Mutational Analysis of *cis*-Acting Packaging Signals in Human Immunodeficiency Virus Type 1 RNA

JEREMY LUBAN¹ AND STEPHEN P. GOFF^{2*}

Department of Medicine¹ and Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics,² Columbia University College of Physicians and Surgeons, New York, New York 10032

Received 8 December 1993/Accepted 7 March 1994

We previously identified blocks of sequence near the 5' end of the human immunodeficiency virus (HIV-1) genome which conferred on RNA the ability to bind specifically to the HIV-1 Gag polyprotein, Pr55^{gag} (J. Luban and S. P. Goff, *J. Virol.* 65:3203–3212, 1991; R. Berkowitz, J. Luban, and S. P. Goff, *J. Virol.* 67:7190–7200, 1993). Here we report the use of an RNase protection assay to quantify the effect of deletion of these sequences on RNA packaging into virions. First, we demonstrated with wild-type HIV-1 sequences that in comparison with spliced viral RNA, full-length viral genomic RNA is enriched 20-fold in virions. A previously described mutation with deletion of sequences between the major splice donor and the first codon of *gag* (A. Lever, H. Gottlinger, W. Haseltine, and J. Sodroski, *J. Virol.* 63:4085–4087, 1989) disrupted these ratios such that different HIV-1 RNA forms were packaged in direct proportion to cytoplasmic concentrations. The effect of deletion mutations preceding and within *gag* coding sequence on packaging was then tested in competition with RNAs containing wild-type packaging sequences. Using this system, we were able to demonstrate significant effects on packaging of RNAs with mutations immediately preceding the first codon of *gag*. The greatest reduction in packaging was seen with RNAs lacking the first 40 nucleotides of *gag* coding sequence, although sequences more 3' had slight additional effects.

The importance of retroviruses as pathogens and as vectors for the delivery and expression of cloned cDNAs has prompted extensive analysis of the genetic requirements for the selective encapsidation of retroviral genomic RNA into virion particles (for a review, see reference 33). In the case of human immunodeficiency virus type 1 (HIV-1), more than 30 alternatively spliced mRNAs are found in an infected cell (for example, see reference 45). Despite the presence in the host cell cytoplasm of equal or greater amounts of these spliced viral RNAs, it is the unspliced viral genomic RNA which is the predominant form packaged into virions.

Many questions remain about the *cis*-acting requirements for retroviral packaging. Pregenomic RNA used for packaging is indistinguishable from the unspliced mRNA from which *gag* and *pol* are translated. Early studies suggested that the RNAs performing these separate functions might be sequestered in nonequilibrating pools (32). Once packaged into virions, the genomic RNA is isolated as a dimer, but it is not known if RNA dimerization is required for the specific incorporation of genomic RNA into virions. Rapid-harvest virus experiments have suggested that the RNA is packaged as a monomer and that the dimer forms only later, after virion formation (12, 26). More recent data suggest that the RNA is packaged as an unstable dimer which subsequently undergoes a viral protease-dependent condensation (19).

The fact that subgenomic viral RNAs share 5' and 3' termini with viral genomic RNA and differ only internally suggests that the *cis*-acting packaging signals are present in sequences which are removed by splicing. The importance for packaging specificity of sequences at the 5' end of the retroviral genome which are removed by splicing was supported by the characterization of a spontaneous mutant of Rous sarcoma virus (RSV) which

produced particles deficient in genomic RNA (46). Subsequently, all retroviruses examined have been shown to require similarly located sequences for efficient packaging of genomic RNA. As a general rule, these *cis*-acting packaging sequences are located 5' of *gag* coding sequences but may extend into *gag* coding sequence (1, 3–5, 7, 11, 16, 22, 24, 27, 38, 41, 42, 47, 48). In the specific case of HIV-1, deletion of sequences between the major splice donor and the *gag* coding region has been reported to decrease the encapsidation of genomic RNA (2, 13, 31).

A generally accepted model ascribes retroviral packaging specificity to the selectivity of Gag polyprotein binding to viral genomic RNA. Previously, we devised two *in vitro* assays for studying this binding interaction. In the first assay, a North-western (RNA-protein) blot, recombinant Pr55^{gag} is separated from bacterial proteins by polyacrylamide gel electrophoresis; the proteins are then immobilized on nitrocellulose membranes and probed with radiolabeled RNAs containing sequences from the 5' end of the HIV-1 genome (35). Under conditions which allowed specific binding of HIV-1 RNAs to Pr55^{gag}, two regions within *gag* coding sequence, which independently promoted the binding of RNA to Pr55^{gag}, were identified. One region was located within the first 168 nucleotides of *gag* coding sequence (nucleotides 789 to 957). The second region spanned the 187 nucleotides 3' to the first region (nucleotides 957 to 1144). Sequences between the major splice donor and the first *gag* codon (nucleotides 742 to 789) were also demonstrated to be important to binding, although more stringent binding conditions were necessary before this became evident.

To study the same binding events in solution, we subsequently developed an RNA gel mobility shift assay with which we were able to demonstrate specific binding of HIV-1 RNA to both Pr55^{gag} and the nucleocapsid protein (NC) (9). By using this assay, several sites of binding activity were demonstrated to be located in blocks of sequence 5' to and within *gag* coding sequence. This was very much in agreement with what we had

* Corresponding author. Mailing address: Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, Columbia University College of Physicians and Surgeons, 630 W. 168th St., New York, NY 10032. Phone: (212) 305-3794. Fax: (212) 305-8692.

observed with the Northwestern blot. In distinct contrast with the first assay, no binding activity was seen within *gag* sequences 3' to nucleotide 957.

To determine the importance for in vivo packaging of the blocks of sequence defined by the Pr55^{gag} binding assays, we made use of a quantitative RNase protection assay to examine the RNA content of virion particles. We and others have selected this method to study retroviral RNA because it is highly sensitive and specific and allows accurate quantitation (5, 15, 23). Here we describe the use of this technique to study the effect of deletion mutations located near the beginning of *gag* coding sequence on packaging of RNA into virions. We conclude that the major *cis*-acting signals for HIV-1 RNA packaging are located within the first 40 nucleotides of *gag* coding sequence but extend 5' into untranslated leader sequences, as well as further 3' into *gag* coding sequence.

MATERIALS AND METHODS

Bacteria, cells, and transfections. Plasmid DNAs were propagated in *Escherichia coli* DH1 α by standard methods (20). Supercoiled plasmid DNAs were purified by banding in cesium chloride gradients (44). HIV-1 RNA and protein was expressed from Cos cells transiently transfected with supercoiled plasmid DNAs by the chloroquine DEAE-dextran method (14). In cotransfection experiments, DNAs were used at a ratio of 1:1. HIV-1 gene expression was monitored by the indicated methods 48 h following transfection.

