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Efficiency and Selectivity of RNA Packaging by Rous Sarcoma Virus Gag Deletion Mutants

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In all retrovirus systems studied, the leader region of the RNA contains a *cis*-acting sequence called Ψ that is required for packaging the viral RNA genome. Since the *pol* and *env* genes are dispensable for formation of RNA-containing particles, the *gag* gene product must have an RNA binding domain(s) capable of recognizing Ψ. To gain information about which portion(s) of Gag is required for RNA packaging in the avian sarcoma and leukemia virus system, we utilized a series of *gag* deletion mutants that retain the ability to assemble virus-like particles. COS cells were cotransfected with these mutant DNAs plus a tester DNA containing Ψ, and incorporation of RNA into particles was measured by RNase protection. The efficiency of packaging was determined by normalization of the amount of Ψ^+ RNA to the amount of Gag protein released in virus-like particles. Specificity of packaging was determined by comparisons of Ψ^+ and Ψ^- RNA in particles and in cells. The results indicate that much of the MA domain, much of the p10 domain, half of the CA domain, and the entire PR domain of Gag are unnecessary for efficient packaging. In addition, none of these deleted regions is needed for specific selection of the Ψ RNA. Deletions within the NC domain, as expected, reduce or eliminate both the efficiency and the specificity of packaging. Among mutants that retain the ability to package, a deletion within the CA domain (which includes the major homology region) is the least efficient. We also examined particles of the well-known packaging mutant SE21Q1b. The data suggest that the random RNA packaging behavior of this mutant is not due to a specific defect but rather is the result of the cumulative effect of many point mutations throughout the *gag* gene.

Assembly and budding of retroviruses depends on the product of a single viral gene, *gag*. For type C retroviruses, this process appears to occur at the plasma membrane. In contrast, type B and D retroviruses assemble capsids in the cytoplasm, which then travel to and bud from the plasma membrane (52). During assembly, in a process that is not understood but for which no viral gene product other than Gag is necessary, two copies of the viral RNA genome are packaged (45). Upon budding, the virus particle undergoes maturation whereby the Gag polyprotein precursor is cleaved into the several structural proteins of the virus. These mature proteins then assume the roles that their names imply: matrix or membrane associated (MA), capsid (CA), nucleocapsid (NC), and in avian sarcoma and leukemia viruses (ASLVs), proteinase (PR) (26). The triggering event for proteolysis is not known.

In all retroviruses analyzed to date, the RNA genome contains in the 5′ untranslated region a sequence element, termed Ψ, that identifies this RNA as the viral RNA to be incorporated into virions (23, 29). In most retroviruses, this element is near to, or perhaps coincident with, the dimer linkage site, a sequence through which the two identical RNA subunits in each virion are held together. At least in some retroviruses, stem-loop structures are important components of Ψ (25, 59). While Ψ is necessary for packaging, in many cases it is not sufficient for efficient incorporation of an RNA into virions. For example, in murine leukemia virus (MuLV), sequences within the 5′ portion of the *gag* gene stimulate packaging (5). These and similar studies by others (reviewed in reference 29) suggest that more than one sequence element in the viral RNA may be recognized by the packaging machinery.

In ASLVs, three classes of elements other than Ψ have been implicated directly or indirectly in packaging of RNA into virus particles. The first is the DR (direct repeat, since some ASLVs carry two of these sequences), located in the 3′ untranslated region, which was shown to be essential for packaging of partially deleted viral RNAs containing Ψ (47). In a subsequent study, this sequence was found to increase the incorporation of a Ψ^+ nonviral RNA by 10-fold (4). The second consists of two very short open reading frames in the 5′ leader sequence before the *gag* gene. Mutation of the AUG initiation codon at the beginning of either short reading frame reduced packaging 50- to 100-fold (12). The observation that the sequence of the predicted oligopeptides encoded by these reading frames is unimportant suggests that ribosome binding and translation are required in order for the RNA to be recognized by the packaging machinery. The third element is the dimer linkage (DL), which in ASLV is atypical in that it occurs in the 5′ region of the *gag* gene (38, 41, 44). However, more recent studies indicate that the DL may not be required for packaging, at least not for heterologous Ψ^+ RNAs (3).

What corresponding determinants in Gag recognize and bind to Ψ RNA? Extensive studies of the NC protein, as a part of the *gag* precursor, have established the importance of this domain in the packaging of genomic RNA. A chimeric ASLV Gag protein carrying an MuLV NC domain substituted for normal ASLV NC preferentially packages MuLV-derived RNAs (14). Also, point mutations within the single Cys-His motif of MuLV NC affect specificity (15, 33). However, the role that the Cys-His motif plays in packaging is controversial. According to early reports with several retrovirus systems,

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deletion of Cys-His motifs abrogates packaging of viral RNA by Gag (10, 15, 16, 34, 35). More recent data suggest that such deleted Gag proteins may still be able package viral RNA but that this RNA is degraded into small fragments (3). Thus, the Cys-His motifs may play a structural role in protecting the RNA rather than in packaging per se. Moreover, their absence in the NC of the spumavirus subclass of retroviruses (32) suggests that these motifs are not essential for packaging in all retroviruses. Recently, specific binding of human immunodeficiency virus type 1 (HIV-1) NC to viral RNA (7) and to a small portion of the HIV-1 Ψ sequence was demonstrated (42). However, there has been no such demonstration of specific binding by ASLV NC to RNA. In addition to its part in packaging, this protein performs other functions essential to the life cycle of the virus. The ability of NC to promote annealing both to form the viral RNA dimer and to position the tRNA on the primer binding site is well described (8, 40).

While the role for NC seems firmly established, it is not clear what roles other domains in avian Gag might have in assisting NC in specific packaging. The ASLV MA protein binds nonspecifically to RNA in vitro and thus might be expected to have a function in packaging (48). The MA protein of bovine leukemia virus (BLV) is reported to specifically bind RNA fragments containing the putative BLV Ψ sequence (22). The PR domain of ASLV Gag also is implicated in packaging. In chicken cells, Gag deleted of PR is reported to assemble into particles devoid of viral RNA (39). Specific, in vitro binding of the intact Gag precursor to viral RNA has been reported only for HIV-1 (7, 30).

Certain domains of the Gag precursor have been shown to be responsible for particle assembly. The effect of deletions within *gag* was studied in an expression system based upon Myr1, a Rous sarcoma virus (RSV) *gag* in which the N-terminal 10 codons have been replaced with those of *src*. The *src* amino acid sequence directs the myristylation and subsequent plasma membrane targeting of the Gag precursor, resulting in efficient particle production in the mammalian COS-1 cell line (57). Deletion analysis has led to the conclusion that this myristylated Gag derivative contains three regions that are essential for assembly of particles with normal density (56). The first is the N-terminal eight amino acids of the Src protein, which directs myristylation (57). The second is in a stretch of polypeptide called p2, between the MA and the p10 coding sequences (54), and the third is in NC. This third region may be present as two functionally equivalent elements within NC. One of these elements is necessary for the dense packing of Gag molecules within the particle (54) and can be replaced with functionally equivalent sequences from the HIV Gag protein (6).

