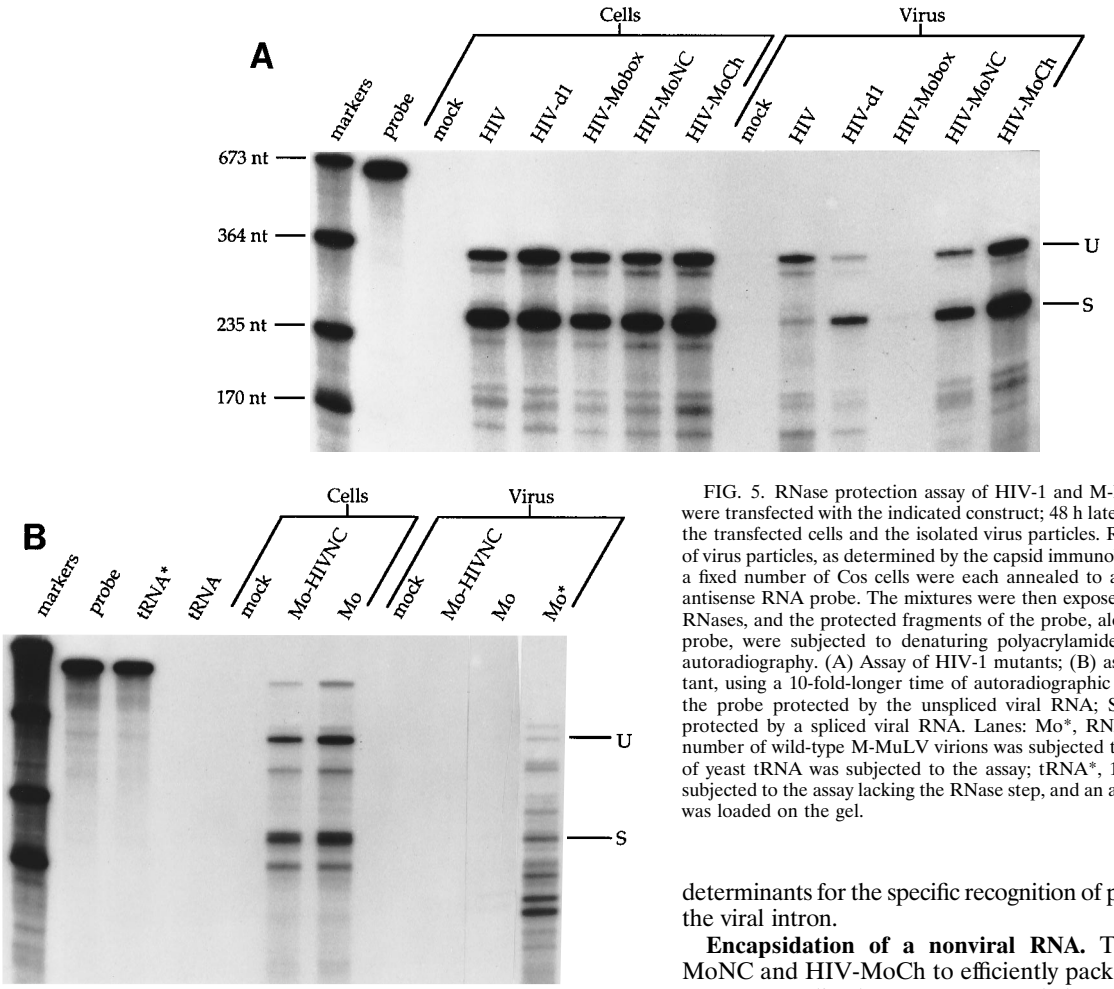

FIG. 5. RNase protection assay of HIV-1 and M-MuLV mutants. Cos cells were transfected with the indicated construct; 48 h later, RNA was isolated from the transfected cells and the isolated virus particles. RNA from a fixed number of virus particles, as determined by the capsid immunoblot assay, and RNA from a fixed number of Cos cells were each annealed to an excess of radiolabelled antisense RNA probe. The mixtures were then exposed to single-strand-specific RNases, and the protected fragments of the probe, along with an aliquot of the probe, were subjected to denaturing polyacrylamide gel electrophoresis and autoradiography. (A) Assay of HIV-1 mutants; (B) assay of the M-MuLV mutant, using a 10-fold-longer time of autoradiographic exposure. U, fragment of the probe protected by the unspliced viral RNA; S, fragment of the probe protected by a spliced viral RNA. Lanes: Mo*, RNA from a 30-fold-greater number of wild-type M-MuLV virions was subjected to the assay; tRNA, 10 μg of yeast tRNA was subjected to the assay; tRNA*, 10 μg of yeast tRNA was subjected to the assay lacking the RNase step, and an aliquot of the final product was loaded on the gel.

shed were assayed for both the HIV-1 mutant RNA (data not shown) and the M-MuLV RNA (Fig. 7A). As expected, wild-type HIV-1, HIV-d1, and HIV-Mobox were unable to encapsidate the M-MuLV RNA, while the HIV-MoNC and HIV-MoCh mutants packaged high levels of the M-MuLV RNA. Interestingly, the spliced M-MuLV RNA was also packaged well by mutants HIV-MoNC and HIV-MoCh. In addition, the extent of packaging of the HIV-1 RNAs was the same in the presence and absence of the M-MuLV RNA.