Cloned DNAs. The retrovirus sequence is numbered with respect to the 5' edge of the 5' long terminal repeat of the HXB2C clone of the HIV-1 DNA provirus. pHIV-gpt (39) was a gift of Ned Landau, Aaron Diamond AIDS Research Center, New York, N.Y. This plasmid contains the complete HXB2C provirus, except that 1,218 nucleotides of *env* coding sequence is replaced by SV-gpt sequences. pHXB Δ P1 (31) was a gift of William A. Haseltine, Harvard Medical School, Boston, Mass. This plasmid contains the complete HXB2C provirus except for a 19-bp deletion located between the 5' major splice donor and the first codon of *gag*. pTZHXB2 contains sequences from the 3' end of the HXB2C provirus extending from the *Sal*I site at nucleotide 5785 to the *Xba*I site in the flanking DNA. This plasmid was a gift of John Dubay and Eric Hunter, University of Alabama, Birmingham. Simian virus 40 (SV40) viral DNA was obtained from Gibco-BRL, Gaithersburg, Md. pGE MEX-1 and pSP72 were obtained from Promega Corp., Madison, Wis., and pBluescript II KS- was obtained from Stratagene Cloning Systems, La Jolla, Calif.

Site-directed mutagenesis. To disrupt translation from the *gag* initiation methionine, we engineered two mutant HXB2C DNAs by the method of Kunkel (30). Mutant AatII789 was constructed as previously described (35) with mutagenic oligonucleotide of sequence 5'-CGCTCTCGACCGACGTCTCTCTCTAGC-3'. It disrupts the sequence encoding the *gag* initiation methionine from 5'-GAG-ATG-3' to 5'-GAC-GTC-3'. The AatII789 mutation was transferred into the pBSHXSS subcloning vector described below and ultimately into pHIV-SVPA for expression in Cos cells.

Construction of the Nde789 mutation has been described previously (35). It changes the sequence preceding the *gag* initiation methionine from 5'-GAG-ATG-3' to 5'-CAT-ATG-3'. This mutation would be predicted to alter the primary sequence context of a methionine codon such that it would not be selected for translation initiation by a scanning ribosome (29). The Nde789 mutation was transferred into the subcloning vectors described below.

Subcloning vectors for mutagenesis. To generate mutant

TABLE 1. Construction of deletion mutants used in packaging assays

Construct	Engineering sites ^a	Sequences created by deletion ^b
Wild type	NA ^c	783-5'-agagagatgggt-3'-794
Nde789	NA	783-5'-agaCaTatgggt-3'-794
Δ 708-789	<i>Bss</i> HII- <i>Nde</i> I	705-5'-gaagcgcg/tatgggt-3'-795
Δ 754-789	<i>Nae</i> I- <i>Nde</i> I	748-5'-tacgcc/tatgggt-3'-795
Δ 754-828	<i>Nae</i> I- <i>Cla</i> I	748-5'-tacgcc/cgatggg-3'-837
Δ 789-828	<i>Nde</i> I- <i>Cla</i> I	783-5'-agacata/cgatggg-3'-837
Δ 828-957	<i>Cla</i> I- <i>Acc</i> I	826-5'-tagatcg/agacaaa-3'-966
Δ 789-957	<i>Nde</i> I- <i>Acc</i> I	783-5'-agacata/agacaaa-3'-966
Δ 957-1144	<i>Acc</i> I- <i>Pvu</i> II	955-5'-gctgtag/ctgaca-3'-1152
Δ 828-906	<i>Cla</i> I- <i>Sac</i> I	826-5'-tagatcg/cgagctcgagcta-3'-906

^a HXB2C DNAs were digested to completion with the indicated restriction enzymes, blunted with the Klenow fragment of DNA polymerase, and cyclized with T4 DNA ligase. The *Nae*I and *Sac*I sites at nucleotides 754 and 906, respectively, were engineered as described in the text.

^b The sequences created at the deletion sites are shown. The numbers refer to the nucleotide positions of the 5' and 3' edges of the sequences shown. Numbering is with respect to the 5' end of the 5' LTR. Point mutations are indicated by capital letters. Slanted bars indicate ligation joints. Sequence 3' of the joint in mutant Δ 828-906 was previously engineered (see the text).

^c NA, not applicable.

proviruses, we first engineered mutations in one of two HXB2C subclones, pBSHXSS-Nde789 or pNSR. To construct the former plasmid, we used a modified pBluescript II KS- which contained deletions from *Nae*I to *Pvu*II (nucleotides 130 to 529) and from *Bam*HI to *Afl*III (nucleotides 689 to 1153). The removed fragments contained restriction sites which would have made subsequent constructions more complicated. The *Sac*I-*Spe*I fragment (nucleotides 691 to 1504) from HXB2C was then subcloned into the polylinker of the modified pBluescript II KS-, yielding pBSHXSS. This plasmid was then modified to produce pBSHXSS-Nde789, which contains an engineered *Nde*I site (5'-CATATG-3') encompassing the *gag* initiator methionine, by exchanging the *Sac*I-*Pst*I fragment (nucleotides 679 to 1416) from the M13 clone in which the mutation was first constructed (35). pNSR is similar to pBSHXSS-Nde789 except that it contains HXB2C sequences from the *Bgl*II site at nucleotide 473 to the *Sac*I site at nucleotide 2752 subcloned into the polylinker of pSP72. Mutations engineered in these plasmids were then transferred into the proviral expression construct, pHIV-SVPA, by replacement of either the *Bss*HII-*Spe*I fragment (nucleotides 708 to 1504) or the *Kas*I-*Spe*I fragment (nucleotides 638 to 1504).

Construction of deletion mutants. For the construction of deletion mutations, either pBSHXSS-Nde789 or pNSR DNA was digested to completion with the pairs of restriction endonucleases indicated in Table 1. Agarose gel electrophoresis was used to purify sequences to be retained from fragments to be deleted. Incompatible termini were made blunt ended with the Klenow fragment of DNA polymerase I, and the DNAs were then cyclized with T4 DNA ligase. Two of the deletion mutations, Δ 754-789 and Δ 754-828, were constructed by taking advantage of a previously engineered *Nae*I site present in pHXB Δ P1 (31). Mutant Δ 828-906 was generated by using the *Sac*I site provided by a previously constructed linker insertion mutation, A906 (36). The identity of the mutations was confirmed by detailed restriction digests and by dideoxy sequencing with an oligonucleotide primer (5'-GGGAAACCA GAGCTCTCTCGACG-3') corresponding to nucleotides 666 to 688 of HXB2C.

Construction of proviral expression plasmids. pHIV-SVPA replaces the proviral 3' long terminal repeat of pHIV-gpt with

SV40 sequences containing a polyadenylation addition sequence. A 237-nucleotide *Bam*HI-*Bcl*I fragment containing the relevant SV40 sequences was transferred into the *Bam*HI site of pGEMEX-1. SV40 sequences were then excised from this plasmid, taking advantage of flanking *Xho*I and *Xba*I restriction enzyme recognition sequences, and cloned into pTZHXB2. The resulting plasmid contained the 3' half of the HXB2C provirus, except that the 3' long terminal repeat and the flanking DNA sequences were replaced with the SV40 polyadenylation addition signal. A *Bam*HI-*Xba*I fragment from this plasmid was then subcloned into pHIV-gpt. The resulting plasmid, pHIV-SVPA, replaced the sequences 3' of the *Xho*I site at 8896 with the SV40 polyadenylation signal.

pHIV-SVPA- Δ P1 was produced by replacing the *Bss*HII-*Spe*I fragment (nucleotides 708 to 1504) from pHIV-SVPA with the analogous fragment from pHXBAP1. pHIV-SVPA- Δ Rev was constructed by deleting the *Bam*HI fragment from nucleotide 8474 to the 5' end of the SV40 poly(A) signal. The resulting plasmid had the majority of *rev* coding sequence deleted. For the construction of pHIV-SVPA-AatII789, the *Bss*HII-*Spe*I fragment of pHIV-SVPA (nucleotides 708 to 1504) was replaced with the corresponding fragment from pBSHXSS-AatII789. For the construction of pHIV-SVPA-Nde789, the *Bss*HII-*Spe*I fragment of pHIV-SVPA (nucleotides 708 to 1504) was replaced with the corresponding fragment from pNSR.