We have utilized the Myr1 variant of Gag expressed in COS cells to investigate the role of avian Gag domains in packaging. In this system, Gag protein is efficiently assembled into virus-like particles that, by electron microscopy, are indistinguishable from authentic RSV virions (53). Since Myr1 produces an RNA without Ψ, we modified the expression system by providing a Ψ^+ RNA in trans. Assembly-competent deletions were examined with the intent to identify domains that contribute to packaging. We found that much of Gag is unnecessary for efficient and specific packaging. In addition, we also examined the Gag of SE21Q1b, an RNA packaging mutant. Because of its ability to package random RNA, it has long been assumed that analysis of this mutant would reveal the domain within Gag responsible for specificity. The sequence of SE21Q1b indicates several amino acid differences with Prague C RSV. A cluster of differences in the C terminus of the NC domain, when tested in a wild-type background, did not reconstitute the mutant phenotype (2). The results reported here are consistent with those of Anderson et al. (2), who suggest that the behavior of SE21Q1b Gag is not due to a single defect.

MATERIALS AND METHODS

DNA constructs. Plasmid pSV.Myr1A, a derivative of plasmid pSV.Myr1 (57), is a simian virus 40 (SV40)-based vector which produces a transient overexpression of *gag* when transfected into COS-1 cells. pSV.Myr1A differs from pSV.Myr1 by the addition of a poly(A) signal inserted within the *Xba*I site 3′ to the *gag* sequences (Fig. 1A). Mutant constructs pSV.Myr1.MA1 (58), pSV.Myr1.R-3J and pSV.Myr1.SmBs (56), and pSV.Myr1.3h (53) are described elsewhere. Plasmid pSV.Myr1.Es-Bg was constructed by digesting pSV.Myr1 with *Esp*I and *Bgl*II followed by treatment with Klenow polymerase before ligation. The resulting construct lacks *gag* codons 349 to 417. Plasmid pSV.Myr1.Bg-Xm was constructed by digesting pSV.Myr1 with *Bgl*II and *Xma*I. Treatment with Klenow polymerase resulted in an out-of-frame alignment but happened to recreate the *Xma*I site. Subsequent digestion with *Xma*I and treatment with S1 nuclease allowed realignment of the *gag* sequences. One foreign arginine residue is inserted between amino acids 418 and 520. Plasmids pSV.Myr1.PR-L1 and pSV.Myr1.SP-S1 were constructed by oligonucleotide-directed mutagenesis as already described (57). The sequences of the mutagenic oligonucleotides were 5′-GCCGTCTCGT AAGCGATG and 5′-GGCCATGTAGTCTGC, respectively. Plasmid pSV.Myr1.PR-L1 is an exact deletion of the PR domain beginning at amino acid 577 and is identical to the CM5 mutant of Oertle and Spahr (39), while pSV.Myr1.SP-S1 is a truncation from the C terminus of the CA domain through the spacer peptide, NC, and all of PR (Fig. 1B). The expressed products of these clones will be referred to by the abbreviation Myr1 or by the designation of the deletion; e.g., Es-Bg refers to Gag protein and particles produced by clone pSV.Myr1.Es-Bg.

A chimeric *gag* gene was constructed by replacing fragments of the *gag* gene of pSV.Myr1A with analogous sequences from plasmid pSE21Q (2), kindly provided by Maxine Linial. pSV.Myr1A.QXE (Fig. 1B) contains the SE21Q1b *Xho*I-*Eco*RI fragment (nucleotides 630 to 2319; see reference 44 for numbering). pSV.Myr1A.QXB contains the *Xho*I-*Bgl*II fragment (nucleotides 630 to 1630), while pSV.Myr1A.QBE contains the *Bgl*II-*Eco*RI fragment (nucleotides 1630 to 2319).

Plasmid pSV.P18A was constructed to provide an encapsidation-competent RNA. Plasmid pSV.Myr1A was digested with *Sac*I (nucleotide 255), and an *Eco*RI linker was inserted to create pSV.Myr1AE. This plasmid was then digested with *Eco*RI to excise the RSV sequences (nucleotides 255 to 2319), which were replaced with the *Eco*RI fragment (nucleotides −53 to 9238) of plasmid P18. P18, provided by Joe Sorge, is a derivative of the plasmid pr770/−273/P#3 (47), in which a set of *Xba*I linkers had been inserted into the *Cla*I site (nucleotide ~850), thus providing a stop codon near the end of the MA encoding region (Fig. 1A).

T7 transcription clones were made by inserting appropriate sequences into the multiple cloning site of the phagemid pBluescript II KS+ (Stratagene). Plasmid pL(−)T7 contains the *Eco*RI-*Sac*I (nucleotides −53 to 255) fragment from P18 (Fig. 1A). pMyr(−)T7 contains the *Xho*I-*Sph*I fragment (nucleotides 630 to 1006) of pSV.Myr1A, blunted with Klenow fragment at the *Sph*I site, between the *Xho*I and *Eco*RV sites in the vector. P3-76 (48) produces a plus-sense RNA that corresponds to the RNA made by pSV.P18A in vivo, with the exception that since this plasmid was made with sequences