The M-MuLV mutant Mo-HIVNC was similarly analyzed for its ability to package the nontranslatable HIV-1 RNA shown in Fig. 1C. Perhaps because HIV-1 RNA expression in Cos cells is approximately 10-fold higher than M-MuLV RNA expression, we were able to detect HIV-1 RNA in the wild-type M-MuLV virions (Fig. 7B), whereas we could not detect the M-MuLV RNA (Fig. 5B). Interestingly, wild-type M-MuLV did not exhibit a preference for the unspliced HIV-1 RNA. Mutant Mo-HIVNC exhibited a 4-fold increase in the packaging efficiency of the unspliced HIV-1 RNA and a 1.5-fold increase in the packaging efficiency of the spliced HIV-1 RNA, indicative of a slight preference for the unspliced RNA. These numbers are likely to be underestimates, since a significant fraction of the HIV-1 RNA packaged by the mutant was partially degraded, as evidenced by the presence of many intense smaller protected fragments (Fig. 7B and data not shown). The ability of mutant Mo-HIVNC to preferentially package the unspliced HIV-1 RNA suggests that HIV-1 NC contains determinants for the specific recognition of packaging elements in the viral intron.

Encapsidation of a nonviral RNA. The ability of HIV-MoNC and HIV-MoCh to efficiently package both HIV-1 and M-MuLV spliced RNAs suggested that these mutants might have contained an increased nonspecific component of their packaging mechanisms. To test this idea, HIV-MoNC, HIV-d1, and wild-type HIV-1, as well as an HIV-1 mutant containing a 19-nt leader deletion (36) (termed here HIV-d19 for simplicity), were each assessed for the ability to encapsidate a nonviral RNA. Each mutant was introduced into Cos cells along with a eucaryotic expression construct containing the human triosephosphate isomerase cDNA (Fig. 8). RNase protection analysis of the virion and cytoplasmic RNA using a probe specific for the 5′ end of the nonviral RNA indicated that the nonviral RNA was packaged at low levels by all of the viruses except HIV-MoNC, which packaged the nonviral RNA 20-fold more efficiently than the rest. This result suggests that the HIV-MoNC chimeric Gag protein is able to interact with RNA nonspecifically to a much greater extent than is wild-type HIV-1 Gag.

DISCUSSION

In retroviruses, the NC domain of the Gag polyprotein is generally believed to contain the binding site specific for the packaging signal in the intron of the unspliced genomic RNA, primarily because mutations in NC often lower the levels of virion-associated viral RNA (1, 8, 14, 15, 21–23, 39–41, 44). However, while these studies have indicated that the NC domain is necessary for RNA packaging, they do not address whether NC actually mediates the selective recognition of the viral genomic RNA. An alternative model suggests that the