Construction of cDNA templates for the synthesis of antisense riboprobes. Two plasmids were constructed to serve as templates for the synthesis of labeled RNAs to be used as probes in an RNase protection assay. pHRAS contains the *Sca*I-*Acc*I fragment (nucleotides 315 to 957) of HXB2C ligated to pSP72 digested with the restriction enzymes *Acc*I and *Pvu*II. pHRCS was constructed by removing a 168-nucleotide *Clal* fragment from pHRAS, such that nucleotides 315 to 828 from HXB2C are remaining. Transcription from the T7 promoter of either plasmid results in an RNA product which is antisense to the corresponding sequences of HXB2C.

Riboprobe synthesis. Antisense RNA probes with a specific activity of 10^8 cpm/ μ g were synthesized as previously described (35). Probe RAS was synthesized with T7 RNA polymerase (Boehringer Mannheim, Indianapolis, Ind.) from pHRAS linearized with *Nde*I. Probe RCS was synthesized with T7 RNA polymerase from pHRCS linearized with *Nde*I. To serve as sizing markers for denaturing polyacrylamide gels, we synthesized a pool of riboprobes from pHRAS linearized with either *Pvu*II, *Hind*III, or *Bss*HII.

RNA isolation from cytoplasm and virions. Cytoplasmic RNA from Cos cells was obtained by rapid lysis at 4°C in Nonidet P-40 buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.5% Nonidet P-40). Cell debris and nuclei were removed by a 15-s centrifugation step in a microcentrifuge. The supernatant was adjusted to 0.2% sodium dodecyl sulfate (SDS) and 150 μ g of proteinase K per ml, incubated at 37°C for 15 min, and extracted twice with phenol-chloroform and once with chloroform, and nucleic acids were then collected by ethanol precipitation. RNA was stored in ethanol at -70°C. RNA recovery was monitored by spectrophotometry. Typically a 100-mm tissue culture plate yielded 100 μ g of RNA.

RNA was isolated from virions purified from 10 ml of Cos cell supernatant by centrifugation through a 25% sucrose cushion in an SW41 rotor at 80,000 $\times g$ for 4 h. Virions were disrupted in proteinase K buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM EDTA, 1% SDS, 100 μ g of proteinase K per ml, 100 μ g of tRNA per ml) for 30 min at 37°C. After two phenol-chloroform extractions and one chloroform extrac-

tion, the RNA was precipitated with ethanol and stored at -70°C.

RNase protection assay. Various amounts of cytoplasmic or virion RNAs were first added to the reactions to establish linearity of signal. For our standard assay as used in the data presented, 10 μ g of Cos cell cytoplasmic RNA, or the complete virion RNA preparation from 10 ml of Cos cell supernatant supplemented with 10 μ g of carrier tRNA, was precipitated with ethanol and resuspended in 30 μ l of hybridization buffer [40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 1 mM EDTA, and 400 mM NaCl in 80% formamide]. A total of 2×10^5 cpm (Cerenkov) of ³²P-labeled antisense RNA (10 to 100 fmol) was added to each sample. Samples were heated to 85°C for 10 min to denature the RNAs and then hybridized at 50°C for 12 h. The samples were cooled to 25°C for 2 h, and 300 μ l of RNase digestion mixture (10 mM Tris-HCl [pH 7.5], 300 mM NaCl, 5 mM EDTA, 2 μ g of RNase T₁ per ml, 40 μ g of RNase A per ml) was added for a 1-h incubation at 30°C. SDS was added to 1%, and proteinase K was added to 300 μ g/ml. Samples were incubated for 30 min at 37°C and then extracted twice with phenol-chloroform and once with chloroform. tRNA (4 μ g) was added, and samples were precipitated with ethanol. Pellets were resuspended in 40 μ l of formamide loading buffer (80% formamide, 10 mM EDTA [pH 8.0]), heated to 95°C for 5 min, and subjected to polyacrylamide gel electrophoresis in the presence of 7 M urea. Gels were dried and subjected to autoradiography. The intensity of given bands on the gel was quantitated with a Betascope model 603 blot analyzer (Betagen Corp., Waltham, Mass.).

RESULTS

Establishment of a quantitative RNase protection assay to study HIV-1 RNA packaging. To determine whether sequences that we had previously demonstrated to be important for binding of HIV-1 RNA to the HIV-1 Gag polyprotein in vitro (9, 35) were necessary for packaging of RNA in vivo, we examined the effect of deletion mutations on packaging by quantitating cytoplasmic and virion RNAs for HIV-1 sequences by an RNase protection assay. Identification of the fragments protected was predicted to be complicated by the presence of sequences in the RNA derived from the two viral long terminal repeats. Therefore we constructed pHIV-SVPA (Fig. 1A), which replaced the 3' long terminal repeat, of pHIV-gpt (39) with SV40 poly(A) sequences. This construct produced pelletable virion particles from transiently transfected Cos cells as efficiently as did the parent plasmid, as evidenced by the production of HIV-1 proteins and reverse transcriptase activity (data not shown) and genomic viral RNA.

The probes used in the RNase protection assay were designed to satisfy several criteria. First, the probes should distinguish contaminating DNA from nascent transcripts. This requirement was satisfied since the probes were synthesized from template sequences which crossed the U3-R boundary of the viral LTR (Fig. 2). All DNA-protected fragments were 140 nucleotides longer than the corresponding RNA-protected fragments. Second, the probes should distinguish full-length from spliced RNA forms. This requirement was met since the probes spanned the splice donor site; spliced RNAs characteristically protected shorter fragments than did full-length RNAs. Third, the probes should distinguish wild-type sequences from those with deletions 5' to or within *gag* coding sequence. This last requirement was met since the probes extended at least 38 nucleotides into *gag* coding sequence.

To accurately determine the relative quantities of specific RNAs contained within a heterogeneous mixture, it was nec-

FIG. 1. pHIV-SVPA and derivative plasmids used in RNase protection assays. The HIV-1 genetic elements contained in these plasmids are shown schematically. pHIV-SVPA was constructed by replacing the 3' LTR of pHIV-gpt (39) with simian virus 40 polyadenylation addition sequences. (A) pHIV-SVPA and the two mutant plasmids, pHIV-SVPA-Nde789 and pHIV-SVPA-AatII789, along with the nucleotide sequence around the *gag* initiator methionine of these plasmids. (B) pHIV-SVPA- Δ Rev. This plasmid was produced from pHIV-SVPA by deletion of *rev* coding sequence.

essary to demonstrate that the probe was added to hybridizations in molar excess over viral target RNAs (23). If viral RNAs had to compete with each other to hybridize with a probe present in limiting quantity, less-stable hybrids would not be adequately represented. Also, if probe is limiting, trimolecular hybrids may form between the probe and the 5' end of one RNA and the 3' end of another, thus producing protection fragments of anomalous size (34). To demonstrate that the probes used in the following experiments were present in molar excess, we performed hybridizations with various amounts of viral or cytoplasmic RNAs from transfected cells while keeping the amount of probe RNA constant. In each case titration of the input RNA produced a linear decrease in signal (reference 15 and data not shown).