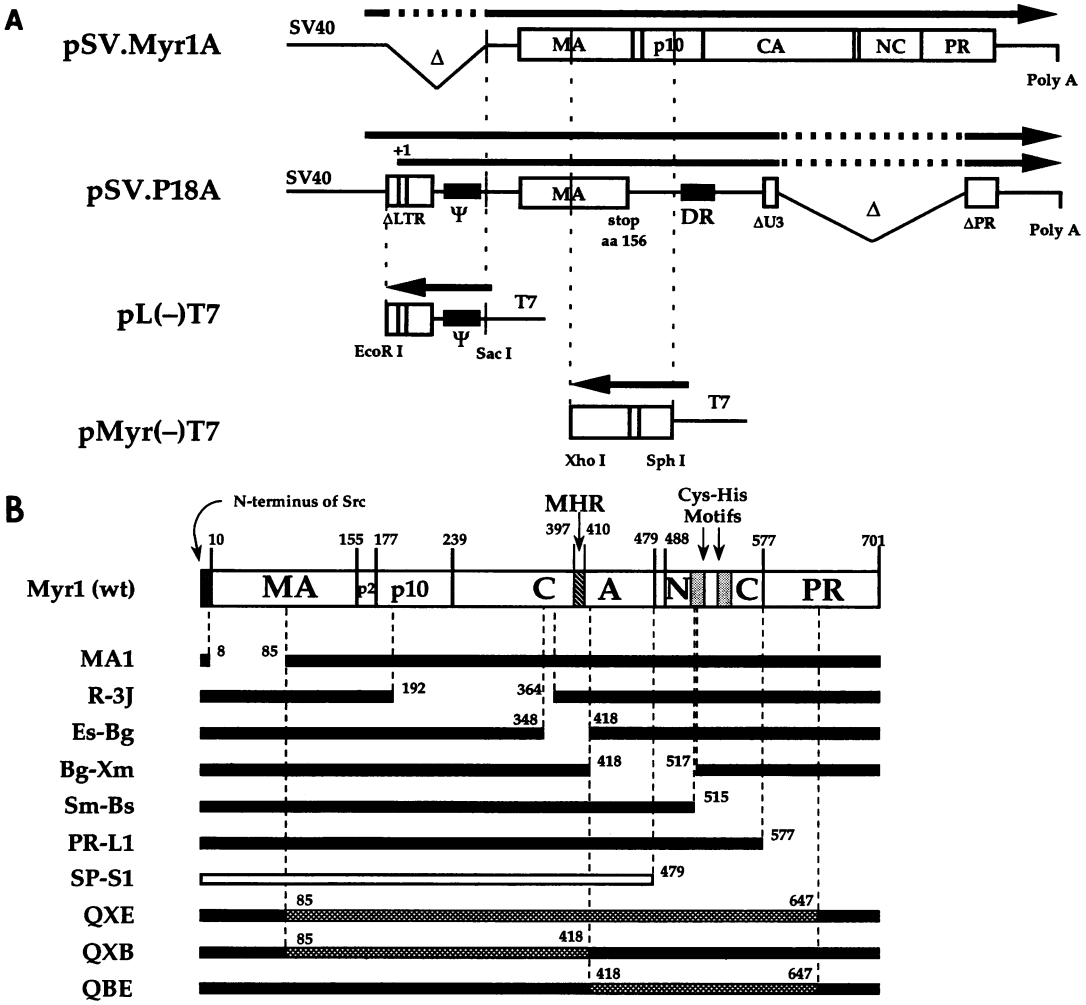

FIG. 1. (A) Transcription units of SV40-based and T7 expression vectors. Transcription of pSV.Myr1A produces an RNA that encodes a full-length *gag* polyprotein precursor but does not contain the Ψ packaging element. Plasmid pSV.P18A produces an RNA that contains all elements known to be associated with packaging. The two T7 transcription clones encode riboprobes to detect the complementary sequences, as indicated by the vertical dashed lines. Dark arrows illustrate transcribed RNAs, plus sense pointing to the right and antisense pointing to the left. Note that transcription initiation on pSV.P18A occurs at two places, at the SV40 late promoter and also at +1 in the long terminal repeat (LTR). aa, amino acid. (B) Polyprotein precursor Myr1 and its derivatives. Myr1 (wild type [wt]) is a modification of the Gag precursor Pr76 in which the N-terminal 10 amino acids have been changed to the sequence of the Src protein. The mature proteins cleaved from the precursor, MA, p2, p10, CA, NC, and PR, are shown in the box, with amino acid numbers defining their ends above. MHR is a conserved amino acid sequence in retrovirus CA proteins. Cys-His motifs are conserved amino acid sequences in most retrovirus NC proteins. Dark bars depict assembly-competent Gag deletion mutants; the open bar depicts an assembly-defective deletion. The numbers indicate amino acid residue numbers that define each deletion. Cross-hatched sections of bars depict regions of SE21Q1b sequence within Myr1-SE21Q1b chimeras.

from pr770/−273/P#3 (47), it does not contain the *Xba* linkers (see above and Fig. 1A).

Cells and virus. COS-1 cells were grown in low-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Life Technologies), 1.0% vitamins, 20 mM L-glutamine, 100 U of penicillin G per ml, and 100 μg of streptomycin sulfate per ml. Schmidt-Ruppin strain A (SR-A) RSV was harvested from turkey embryo fibroblasts that had been stably transfected with plasmid RCAS*neo* (49). These cells, which continuously shed SR-A virus, were maintained in the medium described above plus 25 μg of active G418 per ml.

Transfection and metabolic labeling of COS-1 cells. SV40-based *gag* expression constructs were prepared for transfection by digestion with *Xba*I followed by ligation, as previously described (57), to position the SV40 polyadenylation signal just 3′ to the *gag* sequence. These plasmids were then cotransfected with undigested pSV.P18A into COS-1 cells. Although pSV.Myr1A need not be cut and ligated since it has an additional polyadenylation signal downstream of *gag*, it was nevertheless treated as were the *gag* deletion plasmids which lack this additional signal. The exception was in the experiments to compare Myr1 with SR-A RSV. In these cases, plasmid pSV.Myr1A was cotransfected with pSV.P18A as is. Plasmid pSV.P18A was never cut and ligated in any experiment.

COS-1 cells were transfected by a slight modification of the DEAE-dextran method of Mortlock et al. (37). Briefly, the day before transfection, COS-1 cells were plated in transfection medium (growth medium with fetal bovine serum replaced by 10% Nu-serum IV [Collaborative Research]) at a density of 5×10^5 cells per 60-mm-diameter plate. Cut and ligated *gag*-

expressing DNA (1.25 to 2.0 μg) was combined with 1.5 μg of uncut pSV.P18A in 600 μl of phosphate-buffered saline (PBS) containing 0.2 mg of DEAE-dextran per ml and then added to PBS-washed 75 to 85% confluent 60-mm-diameter plates. After a 60-min incubation at 37°C in 5% CO_2, the DNA was removed by aspiration and replaced with 2 ml of 10% dimethyl sulfoxide in transfection medium. Plates were incubated further for 2.5 min at room temperature, the dimethyl sulfoxide medium was replaced with 4 ml of transfection medium containing 100 μM chloroquine, and the plates were returned to the CO_2 incubator. After 4 h, the chloroquine-containing medium was replaced with transfection medium. Transfection efficiency was monitored by parallel transfection with plasmid pHβAPr-1-βgal, which contains the bacterial β-galactosidase gene driven by the human β-actin promoter (37). Efficiencies were estimated from in situ β-galactosidase assays using 5-bromo-4-choro-3-indolyl-β-D-galactopyranoside (X-Gal) (37) and ranged from 1 to 25%. Transfections with less than 10% efficiency were discarded.

Forty-eight hours posttransfection, cells were labeled with L-[^{35}S]methionine (>1,000 Ci/mmol; ICN Biomedicals, Inc.). After three washes with PBS, cells were labeled with 75 μCi of [^{35}S]methionine in 1.5 ml of serum-free Dulbecco's modified Eagle medium minus methionine. Five hours later, 2.5 ml of transfection medium was added. Labeled particles were collected the next day.

Isolation of virus-like particles. Particles were collected from COS-1 cells either 48 h posttransfection or, if labeled with [^{35}S]methionine, 72 h posttransfection. SR-A RSV produced by turkey embryo fibroblasts was collected after 1 day of production in fresh transfection medium. After preclearing of medium by centrifugation at 10,000 rpm for 10 min, particles were pelleted through a 20% (wt/vol) sucrose cushion in 100 mM NaCl–10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA by ultracentrifugation at 30,000 rpm for 2 h in a Beckman SW60 rotor.

Western blotting (immunoblotting), immunoprecipitation, and protein quantitation. For quantitation by Western blotting, virus pellets were dissolved in sodium dodecyl sulfate (SDS) sample buffer and then loaded onto an SDS–15% polyacrylamide gel. Proteins were transferred to an Immobilon P membrane (Millipore Corp.), probed first with rabbit anti-capsid (αCA) serum at a dilution of 1:2,000, and then probed once more with 2 μCi of [^{125}I]protein A (>30 μCi/μg; ICN Biochemicals). The film exposure was used to locate the protein bands on the blot for excision and counting. A standard curve was constructed with known amounts of purified CA protein. Values of nanograms of CA for virus samples were then read off of this standard curve.