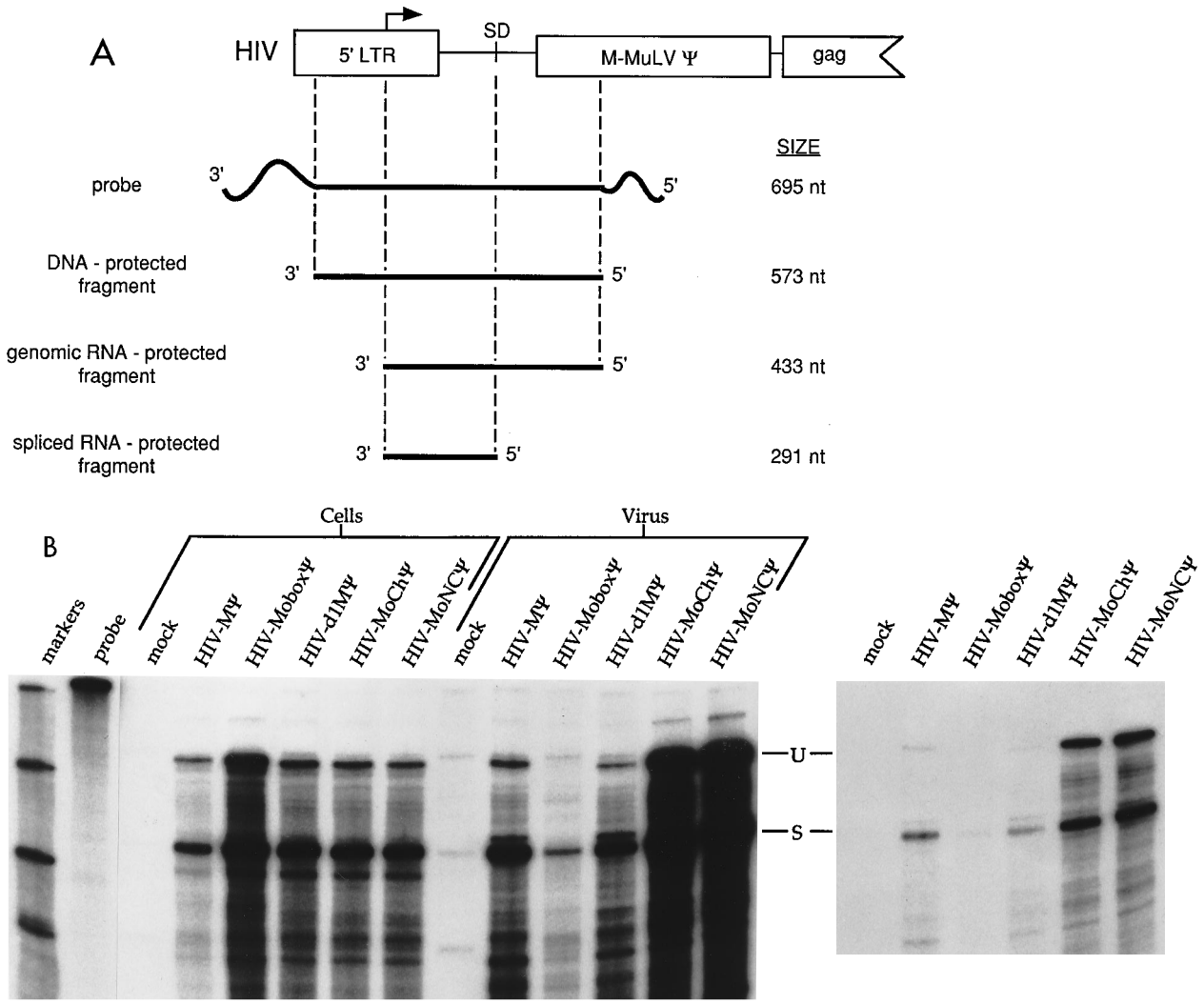

FIG. 6. RNase protection assay of the HIV-1 mutants containing M-MuLV Ψ substitution leader mutations. (A) Schematic of the mutant provirus, modified HIV-MuLV probe, and anticipated protection fragments, depicted as in Fig. 4. (B) RNase protection assay. Each of the indicated constructs was evaluated for cellular and normalized virion-associated RNA levels, as indicated in Fig. 5. (Right) A shorter exposure of the viral RNA section of the gel shown at the left.

specific recognition of the viral genomic RNA occurs elsewhere in the Gag polyprotein but that the NC domain is required to bind nonselectively to the RNA to strengthen the interaction between the two molecules. Indeed, NC is known to bind nonselectively to RNAs, primarily through its large number of basic residues (12, 29, 31, 33, 35).

One way to test these two models is by substituting one retroviral NC domain with another and assaying whether the specificity of RNA packaging has been changed to that of the replacing virus. To assay the specificity of RNA packaging, the packaging efficiency of an RNA containing the packaging signal should be compared with the packaging efficiency of an RNA lacking the packaging signal. We replaced the HIV-1 NC domain with the M-MuLV NC domain and made several observations. First, the mutant, but not wild-type HIV-1, was capable of preferentially encapsidating an RNA containing the M-MuLV Ψ packaging element over an RNA lacking the element; this result indicates that the M-MuLV NC domain specifically recognized the M-MuLV Ψ packaging element. Second, an HIV-1 mutant containing the M-MuLV Cys-His box flanked by the HIV-1 NC terminal regions was unable to package the M-MuLV Ψ-containing RNA, indicating that both the M-MuLV Cys-His box and the M-MuLV flanking regions were required for the specific recognition of the M-MuLV Ψ element. We also found that the packaging specificity of M-MuLV could be changed, at least partially, to that of HIV-1 by replacing the M-MuLV NC domain with the HIV-1 NC domain. These results corroborate recent reports demonstrating that purified HIV-1 NC protein can bind to HIV-1 RNAs with specificity in vitro (5, 6, 9, 11).