Effect on packaging of sequences between the splice donor and the *Gag* initiator methionine. We compared packaging of

wild-type RNA with packaging of RNA containing a previously described mutation, Δ P1 (31). Δ P1 is a deletion of 19 bases (nucleotides 754 to 772) between the 5' splice donor and the *gag* initiator methionine. pHIV-SVPA served as our wild-type expression construct. Sequences containing the Δ P1 mutation were subcloned into pHIV-SVPA, and the two DNAs, wild type and Δ P1 mutant, were transfected into Cos cells. As previously described (31), the deletion mutation had no measurable effect on particle production; reverse transcriptase activity in the supernatant was comparable to that from cells transfected with the parent plasmid (data not shown).

To monitor the levels of viral RNA packaged, we used plasmid pHRAS linearized with the restriction enzyme *Nde*I as a template for the synthesis of the antisense riboprobe. Figure 2 shows the sequences contained in this riboprobe (Fig. 2A) and the expected sizes of the RNase protection fragments for RNAs expressed from the wild-type (Fig. 2B) or mutant (Fig. 2C) constructs. Figure 3 shows the positions of internally labeled RNA markers (lane 1) and the position of migration of the input antisense riboprobe (lane 2). No significant protection fragments were seen with tRNA alone (lane 3) or with RNAs from mock-transfected cells (lanes 5 and 8).

Cytoplasmic RNA obtained from cells transfected with wild-type pHIV-SVPA (Fig. 3, lane 4) protected fragments of the mobility expected for viral full-length (502-nucleotide) and spliced (291-nucleotide) RNAs. Faint bands of the sizes expected for protection by contaminating DNA were seen. In addition, several bands of intermediate size were detected. We believe that these bands result from digestion of the full-length hybrid form at an adenine- and thymidine-rich stretch in the beginning of *gag* coding sequence; titration of RNase caused these bands to shift upward into the full-length form with no change in total counts per minute (data not shown). In quantifying RNAs protected with this probe, radioactivity in both the smaller and the expected hybrid bands were summed. Pretreatment of the samples with RNase, alkali, or DNase confirmed the identity of the various fragments as RNA or DNA.

Virions produced by cells transfected with pHIV-SVPA showed a predominance of full-length RNA over spliced

FIG. 2. Regions of complementarity between the antisense riboprobe RAS and viral RNA or proviral DNA. (A) RAS is complementary to HIV-1 nucleotides 315 to 957 but has a 3' extension of sequence from the transcription vector SP72. (B and C) The boxed numbers are the expected number of RAS nucleotides protected by nucleic acids from wild-type pHIV-SVPA (B) or mutant pHIV-SVPA- Δ P1 (C). Fragments resulting from protections with viral RNA are shown by black bars. Fragments resulting from protections with proviral DNA are shown by hatched bars.

FIG. 3. Packaging of RNA into virions from cells transfected with pHIV-SVPA and pHIV-SVPA- Δ P1. Shown is an autoradiogram of gel-separated fragments of 32 P-labeled riboprobe RAS resulting from RNase protection with cytoplasmic (lanes 4 to 6) or virion (lanes 7 to 9) preparations produced by transfection with pHIV-SVPA (lanes 4 and 7) or pHIV-SVPA- Δ P1 (lanes 6 and 9) or by mock transfection (lanes 5 and 8). Protections with tRNA alone are shown in lane 3, and riboprobe RAS without RNase treatment is shown in lane 2. Size markers are shown in lane 1. The identity of the fragments is indicated on the right of the panel.

RNAs (Fig. 3, lane 7). Quantitation of the intensity of the bands resulting from RNase protection indicated that there is 20-fold enrichment of full-length RNA over spliced RNA during the virion assembly process. Thus our transient-expression assay with detection of RNA by RNase protection provided us with the ability to quantitate packaging specificity and gave us the confidence to proceed with our analysis of the effect of mutations on packaging.

Cytoplasmic RNA from cells transfected with pHIV-SVPA- Δ P1 protected fragments of 298 and 291 nucleotides, which were predicted to result from protection with full-length and spliced RNA forms, respectively (Fig. 3, lane 6). The total amount of RNA per virion was nearly equal to that seen in the virions produced by transfection with wild-type sequences. In contrast to the wild-type virions, there was no significant enrichment of full-length RNA in the virions produced by transfection with pHIV-SVPA- Δ P1 (lane 9); rather, the level of full-length or spliced RNAs in the virions directly reflected the levels present in the cytoplasm. Compared with RNA of wild-type sequence, full-length RNA containing this mutation was packaged four- to fivefold less efficiently. The results suggest that the viral structural proteins do not seem to be able to differentiate between genomic and subgenomic viral RNAs containing this mutation. The incorporation of the spliced RNAs by the Δ P1 deletion mutant results in a moderate reduction in full-length RNA packaging.

Establishment of a competitive packaging assay in Cos cells using *gag* and *pol* proteins provided in trans. By using a Northwestern binding assay, we previously demonstrated that the major in vitro binding activity of HIV-1 RNA was present in two regions within *gag* coding sequence (35). In subsequent studies with RNA gel shifts we confirmed the importance for Gag binding of the first of these RNA sequences around the beginning of *gag* coding sequences (9). Therefore, we chose to study the effect of engineered deletion mutations in this region on RNA packaging in virions by using the RNase protection assay.

Since the mutations we wanted to test were located in *gag* coding sequence, we could not test mutant DNAs alone for their ability to form packaged RNAs. We needed to provide wild-type *gag* in trans and test these particles for the presence of the mutant RNA. To avoid problems with mutant protein that might be produced from the mutant RNA test constructs, we chose to express mutant RNAs from untranslatable constructs. First, we had to develop a parent vector for the construction of the deletion mutations which expressed no protein. In an initial attempt to disrupt translation from the *gag* initiation codon, we mutated the nucleotides at the -3 to +3 positions, changing 5'-GAGATG-3' to 5'-GACGTC-3'. This mutation, AatII789, altered the *gag* initiator methionine and prevented the formation of Pr55^{gag} but surprisingly allowed production of immunoreactive protein of higher mobility on SDS-PAGE (data not shown). We believe that these translation products result from the use of in-frame downstream methionines, the codons for which are located at nucleotides 1213 and 1299. In a second attempt to identify a mutation which would completely disrupt *gag* translation, we tested a previously constructed mutation at the same position which encoded an *Nde*I restriction site, 5'-CATATG-3' (35). When this mutation was subcloned into pHIV-SVPA and expressed in Cos cells, there was no detectable Pr55^{gag} or any other protein reactive with anti-*gag* antibodies in the cell lysate (data not shown), although this construct produces wild-type levels of RNA by our RNase protection assay (see below). This mutation probably blocks translation by altering the context of the initiator codon so that it is no longer functional (29): the purine at the -3 position is changed to a pyrimidine (the sequence of mutant Nde789 is given in Fig. 1A). We therefore chose plasmids with the Nde789 mutation as parents for the deletion mutants in *gag* coding sequence.