For quantitation by immunoprecipitation, [^{35}S]methionine-labeled particles were dissolved in 1 ml of radioimmunoprecipitation assay buffer (17), and viral proteins were precipitated with the addition of 5 μl of αCA, αPR, and/or αMA serum. Antigen-antibody complexes were eluted from protein A-Sepharose beads (Pharmacia LKB Biotechnology, Inc.) in SDS sample buffer and then loaded onto an SDS–15% poly acrylamide gel. After polyacrylamide gel electrophoresis (PAGE), radioactive bands were visualized by fluorography after the gels were impregnated with sodium salicylate. Bands were then excised from the gel and counted without additional scintillant. Quantitation was done by comparing the number of counts in each sample's bands with those in Myr1 control lanes.

RNA purification, RNase protection analysis, and RNA quantitation. Total cellular RNA was purified from COS-1 cells 48 h posttransfection by the acid guanidinium-thiocyanate method of Chomczynski and Sacchi (11). Viral RNA was extracted from viral particle pellets directly without resuspension by the addition of 100 μl of phenol-chloroform, 2.5 μl of 20% SDS, 100 μl of diethyl pyrocarbonate-treated H_2O, and 25 μg of yeast tRNA carrier, in that order. The aqueous phase was then further extracted with chloroform and finally precipitated in ethanol-sodium acetate. Purified RNAs were stored at −70°C until needed. ^{32}P-labeled probes for RNase protection assays were made by transcription reaction using T7 polymerase as instructed by the manufacturer (Promega Corp.). RNA size standards were transcribed by using the RNA Century template set (Ambion, Inc.). RNase protection analysis was performed with slight modification as described by Aronoff and Linial (4). Briefly, approximately 10^6 cpm of probe (not gel purified) and 10 μg of yeast tRNA carrier were added to sample or to standard quantities of in vitro-made RNA in a 10-μl reaction mixture containing 0.4 M NaCl, 0.1 M morpholinepropanesulfonic acid (MOPS), and 10 mM EDTA. Reaction mixtures were heated at 95°C for 2 min, transferred to 65°C for at least 3 h to allow hybridization, and then treated with 90 μl of an RNase cocktail containing 0.3 M NaCl, 10 mM Tris hydrochloride (pH 7.5), 5 mM EDTA, 3 μg of RNase A per ml, and 4,000 U of RNase T_1 (Life Technologies) per ml. After a 30-min incubation at room temperature, the samples were further treated with 0.2 mg of proteinase K (Boehringer Mannheim Biochemicals) per ml for 15 min at 37°C. An additional 10 μg of tRNA carrier was added, and the samples were phenol-chloroform extracted, ethanol-sodium acetate precipitated, and then loaded onto either a 6 or an 8% polyacrylamide–8 M urea sequencing gel. After electrophoresis, the gel was adhered to blot paper and dried in a vacuum gel drier before exposure to film. The region of the gel containing the protected bands was cut out and exposed for 30 to 60 min in a Betagen Betascope 603 blot analyzer. Protected bands were quantitated by comparing the number of counts in each band of sample lanes with those in Myr1 control lanes. For the experiment to compare Myr1 with SR-A RSV, a standard curve was constructed by using the count values corresponding to the nanograms of in vitro-made RNA standards input into the assay.

RESULTS

Adaptation of COS-1 cell *gag* expression system to study RNA packaging. COS-1 cells have been used extensively to study particle assembly of wild-type and partially deleted versions of the ASLV Gag protein (54, 56–58). The parent vector in this system, pSV.Myr1A, contains the ASLV *gag* gene driven by the SV40 late promoter. This vector also includes the SV40 T antigen and the SV40 origin of replication. pSV.Myr1A and all its derivatives lack the core Ψ region of the leader sequence (Fig. 1A), which is essential for viral RNA packaging (3, 23, 50). Thus, particles produced by expression of these vectors would not be expected to contain viral RNA.

To provide a Ψ^+ RNA that would be a substrate for packaging, we constructed the expression vector pSV.P18A (Fig. 1A). Since the parental construct of P18 was shown previously to produce an RNA that could be packaged into virus particles budding from chicken cells (47), we expected the RNA transcribed from pSV.P18A to be also packaged into particles produced by COS cells. This vector contains all viral RNA elements that have been implicated in packaging for ASLV, namely, the entire leader sequence of RSV including the Ψ packaging sequence (23), the three upstream open reading frames (12), and one copy of the DR sequence (4, 47). It also includes part of the *gag* open reading frame correspond-

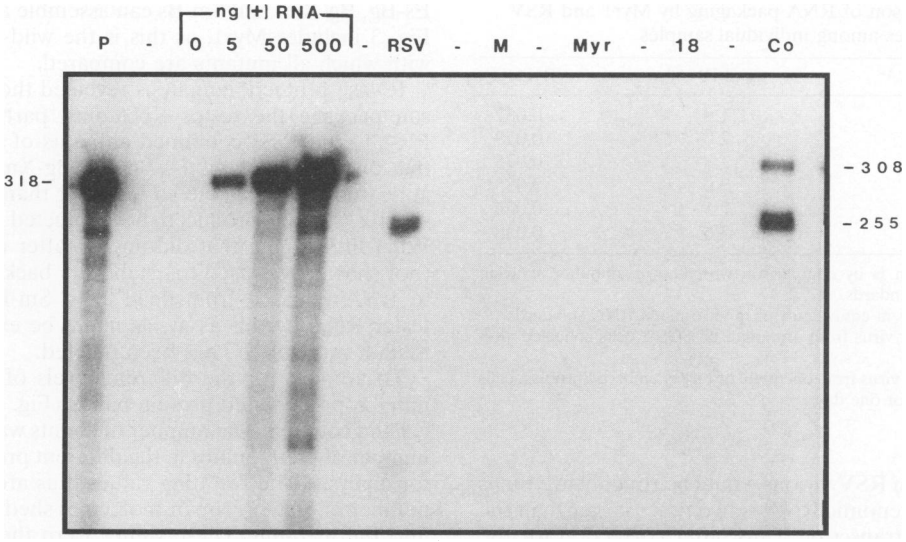

FIG. 2. RNase protection analysis of particle RNA. COS-1 cells were transfected with pSV.Myr1A or pSV.P18A, alone or together. The medium was collected 48 h posttransfection. Alternatively, medium was collected after 24 h of particle production from turkey cells expressing SR-A RSV. RNA extracted from pelleted particles and in vitro-made RNA standards was analyzed by RNase protection, using the probe transcribed from pL(−)T7. Samples were loaded onto a 6% sequencing gel. P, undigested probe; ng (+) RNA, in vitro-made RNA standards, designated by amounts in nanograms; M, mock transfection; Myr, single transfection with pSV.Myr1A; 18, single transfection with pSV.P18A; Co, cotransfection of pSV.Myr1A with pSV.P18A. Empty, unloaded lanes are marked by dashes. The sizes (in nucleotides) of protected fragments are indicated at the left and right.

ing to most of the MA domain of Gag, which contains the dimer linkage.