The ability of the HIV-1 mutant containing the M-MuLV NC domain to form virus particles is in itself interesting. Previous studies have indicated that the presence of either the N-terminal 14 residues of HIV-1 NC or one of the Cys-His boxes is required for virion assembly (3, 17, 19, 28), with at least one Cys-His box required for proper virion density (3, 28). Since the HIV-1 mutant containing the M-MuLV NC domain formed correctly sized immature virus particles, the M-MuLV NC domain appears to be able to substitute for the HIV-1 NC domain during virion assembly. However, since the other HIV-1 mutants exhibited heterogeneous particle sizes and core maturation phenotypes, the regulation of particle size

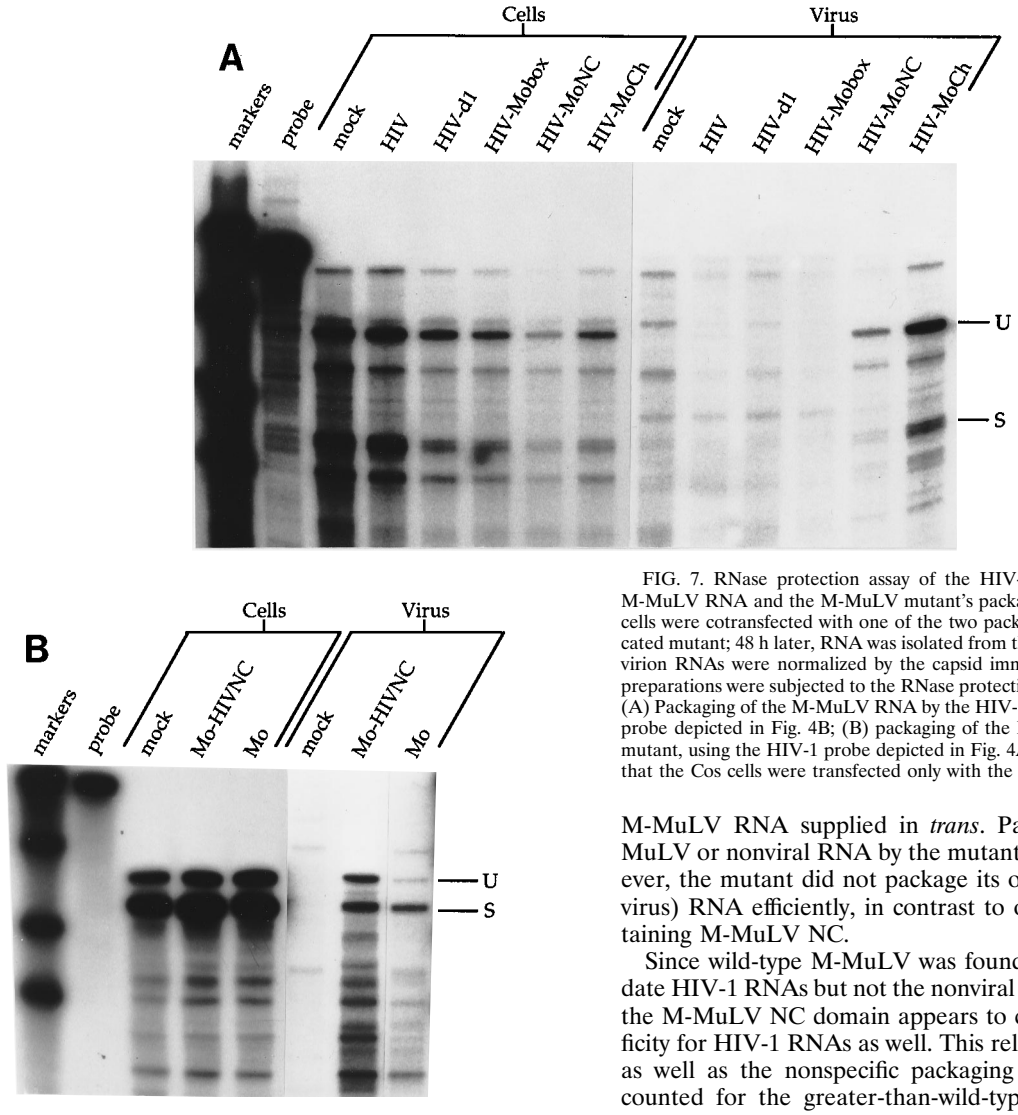

FIG. 7. RNase protection assay of the HIV-1 mutants' packaging of the M-MuLV RNA and the M-MuLV mutant's packaging of the HIV-1 RNA. Cos cells were cotransfected with one of the two packaging constructs and the indicated mutant; 48 h later, RNA was isolated from the cells and virus particles, the virion RNAs were normalized by the capsid immunoblot assay, and the RNA preparations were subjected to the RNase protection assay as indicated in Fig. 5. (A) Packaging of the M-MuLV RNA by the HIV-1 mutants, using the M-MuLV probe depicted in Fig. 4B; (B) packaging of the HIV-1 RNA by the M-MuLV mutant, using the HIV-1 probe depicted in Fig. 4A. Note that "mock" indicates that the Cos cells were transfected only with the packaging construct.

and core maturation appears to be sensitive to the context of the NC domain.