We designed a transient-expression system in which the untranslatable RNAs with mutations in postulated packaging sequences compete for packaging with wild-type RNA (Fig. 4). The two plasmids are designed so that they are codependent for particle production. The first plasmid, pHIV-SVPA-Nde789, with or without deletions in the presumptive packaging sequences, does not make Gag protein and so is dependent on the second plasmid for the production of structural proteins. The second plasmid, pHIV-SVPA- Δ Rev, provides RNA with wild-type packaging sequences as well as *gag* and *pol* translation products. pHIV-SVPA- Δ Rev is nevertheless dependent on pHIV-SVPA-Nde789 for the production of virion particles because it has a mutation which has previously been demonstrated to effectively block *rev* function (17); this viral accessory gene is required for transport out of the nucleus of the unspliced mRNA, which encodes, among other proteins, the Gag polypeptide. This plasmid is therefore dependent on *rev* expression from the first plasmid for the expression of structural proteins. The following experiments involved a modified probe (RCS) which had all the properties of RAS but allowed less nonspecific degradation. The expected protection fragments are shown schematically in Fig. 5.

Figure 6A (lane 3) shows that the vast majority of cytoplasmic HIV RNA in cells transfected with pHIV-SVPA- Δ Rev is spliced. In cotransfections, individual cells can take up only one DNA, only the other DNA, or both DNAs; only the cells receiving a *rev*⁺ DNA can export unspliced RNAs into the cytoplasm (lane 7). It should be pointed out that the ratios of the unspliced cytoplasmic RNAs in the cotransfection experiments are misleading for comparisons with virions RNAs since the cytoplasmic preparations include RNA from singly transfected *rev*⁺ cells which do not contribute to the production of virions.

FIG. 4. Competitive packaging assay. Cos cells are cotransfected with two plasmids, pHIV-SVPA-Nde789 or derivatives and pHIV-SVPA-ΔRev, which are codependent for particle production. The first plasmid provides RNAs with mutations in the presumptive *cis*-acting packaging signal. The latter plasmid provides RNAs with wild-type sequences in the packaging signals. At 48 h posttransfection, RNAs are extracted from the cell cytoplasm and from the virions and analyzed by a quantitative RNase protection assay. The amounts of mutant or wild-type RNAs packaged can then be ascertained relative to cytoplasmic levels.

When cells were transfected with either pHIV-SVPA-Nde789 or pHIV-SVPA-ΔRev alone, no virion particles were produced. This was evidenced by failure to detect reverse transcriptase activity or viral proteins in pelleted supernatant (data not shown). Also, no viral RNA was detected in pelleted supernatants from these transfections (Fig. 6B, lanes 3 and 4). On the other hand, when equal amounts of these two plasmids were cotransfected into the same cells, virions were observed by all the above criteria, including the presence of viral RNA in pelleted supernatant (lane 7). The level of full-length mutant pHIV-SVPA-Nde789 RNA present in the virions was roughly one-fifth that of the wild-type RNA provided by pHIV-SVPA-ΔRev (Table 2). It is noteworthy that the subtle mutation of two nucleotides had such an effect. Despite this effect, we chose to make use of the Nde789 mutant as a parent for further deletions.

Deletion of *gag* coding sequences significantly disrupts RNA packaging. We chose to use the competitive RNase protection assay to test the effect on packaging of deletion of the sequences most important for activity in our Northwestern binding assay (35). DNAs containing the Δ789–957 and Δ957–1144 mutations (Table 1) were subcloned into the parent expression plasmid pHIV-SVPA-Nde789 and transfected into Cos cells. Each mutant produced the expected protection fragments in the cytoplasmic RNA fractions (Fig. 6A, lanes 5 and 6) but was unable to independently produce virion particles (Fig. 6B, lanes 5 and 6). With Δ789–957 one spurious protection fragment was observed, which we have been unable to explain. This extra band results from protection at the 5' end of the riboprobe since it persisted when we linearized the template for probe synthesis at more proximal 3' sites.

When cells were cotransfected with pHIV-SVPA-Δ789–957 and pHIV-SVPA-ΔRev, virion particles in which wild-type RNA was packaged preferentially over the mutant RNA were produced (Fig. 6B, lane 8); the ratio of wild-type to mutant RNA in the virions was 0.02 ± 0.01 (Table 2). Thus, the Δ789–957 mutation greatly impaired the ability of RNA to be packaged. This result was in marked contrast to the minimal reduction in packaging caused by the Δ957–1144 mutation (Fig. 6B, lane 9). In the latter case the ratio of mutant to wild-type RNA present in the virion was 0.16 ± 0.02 . This

FIG. 5. Regions of complementarity between riboprobe RCS and viral or proviral DNA. (A) The 623-nucleotide antisense riboprobe complements HIV-1 nucleotides 315 to 828. (B and C) The sizes of fragments (numbers in boxes) expected from RNase protection with pHIV-SVPA-ΔRev (B) or pHIV-SVPA-Nde789 and its derivatives (C) are shown. Fragments resulting from protections with viral RNA are shown by black bars. Fragments resulting from protections with proviral DNA are shown by hatched bars.

FIG. 6. Effect on RNA packaging into virions of deletions in *gag* coding sequence. Shown is an autoradiogram of gel-separated fragments of ^{32}P -labeled riboprobe RCS resulting from RNase protection with cytoplasmic (A) or virion (B) preparations produced by transfection with pHIV-SVPA- Δ Rev (lanes 3), pHIV-SVPA-Nde789 (lanes 4), pHIV-SVPA- Δ 789-957 (lanes 5), pHIV-SVPA- Δ 957-1144 (lanes 6), or pHIV-SVPA (lanes 10) or by mock transfection (lanes 11). Protections were also performed with preparations from cells cotransfected by pHIV-SVPA- Δ Rev with either pHIV-SVPA-Nde789 (lanes 7), pHIV-SVPA- Δ 789-957 (lanes 8), or pHIV-SVPA- Δ 957-1144 (lanes 9). Protection with tRNA alone is shown in lane 12, and riboprobe RAS without RNase treatment is shown in lanes 2. Size markers are shown in lanes 1. Fragment sizes in nucleotides are indicated at the right of the panels. Figure 5 provides a guide for the expected protection fragments.

number differed little from the ratio obtained from cotransfection experiments with the parent plasmid pHIV-SVPA-Nde789, indicating that sequences between nucleotides 957 and 1144 are of little importance to RNA packaging. The experiments described in this section demonstrate that the block of sequence from the 5' end of *gag* coding sequence which we had previously identified by its ability to bind to HIV-1 Gag polypeptide in vitro (35) is indeed of great importance to RNA packaging in vivo. On the other hand, the more 3' region of *gag* coding sequence that we had previously identified for its binding activity was shown here to be of minimal importance to RNA packaging.