To test if this system indeed could be used to study packaging, we transfected COS-1 cells, singly or in combination, with pSV.P18A and pSV.Myr1A. Forty-eight hours posttransfection, medium was harvested, particles were collected by ultracentrifugation, and RNA was extracted. Packaged RNA was then analyzed by RNase protection. This method has the advantage of being both more sensitive than Northern (RNA) blot analysis and more tolerant of partial RNA degradation. The probe was transcribed from vector pL(−)T7 (Fig. 1A), which hybridizes to the 5′ part of the viral RNA genome, from the R and U5 sequences of the long terminal repeat to the *Sac*I site at nucleotide 255. Only the RNA produced by pSV.P18A can be recognized by this probe, because the *gag*-encoding RNA from pSV.Myr1A does not contain any viral sequences upstream of the *Sac*I site. As a positive control, RNA from virus produced by turkey embryo fibroblast cells stably expressing RCAS*neo*, an infectious clone of SR-A RSV, was tested in parallel. Also included was in vitro-synthesized (plus)-stranded RNA from plasmid P3-76, which corresponds to the P18 RNA made in vivo, in order to gauge the sensitivity of the assay.

Analysis of RNA from single transfections with either pSV.Myr1A or pSV.P18A resulted in no protected bands, indicating that there was no Ψ^+ RNA in these samples (Fig. 2, lanes Myr and 18). In the case of pSV.Myr1A, $Pr76^{gag}$ was expressed, assembled into particles, and then shed into the medium, but no Ψ^+ RNA was available to be packaged. In the case of pSV.P18A, Ψ^+ RNA was made in the cells, but the short N-terminal portion of Gag did not assemble into particles (data not shown). In contrast, cotransfection of the two plasmids (Fig. 2, lane Co) yielded two clusters of protected fragments. (Digestion of the single-stranded regions of the annealed probe by the combination of RNases A and T_1 results in a cluster of similar-size protected fragments). Both Gag and Ψ^+ RNA were produced in the cell at the same time, and so particles assembled, packaged RNA, and were released into the medium. The presence of two clusters of protected fragments is a consequence of the utilization of both the SV40 and the RSV initiation sites by the SV40 late promoter. The antisense probe detects both transcripts because it extends upstream of the RSV initiation site (Fig. 1A). Identification of the larger fragment cluster, at 308 bases in length, is based on comparison with the fragments protected by the in vitro-made RNA (Fig. 2, lanes 0 through 500). These bands are not due to undigested probe, which is 318 bases in length; the size difference is more clearly evident after electrophoresis for longer times (data not shown). Identification of the smaller protected fragment cluster, at 255 bases, is based on comparison with the fragment of identical size produced by authentic RSV (lanes RSV and Co). That the two P18-derived RNA species are present in transfected cells in approximately the same relative proportion as in Myr1 particles, as determined by RNase protection analysis of whole cell RNA, indicates that one RNA is not preferentially packaged (data not shown).

Myr1 particles and authentic RSV package comparable levels of RNA. Although the only difference between wild-type and myristylated $Pr76^{gag}$ resides at the N terminus of the protein and not near the NC domain, it seemed possible that this difference nevertheless might influence packaging. In addition, Myr1 and its derivatives are derived from Prague C RSV sequences (57), while the Ψ^+ tester RNA is derived from the SR-A strain of RSV (47). These considerations led us to directly compare the ability of Myr1 to package RNA with infectious SR-A RSV (Table 1). The data for individual samples in this experiment are shown to illustrate the reproducibility of results. RNA was quantitated by RNase protection followed by exposure in a Betagen blot analyzer. The amount of RNA is expressed in nanograms of the P3-76-derived T7 transcripts used as standards in the assay. The

TABLE 1. Comparison of RNA packaging by Myr1 and RSV particles among individual samples

Virus	ng of CA[a]	ng of RNA[b]	RNA/CA
Myr1[c]	15	1.0	0.067
	25	2.0	0.079
	24	1.8	0.075
RSV[d]	86	3.0	0.035
	104	2.5	0.024
	95	3.6	0.038

[a] Quantitation of protein is by comparison with known amounts of avian myeloblastosis virus CA standards.
[b] Quantitation of RNA is in equivalents of in vitro-made RNA standards.
[c] Samples each represent virus from one plate of COS-1 cells collected 48 h posttransfection.
[d] Samples each represent virus from one plate of turkey embryo fibroblast cells expressing RSV collected for one day.

actual mass of RNA in RSV virions would be roughly five times higher since the full genomic RNA is five times larger than the P18 RNA or the T7 transcript. Thus, after correction for the slight difference in size (255 versus 308) and conversion of the number of counts to nanograms of P3-76 RNA, the values are directly proportional to the number of molecules of RNA packaged (Table 1).

To control for the difference in particle production, half of each medium sample was analyzed for RNA as described above, and the other half was analyzed for the amount of CA by Western blotting. The membrane was probed with αCA serum followed by [^{125}I]protein A, and the radioactivity in excised bands was quantified. Avian myeloblastosis virus CA was used as a standard to allow conversion of counts per minute to nanogram quantities. The amount of RNA packaged is expressed by the ratio of nanograms of RNA to nanograms of CA. By this analysis, Myr1 particles and RSV contained 0.074 ± 0.006 and 0.032 ± 0.007 g of RNA, respectively, per g of CA. Thus, Myr1 particles produced in COS cells are at least as efficient in packaging RNA as infectious virus produced in avian cells (see Discussion).

Efficiency of packaging by partial *gag* deletion mutants. To investigate the possible role of the several domains of the Gag precursor in packaging, we assayed the ability of a set of Gag deletion mutants (Fig. 1B) to incorporate RNA into virus-like particles. Deletions MA1, R-3J, Es-Bg, Bg-Xm, Sm-Bs, and PR-L1 span the entire length of the precursor, with the exception of a region encompassing the C-terminal half of MA, p2, and the N-terminal portion of p10, which contains a domain essential for assembly (54, 56). All but one of these mutants are capable of assembly and release as virus-like particles (53, 56, 58); mutant SP-S1 is assembly defective and was used as a negative control. Particles shed from transfected, [^{35}S]methionine-labeled COS-1 cells were collected and analyzed for protein and RNA.

The protein in virus-like particles was analyzed by immunoprecipitation with appropriate antisera followed by SDS-PAGE (Fig. 3). The selection of antiserum was made on the basis of the location of the deletion. For MA1, the mature CA was precipitated, and for R-3J, Es-Bg, and Bg-Xm, the mature PR was chosen since these constructs contain deletions in CA. For PR-L1 and Sm-Bs, the Gag precursor protein was precipitated since these constructs lack the PR domain and thus no cleavage occurs. Precipitations using αCA serum (Fig. 3A) showed that MA1 and PR-L1 can assemble into virus-like particles whereas SP-S1 cannot. Precipitations with αPR serum or a combination of αPR plus αMA sera, in the case of the experiment including Sm-Bs (Fig. 3B), showed that R-3J, Es-Bg, Bg-Xm, and Sm-Bs can assemble as well. Each panel in Fig. 3 includes Myr1, as this is the wild-type positive control with which all mutants are compared.