We also observed that the HIV-1 mutant containing the M-MuLV NC domain was able to encapsidate a nonviral RNA 20-fold more efficiently than was wild-type HIV-1, indicative of an increased nonspecific packaging activity in the mutant. This phenotype is reminiscent of the avian *SE*21Q1b mutant, which packages cellular RNAs because of ill-defined mutations in the Gag polyprotein (2, 34). However, the HIV-1 mutant containing the M-MuLV NC domain appears not to package RNAs strictly on a nonspecific basis, as the mutant also displayed an ability to specifically recognize the M-MuLV Ψ element.

When we assayed the ability of this mutant to package M-MuLV RNAs supplied in *trans*, we found that the mutant, but not wild-type HIV-1, encapsidated the M-MuLV RNAs efficiently. However, genomic and spliced M-MuLV RNAs were packaged with equal efficiency, suggesting that the mutant's nonspecific packaging activity mediated the packaging of the M-MuLV RNAs. In the only other published study of NC chimeras (16), the Rous sarcoma virus NC domain was replaced with the M-MuLV NC domain, and the resulting chimeric Gag protein, but not the wild-type Rous sarcoma virus parent, was found by slot blot analysis to be able to package a M-MuLV RNA supplied in *trans*. Packaging of spliced M-MuLV or nonviral RNA by the mutant was not assessed; however, the mutant did not package its own (i.e., Rous sarcoma virus) RNA efficiently, in contrast to our HIV-1 mutant containing M-MuLV NC.

Since wild-type M-MuLV was found to be able to encapsidate HIV-1 RNAs but not the nonviral RNA (data not shown), the M-MuLV NC domain appears to contain a relaxed specificity for HIV-1 RNAs as well. This relaxed packaging activity, as well as the nonspecific packaging activity, may have accounted for the greater-than-wild-type packaging of HIV-1 RNAs by the HIV-1 mutant containing the M-MuLV NC domain. Furthermore, the ability of this HIV-1 mutant to package two- to threefold more total viral RNA than wild-type HIV-1 suggests either that the mutant packaged more than two RNAs per virion or that a significant fraction of the wild-type HIV-1 particles lack HIV-1 RNA. Careful studies to assess the percentage of virus particles lacking viral genomes have not been performed, but it seems likely that not all particles contain genomes.

It is important to note that since wild-type HIV-1 and wild-type M-MuLV were unable to package the nonviral RNA, it appears that the ability of the HIV-1 mutant containing the M-MuLV NC domain to package the nonviral RNA was due neither to the M-MuLV NC domain nor to the HIV-1 portion of the polyprotein but instead to the juxtaposition of the two. Although we do not know if the region of the chimeric Gag polyprotein that interacts with the nonviral RNA is located in the M-MuLV NC domain, it is clear that the non-NC residues do influence the Gag polyprotein's packaging specificity.

Evidence for the interaction between NC and an element in the viral intron was observed not only in the chimeric Gag polyproteins but also in the HIV-1 mutant containing a deletion in the N-terminal Cys-His box. This mutant exhibited a fourfold decrease in the packaging of the genomic RNA and a