Sequences around the *gag* initiator methionine are impor-

TABLE 2. Relative packaging of mutant versus wild-type RNAs

Mutant ^a	Ratio ^b
Nde789.....	0.19 $\pm$ 0.02
Δ 789-957.....	0.02 $\pm$ 0.01
Δ 957-1144.....	0.16 $\pm$ 0.02
Δ 708-789.....	0.21 $\pm$ 0.02
Δ 754-789.....	0.16 $\pm$ 0.01
Δ 754-828.....	0.04 $\pm$ 0.03
Δ 789-828.....	0.04 $\pm$ 0.01
Δ 828-957.....	0.12 $\pm$ 0.02

^a All mutants were expressed from plasmid pHIV-SVPA.

^b Calculations are based on results of two to four RNase protection experiments as described in the text. The relative amount of mutant to wild-type RNA was determined and then normalized to cytoplasmic levels of the two RNAs.

tant for packaging. Six additional deletion mutations were constructed in an attempt to more narrowly define the *cis*-acting sequences necessary for packaging. These mutant DNAs were subcloned into pHIV-SVPA-Nde789. RNAs obtained from cells transfected with each of these expression plasmids gave the expected protection fragments (Fig. 7). As before, there was roughly a fivefold reduction in packaging compared with the wild-type sequence, simply as a result of the Nde789 mutation (Fig. 7, lane 10) present in these six mutants.

The bulk of the deletion mutants tested fell within one of two classes, in terms of the magnitude of the effect on packaging. Minimal effects on packaging were seen with RNAs containing deletions Δ 708-789, Δ 754-789, and Δ 828-957 (Fig. 7B, lanes 11, 12, and 16). The ratios in the virion of these mutant RNAs compared with the wild-type RNA were 0.21 ± 0.02 , 0.16 ± 0.01 , and 0.12 ± 0.02 , respectively (Table 2). Significantly greater effects on packaging were observed with mutants Δ 754-828 and Δ 789-828 (Fig. 7B, lanes 13 and 14), for which the packaging ratios when compared with wild-type RNAs were 0.04 ± 0.03 and 0.04 ± 0.01 , respectively (Table 2). The large error of the mean with mutant Δ 754-828 was due to the close overlap between protection fragments obtained with the mutant and spliced RNA forms (Fig. 7, lanes 13). For the same reason, the effect of this mutant on packaging may be greater than reported here, but we were precluded from making the longer exposure necessary for such an assessment.

From analysis of the deletion mutants reported here, we conclude that the greatest reduction in packaging was seen with RNAs lacking the first 40 nucleotides of *gag* coding sequence. Effects of lesser magnitude were also seen with deletion of sequence immediately preceding the first codon of *gag*, as well as with deletion of sequences up to 168 nucleotides into *gag* coding sequence.

Cells transfected with pHIV-SVPA- Δ 828-906 alone were capable of producing virions (Fig. 7B, lane 6). That this mutant RNA would be translationally active was surprising since it also contained the Nde789 mutation. Since this mutant did not require pHIV-SVPA- Δ Rev for the production of particles, we could not accurately quantitate the packaging of this mutant RNA in the competition assay (Fig. 7, lanes 15). In single-plasmid transfections with pHIV-SVPA- Δ 828-906, the unspliced and the spliced RNAs were packaged equally well (lane 6), much as with the Δ P1 mutation. We estimate that this mutant RNA is packaged 10- to 20-fold less efficiently than is wild-type RNA.

DISCUSSION

Application of an RNase protection assay to the analysis of HIV-1 *cis*-acting packaging signals. We have used an RNase

FIG. 7. Effect on RNA packaging into virions of deletions around the *gag* initiation codon. Protections of riboprobe RCS were performed with cytoplasmic (A) and virion (B) preparations. Cells were transfected with the single plasmids indicated in lanes 2 to 8. "Wild Type" refers to the parent plasmid pHIV-SVPA. Cells were cotransfected with pHIV-SVPA-ΔRev and the indicated plasmids in lanes 10 to 16. Cells were mock transfected in lanes 9. Protections were performed with tRNA alone in lanes 17. Fragment sizes in nucleotides are indicated at the right of the panels. Expected protection fragments for pHIV-SVPA-ΔRev are shown in Fig. 5. Diagnostic bands for mutants Δ789-828, Δ828-906, and Δ828-957 are the same as those shown for the parental pHIV-SVPA-Nde789 in Fig. 5. The diagnostic band at 334 nucleotides is replaced by one at 253 nucleotides for mutant Δ708-789 and at 299 nucleotides for mutants Δ754-789 and Δ754-828.

protection assay with a transient-expression system to study the effects on packaging of a series of deletion mutations at the 5' end of the HIV-1 genome. This method allowed us to distinguish (i) viral RNA from transfected DNA, which commonly complicates transfection assays, (ii) spliced RNAs from full-length RNAs, and (iii) mutant RNA from wild-type RNA.

In our initial analysis we found that full-length wild-type HIV-1 RNA is selected for packaging about 20-fold over spliced RNA forms present in the cytoplasm. How this compares with packaging of completely heterologous sequences we cannot say from our data. It remains a possibility that some packaging sequences exist 5' of the splice donor site and that spliced mRNAs are packaged more efficiently than random

sequences. Several groups have found that RNAs with deletions of the primary encapsidation sequences 3' of the splice donor are packaged more efficiently than are irrelevant RNAs (16, 37). In Rous sarcoma virus the major encapsidation sequence is present in both spliced and unspliced RNAs (24, 27), suggesting that other regions of the Rous sarcoma virus genome contain *cis*-acting packaging signals. Recently it has been shown by RNA pseudotyping experiments that simian immunodeficiency virus RNA encapsidation into HIV virion particles was blocked by deletion of sequences 5' of the splice donor (42).

Although our results are in complete agreement with what some have reported (13), we find that deletion of sequences between the splice donor and the start of the *gag* gene of HIV-1 reduces the RNA content of virions to a lesser extent than others have reported (2, 31). The basis for these quantitative differences is not clear. High-level expression in Cos cells might suppress the effect of otherwise disruptive mutations, but these other researchers also used Cos cells in their analysis (2, 31). It should be noted that we have used proteinase K in the preparation of our virion RNAs; others have suggested that this may help RNA recovery (4).

Deletions in sequence 5' of the *gag* coding region in retroviruses distantly related to HIV-1 can impair packaging up to 1,000-fold (24, 27, 38, 48), indicating that the major *cis*-acting packaging signals of these viruses lie within the leader sequences. The quantitatively greater effect of the leader sequence deletions in these viruses is interesting in light of primary structural differences with HIV-1. For example, Moloney murine leukemia virus has 350 nucleotides between the splice donor and the beginning of *gag* coding sequence. HIV-1 has only 47 nucleotides in this location. Thus, it might be expected that important signals exist in other locations in HIV-1.