RNase protection analysis revealed those mutant Gags that can package the tester RNA into particles (Fig. 4). MA1, PR-L1, and R-3J contained amounts of RNA comparable to that of Myr1. Mutants Es-Bg and Bg-Xm packaged RNA but appeared to have done so less well than Myr1. The negative control, SP-S1, produced no protected bands in the assay. What little signal that did appear after a very long exposure (not shown) was used to establish a background, perhaps due to RNA released from dead cells. Sm-Bs also packaged no tester RNA by this assay, as might be expected, since in this mutant most of NC has been deleted.

To account for the different levels of particle production, immunoprecipitated protein bands (Fig. 3) were cut from the gel and counted. The number of counts was then divided by the number of methionines in the different protein species selected for analysis. The resulting values thus are proportional to the number of Gag precursor molecules shed in a particulate form into the medium. These values were then normalized to the value for Myr1 to give a relative measure of particle production (Fig. 5A). The efficiency of packaging was then calculated by dividing the quantity of protected RNA by the relative level of particle production. The packaging efficiency for Myr1 was defined as 1.0 (Fig. 5B). Note that no value for SP-S1 is given in Fig. 5B. SP-S1 is the background control, and so the amount of RNA signal that it gives relative to Myr1 has been subtracted from all other values.

By this measure of efficiency, only two regions of Gag were found to influence packaging. Even after taking into account its low level of virus production, Sm-Bs is still incapable of packaging the tester RNA (Fig. 5B). This mutant is similar to one made by Méric and Spahr (PrC 10.8), in which NC is deleted from within the proximal through the distal Cys-His motif, and gives the same result—particles devoid of viral RNA (35). However, our mutation also deletes PR, while PrC 10.8 does not. Es-Bg is only 25% as efficient as Myr1. This deletion is entirely within CA, yet it is the most severely affected of all the mutants that retain the ability to incorporate RNA into particles.

In contrast, the remaining mutants package RNA relatively efficiently. Even Bg-Xm, which deletes the proximal Cys-His motif of NC (Fig. 1B), was found to have RNA levels within a factor of 2 of RNA levels of Myr1. This result is consistent with the work of Méric et al., which showed that single deletions of either of the Cys-His motifs reduced but did not abrogate RNA encapsidation in chicken cells (34). Deletions MA1 and R-3J each have a modest effect upon packaging, and PR-L1 can package efficiently as well. We attribute the observation that PR-L1 particles contain 1.5 times more RNA than Myr1 particles to a technical artifact. In our hands, the Gag polyprotein product of PR-L1 smears in SDS-PAGE (Fig. 3A). Thus, when the band is excised to be counted, some of the signal is lost, leading to an underreporting of the number of particles produced. We estimate that this discrepancy contributes no more than a twofold error, and hence PR-L1 is certainly efficient at packaging. This conclusion is in direct contrast to the results of others, who reported that a mutant Gag deleted of the PR domain failed to package viral RNA (3, 39).

Efficiency of packaging by Myr1-SE21Q1b chimeras. Although virus particles of SE21Q1b package Ψ^+ RNA, when produced in avian cells, this mutant Gag protein is roughly fivefold less discriminating than the wild type in that it also packages random RNAs (4). To compare the packaging effi-

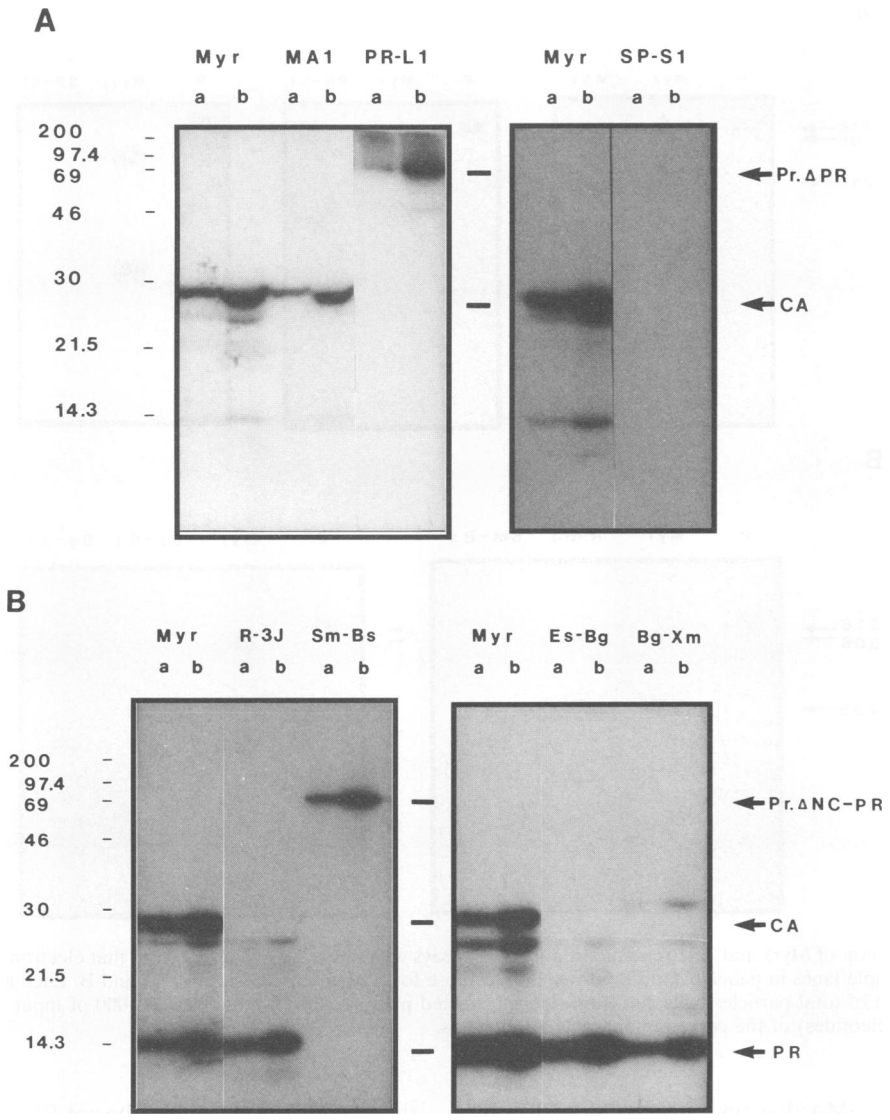

FIG. 3. Immunoprecipitation of Myr1 and deletion mutant virus-like particles. Precipitated proteins were electrophoresed on an SDS–15% polyacrylamide gel. Pairs of lanes are as marked for cotransfections of *gag* expression constructs with pSV.P18A. Lanes a and b are 1/12 and 1/6, respectively, of total pelleted particles per transfected plate. Numbers indicate the positions of molecular size standards in kilodaltons. (A) Precipitations using αCA serum. CA, position of the mature capsid protein; Pr.ΔPR, position of precursor deleted in the proteinase domain (lanes PR-L1). CA and Pr.ΔPR bands were excised for subsequent quantitation. (B) Precipitations using αPR plus αMA sera (left) or αPR serum alone (right). PR, position of proteinase; Pr.ΔNC-PR, position of precursor deleted in nucleocapsid and proteinase domains (lanes Sm-Bs). PR and Pr.ΔNC-PR bands were excised for quantitation.

ciency of the SE21Q1b Gag precursor with that of Myr1, we made a series of clones in which Myr1 sequences were replaced with the corresponding ones from SE21Q1b. The largest replacement, clone pSV.Myr1A.QXE, produces a Gag precursor in which amino acid 85 in the MA domain to amino acid 647 in the PR domain is derived from SE21Q1b sequences (Fig. 1B). Two complementary half replacement clones centered around amino acid 418 in the CA domain, pSV.Myr1A.QXB and pSV.Myr1A.QBE, were also made. Cotransfections, metabolic labeling, and assays were performed as before for the Gag deletion mutants (not shown).