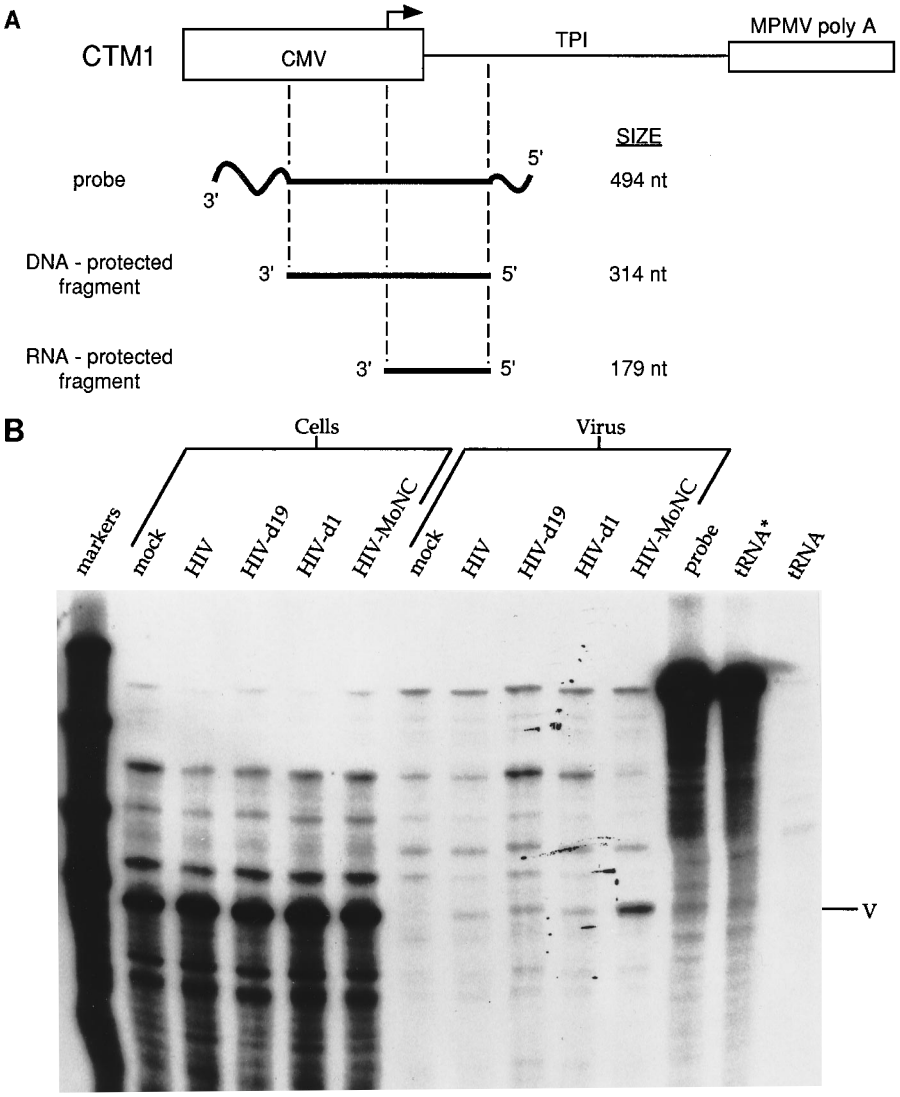

FIG. 8. RNase protection assay of the CTM1 nonviral RNA packaged by the HIV-1 mutants. (A) Schematic of the CTM1 construct, probe, and anticipated protection fragments, depicted as in Fig. 4; (B) RNase protection assay. Cos cells were cotransfected with the CTM1 construct and the indicated provirus; 48 h later, RNA was isolated from the cells and virus particles, the virion RNAs were normalized by the capsid immunoblot assay, and the RNA preparations were subjected to the RNase protection assay as indicated in Fig. 5. V, fragment of the probe protected by the CTM1 vector RNA. tRNA and tRNA*, same as in Fig. 5B.

fourfold increase in the packaging of the spliced RNAs, exactly like that of a previously described mutant (HIV-SVPA-ΔP1) containing a 19-nt deletion just upstream of the *gag* start codon (36). The ability of the Gag polyprotein to preferentially package genomic RNAs over spliced RNAs can thus be abrogated by a mutation in the viral intron or a mutation in the Cys-His box. This result suggests that the preferential packaging of the viral genomic RNA is mediated by an interaction involving the Cys-His box and an element in the viral intron.

The demonstration that NC specifically recognizes the genomic viral RNA does not exclude the possibility that NC also forms nonspecific contacts with the RNA. The nonspecific RNA-binding activity of NC is mediated by its basic residues (33), and the basic residues, at least those near the Cys-His box(es), are also required for viral RNA packaging in vivo (18, 26, 44) and specific viral RNA binding in vitro (5, 11).

Finally, the results indicate that HIV-1 may contain two packaging elements, one in the viral intron and one in a region contained within the spliced RNAs. The efficiency of packaging of the spliced viral RNAs, at least in Cos cells, is only 10-fold lower than that of the genomic viral RNA. In addition, elimination of the preferential packaging of the genomic RNA via deletions in the viral intron or NC box 1 reduces genomic RNA packaging only 4-fold, not 100-fold as in the case of M-MuLV (38, 46) or Rous sarcoma virus (30, 32, 34), and actually increases spliced RNA packaging. Therefore, the exonic packaging element can function in the absence of the intronic packaging element, causing the spliced RNAs to compete with the genomic RNA for the apparently limited packaging mechanism. A prediction of this model is that deletion of the exonic element should abrogate the encapsidation of spliced RNAs and moderately impair the encapsidation of the genomic RNA. Of course, each element could be composed of multiple independent subelements. In vitro binding studies have detected multiple specific binding elements in the HIV-1 genome (5, 6, 9). Future attempts at mapping the HIV-1 packaging signal

should perhaps be performed one element at a time, using a parent in which the other element has been deleted.