To examine the effect of mutations in 5' *gag* coding sequence, we devised a cotransfection assay in which the effect on packaging of deletion mutations preceding and within *gag* coding sequence was tested in competition with RNAs containing wild-type packaging sequences. The deletion mutants were expressed from RNAs with untranslatable *gag* and *pol* sequences. This was done because of concerns about the effect on particle production of mutant *gag* proteins. Wild-type RNA and *gag* and *pol* gene products were expressed from a cotransfected *rev* mutant plasmid. Thus the two plasmids were codependent on each other for virion particle production. We have found that deletions which encompassed the first 40 nucleotides of *gag* coding sequence were the most disruptive of packaging. Deletions extending further into *gag* coding sequence had slight additional effects. By using this cotransfection system, deletions between the splice donor and the first codon of *gag* were again found to have modest effects.

Correlation of RNA packaging in vivo with RNA binding to Gag polyprotein in vitro. The sequences that we identified as important for binding to Gag polyprotein by our RNA gel shift assay (9) correlate quite well with those which we demonstrate here to be important for packaging. These include sequences between the splice donor and the start of *gag*, sequences within the first 40 nucleotides of *gag*, and slight additional activity extending 168 nucleotides into *gag* coding sequence. It therefore seems likely that binding to Gag polyprotein is the major requirement for specific RNA packaging.

We confirmed the importance for packaging in vivo of two of the regions identified from our Northwestern binding assay (35): first, sequences between the major splice donor and the start of *gag* (nucleotides 742 to 789), and, second, the first 168 nucleotides of *gag* coding sequence (nucleotides 789 to 957).

FIG. 8. Proposed RNA secondary structure within *gag* coding sequences demonstrated to be important for HIV-1 RNA packaging. The *gag* initiator methionine is enclosed within a box.

Deletion of the third block of sequence (nucleotides 957 to 1144) had no significant effect on packaging of RNA into virions. Thus, although this region binds to Gag polyprotein in vitro, it is not necessary for packaging in vivo. It may be that there are redundant binding activities within the HIV-1 sequence and that the presence of sequences 789 to 957 can substitute for this deletion.

It is important to note that our analysis of the effect of the deletion mutations reported here is restricted to the late events of the retroviral life cycle and does not address the effect of our mutations on viral infectivity. We have merely studied the requirements for bringing the RNA into the virion. These RNAs may nonetheless be incapable of some other function necessary for infectivity, such as annealing of the tRNA primer.

Importance of RNA secondary structure for packaging. The fact that there are no obvious primary sequence similarities between different retroviral packaging regions has suggested that the Gag polyprotein recognizes a secondary structure in the genomic RNA. Computer modeling has identified a conserved hairpin pair motif present between the splice donor and the first codon of *gag* in Moloney murine leukemia virus and related viruses (25). The importance of these structures for RNA packaging has been demonstrated for these viruses (18, 49); disruption of either loop alone reduces packaging 10- to 100-fold, while disruption of both reduces it at least 1,000-fold.

Chemical mapping studies of the 5' end of HIV-1 RNA have identified secondary structures (6, 21), whose functional significance has yet to be demonstrated by classical stem-loop mutagenesis experiments. Sakaguchi et al. (43) have shown that a 44-nucleotide RNA fragment contains two stem-loops important for binding *gag* protein. Both of these stem-loops are in the sequence 5' of the HIV-1 *gag* initiation codon. Another potential stem-loop exists on the other side of the *gag* initiator codon (6), within sequences that we have found to be important for packaging (Fig. 8). Thus, there may be functionally important stem-loops within leader sequences and within the first 40 nucleotides of *gag* coding sequence. The one mutation we examined which deletes both leader and *gag* sequences ($\Delta 754-828$) decreased packaging at least as much as did any of the other mutations and possibly much more.

Comparison with heterologous packaging data. At least two groups have used sequences from the 5' end of HIV-1 to package heterologous RNAs into HIV-1 virions (11, 40). Both of these groups have included sequences in their vectors that we have shown to be important for binding to Gag polyprotein

(9, 35) or that we have demonstrated here to be important for packaging of RNA in vivo. Each of these packaging vectors contained sequences 5' of *gag* but also included the first 40 nucleotides of *gag* coding sequence. Vectors which extended further into *gag* increased packaging roughly fivefold (11). Again, this result is consistent with the data reported here.

Importance of dimerization for packaging. In addition to disrupting binding to Gag polyprotein, the deletions that we have assayed here may disrupt RNA dimerization. We have not directly assayed the effect of our deletion mutations on RNA dimerization, but a limited analysis of the dimerization of RNAs containing fragments of HIV sequences suggests that the major HIV-1 RNA dimerization signals are 5' of the region that we are analyzing here (8). Therefore, we would predict that our deletions which are disrupted for packaging are still able to dimerize.

Effect of the Nde789 mutation on translation. It is of interest that the Nde789 mutation caused a reduction in packaging. Perhaps the same signals necessary for ribosome recognition of mRNA are used by the Gag polyprotein to package the RNA. Interesting, dimerization of retroviral RNA, believed by some to be necessary for RNA packaging, is inhibitory for translation (10).

It was not surprising that the Nde789 mutation should disrupt translation from the *gag* initiator methionine. This mutation changes a nucleotide in the -3 position relative to the translational start, which is important for initiator codon recognition by scanning ribosomes (29). We cannot explain why the downstream translation products that we observed with the AatII789 mutation were not seen with the Nde789 mutation. Although it seems unlikely that such a drastic change in the secondary structure could be caused by mutation of two nucleotides, perhaps a scanning ribosome cannot negotiate the structure created by the Nde789 mutation (28). That one of our deletion mutations ($\Delta 828-906$) restored translation and particle production to an RNA containing the Nde789 mutation was also surprising. This result suggests that secondary RNA structure may override the primary RNA sequence requirements for recognition by a ribosome.

ACKNOWLEDGMENTS

We thank Robert Berkowitz, John Dubay, John Fisher, Ettaly Franke, Heinrich Gottlinger, William Haseltine, Eric Hunter, Ned Landau, and Caterina Strambio de Castillia for their advice, reagents, and technical assistance.

This work was supported by grant AI 24845 from the National Institute of Allergy and Infectious Diseases (NIAID) to S.P.G. and by grant AI 00988 from the NIAID and grant 91-49 from the James S. McDonnell Foundation to J.L. S.P.G. is an Investigator of the Howard Hughes Medical Institute.

REFERENCES

1. Adam, M. A., and A. D. Miller. 1988. Identification of a signal in a murine retrovirus that is sufficient for packaging of nonretroviral RNA into virions. *J. Virol.* **62**:3802-3806.
2. 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.
3. Armentano, D., S. Yu, P. W. Kantoff, T. von Ruden, W. F. Anderson, and E. Gilboa. 1987. Effect of internal viral sequences on the utility of retroviral vector. *J. Virol.* **61**:1647-1650.
4. Aronoff, R., A. M. Hajjar, and M. L. Linial. 1993. Avian retroviral RNA encapsidation: reexamination of functional 5' RNA sequences and role of the nucleocapsid Cys-His motifs. *J. Virol.* **67**:178-188.
5. Aronoff, R., and M. Linial. 1991. Specificity of retroviral RNA packaging. *J. Virol.* **65**:71-80.
6. Baudin, F., R. Marquet, C. Isel, J.-L. Darlix, B. Ehresmann, and