All three chimeric clones produced somewhat more particles than Myr1 (Fig. 6A). The packaging efficiencies of the two half replacements, QXB and QBE, were higher than that of the full replacement but lower than that of Myr1.

Specificity of packaging. While efficiency is a measure of how much RNA is packaged into a particle, it does not provide information about the ability of Gag to discriminate between RNA species. To investigate the capacity of each of the mutants to specifically recognize and select the Ψ^+ tester RNA, we compared the levels of incorporation of this RNA and the Ψ^- RNA that encodes Gag for each mutant (Fig. 1A). To detect both the *gag* and the Ψ^+ tester RNAs simultaneously in the RNase protection assay, we constructed the T7 transcription vector pMyr(−)T7 (Fig. 1A). The riboprobe made from this construct can base pair with 376 bases of the *gag*

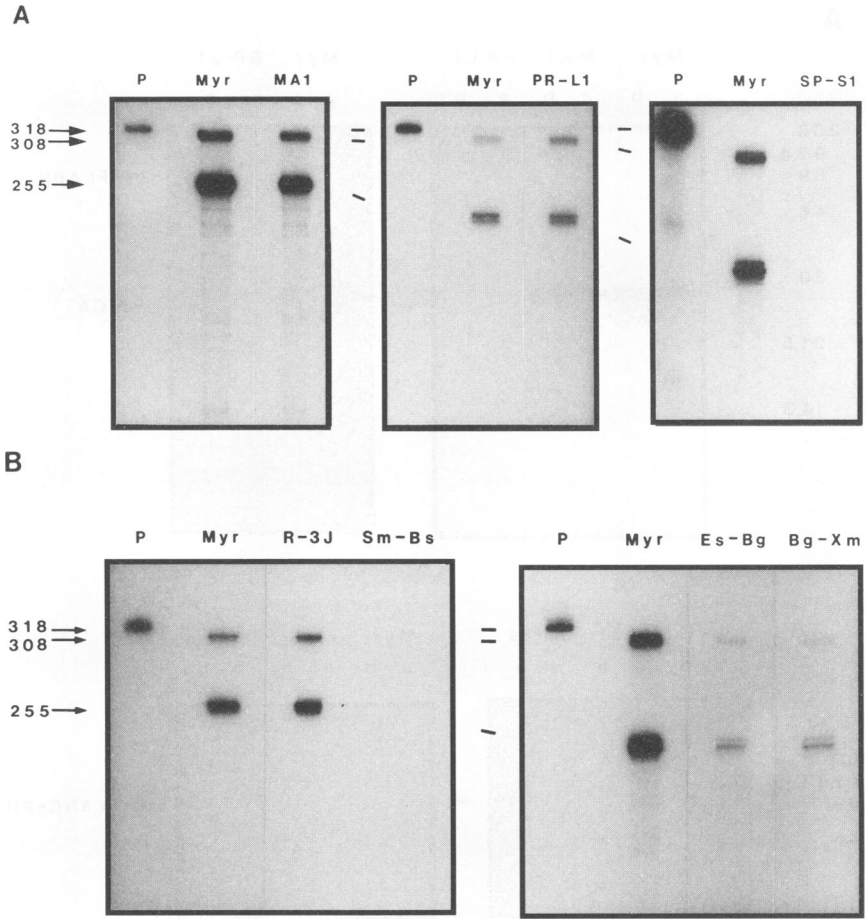

FIG. 4. RNase protection of Myr1 and deletion mutant particles. Assays were done as for Fig. 2 except that electrophoresis was performed on 8% sequencing gels. Sample lanes in panels A and B correspond to those for protein analysis in Fig. 3A and B. Each lane represents protection of RNA from one-fourth of total particles collected from one transfected plate of cells. Lanes P are 1/1,000 of input probe. Numbered arrows indicate the sizes (in nucleotides) of the corresponding RNA fragments.

RNA (from *Xho*I in the MA domain sequence to *Sph*I in the p10 domain sequence) but only 220 bases of Ψ^+ RNA. Thus, two distinct protected fragments should be produced by a mixture of these two RNAs in a protection assay. COS-1 cells were again transfected with pSV.P18A singly or in combination with each of the Gag-encoding plasmids. RNase protection analysis was then performed on both cellular RNA and RNA from virus-like particles.

Single transfections of the Myr1- and P18-expressing constructs produced the expected protected fragments in both cells and particles (Fig. 7, lanes Myr and 18 at the left). No RNA signal was seen in medium from a single transfection with the P18-expressing construct (Fig. 7B, lane 18) because, as discussed for Fig. 2, no particles were produced. In contrast, a single transfection with the Myr1-expressing construct did produce a signal since some *gag* RNA was packaged.

Analysis of cotransfections showed that although all of the mutants incorporated the *gag* RNA along with the Ψ^+ RNA, the Ψ^+ RNA was enriched in particles compared with cells, indicating that this RNA is preferentially selected (compare Fig. 7, lanes Myr, PR-L1, MA1, QXE, 3h, Es-Bg, and Bg-Xm). Some mutants failed to package any of either RNA, as expected from the earlier results to measure efficiency (Figs. 4 and 7B, lanes SP-S1 and Sm-Bs). Note that for mutant R-3J, the fragment protected by the *gag* RNA is smaller. This is due to the deletion of some of the p10 domain in this construct, such that a slightly smaller fragment of the probe would be protected (compare Fig. 1A and B). Note also that an additional mutant, 3h, was tested in these experiments. This mutant is similar to PR-L1 except that 3h retains the first seven amino acids of PR plus a C-terminal lysine residue not normally found in that position (53).