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REFERENCES

1. **Aldovini, A., and R. Young.** 1990. Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. J. Virol. **64:**1920–1926.
2. **Anderson, D. J., P. Lee, K. L. Levine, J. Sang, S. A. Shah, O. O. Yang, P. R. Shank, and M. L. Linial.** 1992. Molecular cloning and characterization of the RNA packaging-defective retrovirus *SE*21Q1b. J. Virol. **66:**204–216.
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. D., and S. P. Goff.** 1993. Point mutations in Moloney murine leukemia virus envelope protein: effects on infectivity, virion association, and superinfection resistance. Virology **196:**748–757.
5. **Berkowitz, R. D., and S. P. Goff.** 1994. Analysis of binding elements in the human immunodeficiency virus type 1 genomic RNA and nucleocapsid protein. Virology **202:**233–246.
6. **Berkowitz, R. D., J. Luban, and S. P. Goff.** 1993. Specific binding of human immunodeficiency virus type 1 *gag* polyprotein and nucleocapsid protein to viral RNAs detected by RNA mobility shift assays. J. Virol. **67:**7190–7200.
7. **Blain, S. W., and S. P. Goff.** 1993. Nuclease activities of Moloney murine leukemia virus reverse transcriptase. J. Biol. Chem. **268:**23585–23592.
8. **Bowles, N. E., P. Damay, and P. Spahr.** 1993. Effect of rearrangements and duplications of the Cys-His motifs of Rous sarcoma virus nucleocapsid protein. J. Virol. **67:**623–631.
9. **Clever, J., C. Sassetti, and T. G. Parslow.** 1995. RNA secondary structure and binding sites for *gag* gene products in the 5′ packaging signal of human immunodeficiency virus type 1. J. Virol. **69:**2101–2109.
10. **Colicelli, J., and S. P. Goff.** 1988. Sequence and spacing requirements of a retrovirus integration site. J. Mol. Biol. **199:**47–59.
11. **Dannull, J., A. Surovoy, G. Jung, and K. Moelling.** 1994. Specific binding of HIV-1 nucleocapsid protein to PSI RNA in vitro requires N-terminal zinc finger and flanking basic amino acid residues. EMBO J. **13:**1525–1533.
12. **Davis, J., M. Scherer, W. P. Tsai, and C. Long.** 1976. Low-molecular-weight Rauscher leukemia virus protein with preferential binding for single-stranded RNA and DNA. J. Virol. **18:**709–718.
13. **Dorfman, T., A. Bukovsky, Å. Öhagen, S. Höglund, and H. G. Göttlinger.** 1994. Functional domains of the capsid protein of human immunodeficiency virus type 1. J. Virol. **68:**8180–8187.
14. **Dorfman, T., J. Luban, S. P. Goff, W. A. Haseltine, and H. G. Göttlinger.** 1993. Mapping of functionally important residues of a cysteine-histidine box in the human immunodeficiency virus type 1 nucleocapsid protein. J. Virol. **67:**6159–6169.
15. **Dupraz, P., S. Oertle, C. Meric, P. Damay, and P. Spahr.** 1990. Point mutations in the proximal Cys-His box of Rous sarcoma virus nucleocapsid protein. J. Virol. **64:**4978–4987.
16. **Dupraz, P., and P. Spahr.** 1992. Specificity of Rous sarcoma virus nucleocapsid protein in genomic RNA packaging. J. Virol. **66:**4662–4670.
17. **Franke, E. K., H. E. H. Yuan, K. L. Bossolt, S. P. Goff, and J. Luban.** 1994. Specificity and sequence requirements for interactions between various retroviral Gag proteins. J. Virol. **68:**5300–5305.
18. **Fu, X., R. A. Katz, A. M. Skalka, and J. Leis.** 1988. Site-directed mutagenesis of the avian retrovirus nucleocapsid protein, pp12. J. Biol. Chem. **263:**2140–2145.
19. **Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. DeWilde.** 1989. Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. Cell **59:**103–112.
20. **Goff, S. P., P. Traktman, and D. Baltimore.** 1981. Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase. J. Virol. **38:**239–248.
21. **Gorelick, R. J., D. J. Chabot, A. Rein, L. E. Henderson, and L. O. Arthur.** 1993. The two zinc fingers in the human immunodeficiency virus type 1 nucleocapsid protein are not functionally equivalent. J. Virol. **67:**4027–4036.
22. **Gorelick, R. J., L. E. Henderson, J. P. Hanser, and A. Rein.** 1988. Point mutants of Moloney murine leukemia virus that fail to package viral RNA: evidence for specific RNA recognition by a "zinc finger-like" protein sequence. Proc. Natl. Acad. Sci. USA **85:**8420–8424.
23. **Gorelick, R. J., S. M. Nigida, J. R. Bess, L. O. Arthur, L. E. Henderson, and A. Rein.** 1990. Noninfectious human immunodeficiency virus type 1 mutants deficient in genomic RNA. J. Virol. **64:**3207–3211.
24. **Hansen, M. S. T., and E. Barklis.** 1995. Structural interactions between retroviral Gag proteins examined by cysteine cross-linking. J. Virol. **69:**1150–1159.