- C. Ehresmann. 1993. Functional sites in the 5' region of human immunodeficiency virus type 1 RNA form structural domains. *J. Mol. Biol.* **229**:382-397.
7. Bender, M. A., T. D. Palmer, R. E. Gelinas, and A. D. Miller. 1987. Evidence that the packaging signal of Moloney murine leukemia virus extends into the gag region. *J. Virol.* **61**:1639-1646.
8. Berkowitz, R., and S. P. Goff. Unpublished data.
9. Berkowitz, R., 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 assay. *J. Virol.* **67**:7190-7200.
10. Bieth, E., C. Gabus, and J. Darlix. 1990. A study of the dimer formation of Rous sarcoma virus RNA and of its effect on viral protein synthesis *in vitro*. *Nucleic Acids Res.* **18**:119-127.
11. Buchschacher, G. L., and A. T. Panganiban. 1992. Human immunodeficiency virus vectors for inducible expression of foreign genes. *J. Virol.* **66**:2731-2739.
12. Canaani, E., K. V. D. Helm, and P. Duesberg. 1973. Evidence for 30-40S RNA as precursor of the 60-70S RNA of Rous sarcoma virus particles. *Proc. Natl. Acad. Sci. USA* **70**:401-405.
13. Clavel, F., and J. M. Orenstein. 1990. A mutant of human immunodeficiency virus with reduced RNA packaging and abnormal particle morphology. *J. Virol.* **64**:5230-5234.
14. Cullen, B. 1987. Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol.* **152**:684-704.
15. Dorfman, T., J. Luban, S. P. Goff, W. A. Haseltine, and H. G. Gottlinger. 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.
16. Embretson, J. E., and H. M. Temin. 1987. Lack of competition results in efficient packaging of heterologous murine retroviral RNAs and reticuloendotheliosis virus encapsidation-minus RNAs by the reticuloendotheliosis virus helper cell line. *J. Virol.* **61**:2675-2683.
17. Feinberg, M. B., R. F. Jarrett, A. Aldovini, R. C. Gallo, and F. Wong-Staal. 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. *Cell* **46**:807-817.
18. Fisher, J., and S. P. Goff. Unpublished data.
19. Fu, W., and A. Rein. 1993. Maturation of dimeric viral RNA of Moloney murine leukemia virus. *J. Virol.* **67**:5443-5449.
20. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**:557-580.
21. Harrison, J. P., and A. M. L. Lever. 1992. The human immunodeficiency virus type 1 packaging signal and major splice donor region have a conserved stable secondary structure. *J. Virol.* **66**:4144-4153.
22. Hayashi, T., T. Shioda, Y. Iwakura, and H. Shibuta. 1992. RNA packaging signal of human immunodeficiency virus type 1. *Virology* **188**:590-599.
23. Herman, S. A., and J. M. Coffin. 1986. Differential transcription from the long terminal repeats of integrated avian leukosis virus DNA. *J. Virol.* **60**:497-505.
24. 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.
25. Konings, D. A. M., M. A. Nash, J. V. Maizel, and R. B. Arlinghaus. 1992. Novel GACG-hairpin pair motif in the 5' untranslated region of type C retroviruses related to murine leukemia virus. *J. Virol.* **66**:632-640.
26. Korb, J., M. Travnicek, and J. Riman. 1976. The oncornavirus maturation process: quantitative correlation between morphological changes and conversion of genomic virion RNA. *Intervirology* **7**:211-224.
27. 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.
28. Kozak, M. 1986. Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. *Proc. Natl. Acad. Sci. USA* **83**:2850-2854.
29. Kozak, M. 1991. An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* **115**:887-903.
30. Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**:488-492.
31. Lever, A., H. Gottlinger, W. Haseltine, and J. Sodroski. 1989. Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. *J. Virol.* **63**:4085-4087.
32. Levin, J. G., and M. J. Rosenak. 1976. Synthesis of murine leukemia virus proteins associated with virions assembled in actinomycin D-treated cells: evidence for persistence of viral messenger RNA. *Proc. Natl. Acad. Sci. USA* **73**:1154-1158.
33. Linial, M., and A. D. Miller. 1990. Retroviral RNA packaging: sequence requirements and implications. *Curr. Top. Microbiol. Immunol.* **157**:125-152.
34. Lopata, M. A., B. Sollner-Webb, and D. W. Cleveland. 1985. Surprising S1-resistant trimolecular hybrids: potential complication in interpretation of S1 mapping analyses. *Mol. Cell. Biol.* **5**:2842-2846.
35. Luban, J., and S. P. Goff. 1991. Binding of human immunodeficiency virus type 1 (HIV-1) RNA to recombinant HIV-1 gag polyprotein. *J. Virol.* **65**:3203-3212.
36. Luban, J., C. Lee, and S. P. Goff. 1993. Effect of linker insertion mutations in the human immunodeficiency virus type 1 gag gene on activation of viral protease expressed in bacteria. *J. Virol.* **67**:3630-3634.
37. Mann, R., and D. Baltimore. 1985. Varying the position of a retrovirus packaging sequence results in the encapsidation of both unspliced and spliced RNAs. *J. Virol.* **54**:401-407.
38. Mann, R., R. C. Mulligan, and D. Baltimore. 1983. Construction of retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33**:153-159.
39. Page, K. A., N. R. Landau, and D. R. Littman. 1990. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. *J. Virol.* **64**:5270-5276.
40. Poznansky, M., A. Lever, L. Bergeron, W. Haseltine, and J. Sodroski. 1991. Gene transfer into human lymphocytes by a defective human immunodeficiency virus type 1 vector. *J. Virol.* **65**:532-536.
41. Pugatsch, T., and D. W. Stacey. 1983. Identification of a sequence likely to be required for avian retroviral packaging. *Virology* **128**:505-511.
42. Rizvi, T. A., and A. T. Panganiban. 1993. Simian immunodeficiency virus RNA is efficiently encapsidated by human immunodeficiency virus type 1 particles. *J. Virol.* **67**:2681-2688.
43. Sakaguchi, K., N. Zambrano, E. T. Baldwin, B. A. Shapiro, J. W. Erickson, J. G. Omichinski, G. M. Clore, A. M. Gronenborn, and E. Appella. 1993. Identification of a binding site for the human immunodeficiency virus type nucleocapsid protein. *Proc. Natl. Acad. Sci. USA* **90**:5219-5223.
44. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. Schwartz, S., B. K. Felber, D. M. Benko, E. Fenyo, and G. N. Pavlakis. 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. *J. Virol.* **64**:2519-2529.
46. Shank, P. R., and M. Linial. 1980. Avian oncovirus mutant (SE21Q1b) deficient in genomic RNA: characterization of a deletion in the provirus. *J. Virol.* **36**:450-456.
47. Vile, R. G., M. Ali, E. Hunter, and M. O. McClure. 1992. Identification of a generalized packaging sequence for D-type retroviruses and generation of D-type retroviral vector. *Virology* **189**:786-791.
48. Watanabe, S., and H. M. Temin. 1982. Encapsidation sequences for spleen necrosis virus, an avian retrovirus, are between the 5' long terminal repeat and the start of the gag gene. *Proc. Natl. Acad. Sci. USA* **79**:5986-5990.
49. Yang, S., and H. M. Temin. 1994. A double hairpin structure is necessary for the efficient encapsidation of spleen necrosis virus retroviral RNA. *EMBO J.* **13**:713-726.