25. **Höglund, S., L. G. Öfverstedt, Å. Nilsson, P. Lundqvist, H. Gelderblom, and M. Özel.** 1992. Spatial visualization of the maturing HIV-1 core and its linkage to the envelope. AIDS Res. Hum. Retroviruses **8:**1–7.
26. **Housset, V., H. De Rocquigny, B. Roques, and J.-L. Darlix.** 1993. Basic amino acids flanking the zinc finger of Moloney murine leukemia virus nucleocapsid protein NCp10 are critical for virus infectivity. J. Virol. **67:**2537–2545.
27. **Jacks, T., H. D. Madhani, F. R. Masiarz, and H. E. Varmus.** 1988. Signals for ribosomal frameshifting in the Rous sarcoma virus *gag-pol* region. Cell **55:**447–458.
28. **Jowett, J. B., D. J. Hockley, M. V. Nermut, and I. M. Jones.** 1992. Distinct signals in human immunodeficiency virus type 1 Pr55 necessary for RNA binding and particle formation. J. Gen. Virol. **73:**3079–3086.
29. **Karpel, R. L., L. E. Henderson, and S. Oroszlan.** 1987. Interactions of retroviral structural proteins with single-stranded nucleic acids. J. Biol. Chem. **262:**4961–4967.
30. **Katz, R. A., R. W. Terry, and A. M. Skalka.** 1986. A conserved *cis*-acting sequence in the 5′ leader of avian sarcoma virus RNA is required for packaging. J. Virol. **59:**163–167.
31. **Khan, R., and D. P. Giedroc.** 1994. Nucleic acid binding properties of recombinant Zn_2 HIV-1 nucleocapsid protein are modulated by COOH-terminal processing. J. Biol. Chem. **269:**22538–22546.
32. **Koyama, T., F. Harada, and S. Kawai.** 1984. Characterization of a Rous sarcoma virus mutant defective in packaging its own genomic RNA: biochemical properties of mutant TK15 and mutant-induced transformants. J. Virol. **51:**154–162.
33. **Leis, J., and J. Jentoft.** 1983. Characteristics and regulation of interaction of avian retrovirus pp12 protein with viral RNA. J. Virol. **48:**361–369.
34. **Linial, M., E. Medeiros, and W. S. Hayward.** 1978. An avian oncovirus mutant (*SE*21Q1b) deficient in genomic RNA: biological and biochemical characterization. Cell **15:**1371–1381.
35. **Long, C. W., L. E. Henderson, and S. Oroszlan.** 1980. Isolation and characterization of low-molecular-weight DNA-binding proteins from retroviruses. Virology **104:**491–496.
36. **Luban, J., and S. P. Goff.** 1994. Mutational analysis of *cis*-acting packaging signals in human immunodeficiency virus type 1 RNA. J. Virol. **68:**3784–3793.
37. **Mammano, F., Å. Öhagen, S. Höglund, and H. G. Göttlinger.** 1994. Role of the major homology region of human immunodeficiency virus type 1 in virion morphogenesis. J. Virol. **68:**4927–4936.
38. **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.
39. **Meric, C., and S. P. Goff.** 1989. Characterization of Moloney murine leukemia virus mutants with single-amino-acid substitutions in the Cys-His box of the nucleocapsid protein. J. Virol. **63:**1558–1568.
40. **Meric, C., E. Gouilloud, and P. Spahr.** 1988. Mutations in Rous sarcoma virus nucleocapsid protein p12 (NC): deletions of Cys-His boxes. J. Virol. **62:**3328–3333.
41. **Meric, C., and P. Spahr.** 1986. Rous sarcoma virus nucleic acid-binding protein p12 is necessary for viral 70S RNA dimer formation and packaging. J. Virol. **60:**450–459.
42. **Morellet, N., H. de Rocquigny, Y. Mély, N. Jullian, H. Déméné, M. Ottman, D. Gérard, J.-L. Darlix, M. C. Fournie-Zaluski, and B. P. Roques.** 1994. Conformational behaviour of the active and inactive forms of the nucleocapsid NCp7 of HIV-1 studied by ^{1}H NMR. J. Mol. Biol. **235:**287–301.
43. **Oertle, S., and P. Spahr.** 1990. Role of the *gag* polyprotein precursor in packaging and maturation of Rous sarcoma virus genomic RNA. J. Virol. **64:**5757–5763.
44. **Rein, A., D. P. Harvin, J. Mirro, S. M. Ernst, and R. J. Gorelick.** 1994. Evidence that a central domain of nucleocapsid protein is required for RNA packaging in murine leukemia virus. J. Virol. **68:**6124–6129.
45. **Sakalian, M., J. W. Wills, and V. M. Vogt.** 1994. Efficiency and selectivity of RNA packaging by Rous sarcoma virus Gag deletion mutants. J. Virol. **68:**5969–5981.
46. **Sorge, J., W. Ricci, and S. H. Hughes.** 1983. *cis*-acting RNA packaging locus in the 115-nucleotide direct repeat of Rous sarcoma virus. J. Virol. **48:**667–675.
47. **Summers, M. F., L. E. Henderson, M. R. Chance, J. W. Bess, Jr., T. L. South, P. R. Blake, I. Sagi, G. Peret Alvarado, R. C. Sowder III, D. R. Hare, and L. O. Arthur.** 1992. Nucleocapsid zinc finger detected in retroviruses: EXAFS studies of intact viruses and the solution-state structure of the nucleocapsid protein from HIV-1. Protein Sci. **1:**563–574.