To obtain a quantitative comparison of the ability of each mutant to specifically select the Ψ^+ RNA, the data were analyzed in the following manner. Each protected band was counted, and a ratio of Ψ^+ to *gag* RNA was calculated. This ratio for RNA in the particle was then divided by the same ratio for RNA in the cell to give a value that we define as selectivity [selectivity = $(\Psi_v/G_v)/(\Psi_c/G_c)$, where Ψ_v or Ψ_c and G_v or G_c are the quantities of Ψ^+ RNA and *gag* RNA in particles or cells, respectively]. Qualitatively, selectivity is the ability of a virus, on a molar basis, to select and package one RNA over another. For instance, in our experiments, a selectivity of 10 would mean that when the virus is presented with a pool of RNAs including the Ψ^+ tester and the Ψ^- *gag* RNAs, it has a 10-fold-higher propensity to choose the Ψ^+ RNA over the *gag* RNA. A selectivity of one indicates completely random packaging. We found it necessary to calculate specificity in this

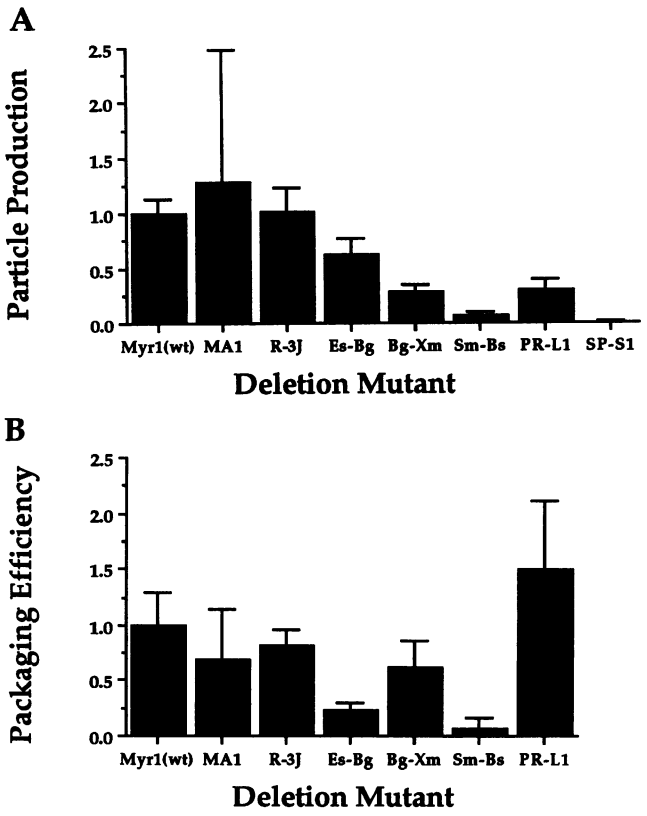

FIG. 5. Summary of deletion mutant analyses. Values represent the averages of four experiments done in two separate sets of two for a total of 4 times except those for mutants Sm-Bs and SP-S1, which were done a total 6 times, and Myr1 (wild type [wt]), which was done a total of 26 times. Error bars represent the standard deviation from the mean for each mutant. Scales are relative to Myr1 set at 1.0. (A) Relative measure of particle production. [^{35}S]methionine-labeled protein bands were excised from the gel (Fig. 3) and counted. The relative amount of protein was determined by dividing the number of counts by the number of methionines in the selected protein species and comparing the result with that for Myr1. (B) Relative measure of packaging efficiency. Protected RNA bands were quantitated by Betascope image analysis of the exposure. The number of counts per sample was divided by the relative particle production and then compared with the value for Myr1. SP-S1 is excluded, as it is the RNA background control.

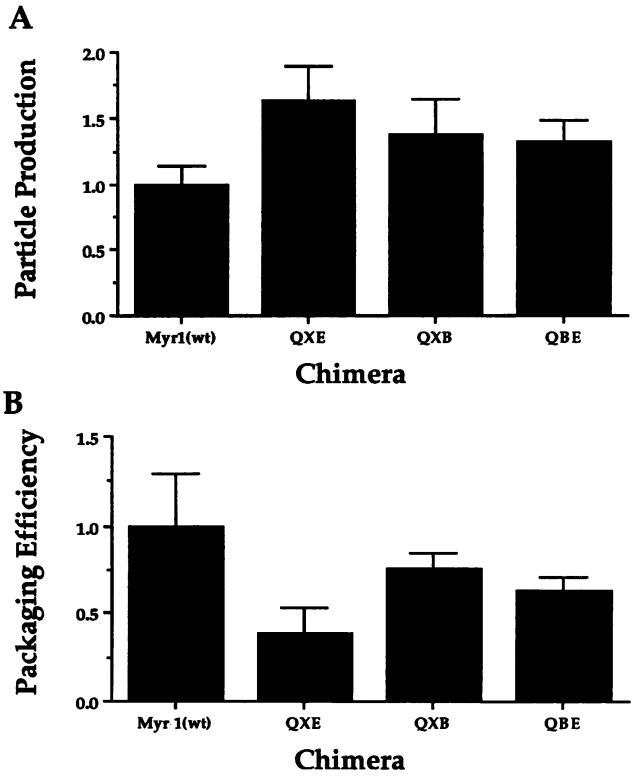

FIG. 6. Summary of analyses of Myr1-SE21Q1b chimeras. Values for chimeras represent the averages of four experiments done in sets of two on two separate occasions. Error bars represent the standard deviation from the mean. Scales are relative to Myr1 set at 1.0. Quantitation was performed as for Fig. 5. (A) Relative measure of particle production. (B) Relative measure of packaging efficiency.

manner since the relative expression of the two cotransfected constructs cannot be precisely controlled. An advantage of measuring packaging by selectivity is that the assay is independent of the amount of protein and of total RNA within particles.

As found for the efficiency of packaging, few regions of Gag influence the specificity of RNA selection. Myr1 has an average selectivity of about 25 (Fig. 8). Most deletions within *gag* had little effect on this value and produce a selectivity of between 15 and 20. These include the near total replacement of Myr1 with SE21Q1b sequences (clone QXE). That this chimera is not grossly deficient in specific packaging of Ψ^+ RNA is consistent with previously published data (4). In contrast, Es-Bg is approximately fourfold reduced in packaging selectivity. However, the mutant most deficient in selectivity, at only about one-eighth the level of Myr1, is Bg-Xm, suggesting that the portion of the Gag protein deleted contains information for specific recognition of viral RNA.

DISCUSSION

We have shown that virus-like particles produced by the Myr1 avian *gag* expression system are capable of efficiently and specifically packaging viral RNA containing Ψ. Production of RNA-containing particles was achieved by cotransfection, analogous to the production of helper-free retroviral vectors by using packaging cell lines (31, 36, 43, 50) except that in our system, the cells were not already expressing the viral proteins. That Myr1 can efficiently incorporate RNA during particle assembly is not surprising given that wild-type Gag can do so in the absence of *pol* and *env* gene products (39, 45) and that in chicken cells, the tester RNA appears fully competent for packaging by RSV (47). Deletion mutants that were still capable of assembling into particles were then examined. Both the efficiency and specificity of RNA encapsidation were determined by standardizing the results against the amount of protein and a nonspecific Ψ^- RNA in released particles. We also similarly examined the Gag of SE21Q1b, a mutant that incorporates random RNAs (28).

Myr1 apparently can package RNA about as well as SR-A RSV. However, we found that the number of RNA molecules in Myr1 particles was approximately twice that in RSV. We believe this difference to be significant given the precision of the measurements. The dispersion within the data for each particle type is small compared with the difference between Myr1 and RSV (Table 1). Under the conventional assumption that wild-type retroviruses contain two molecules of genomic RNA, Myr1 particles therefore contain four tester RNA

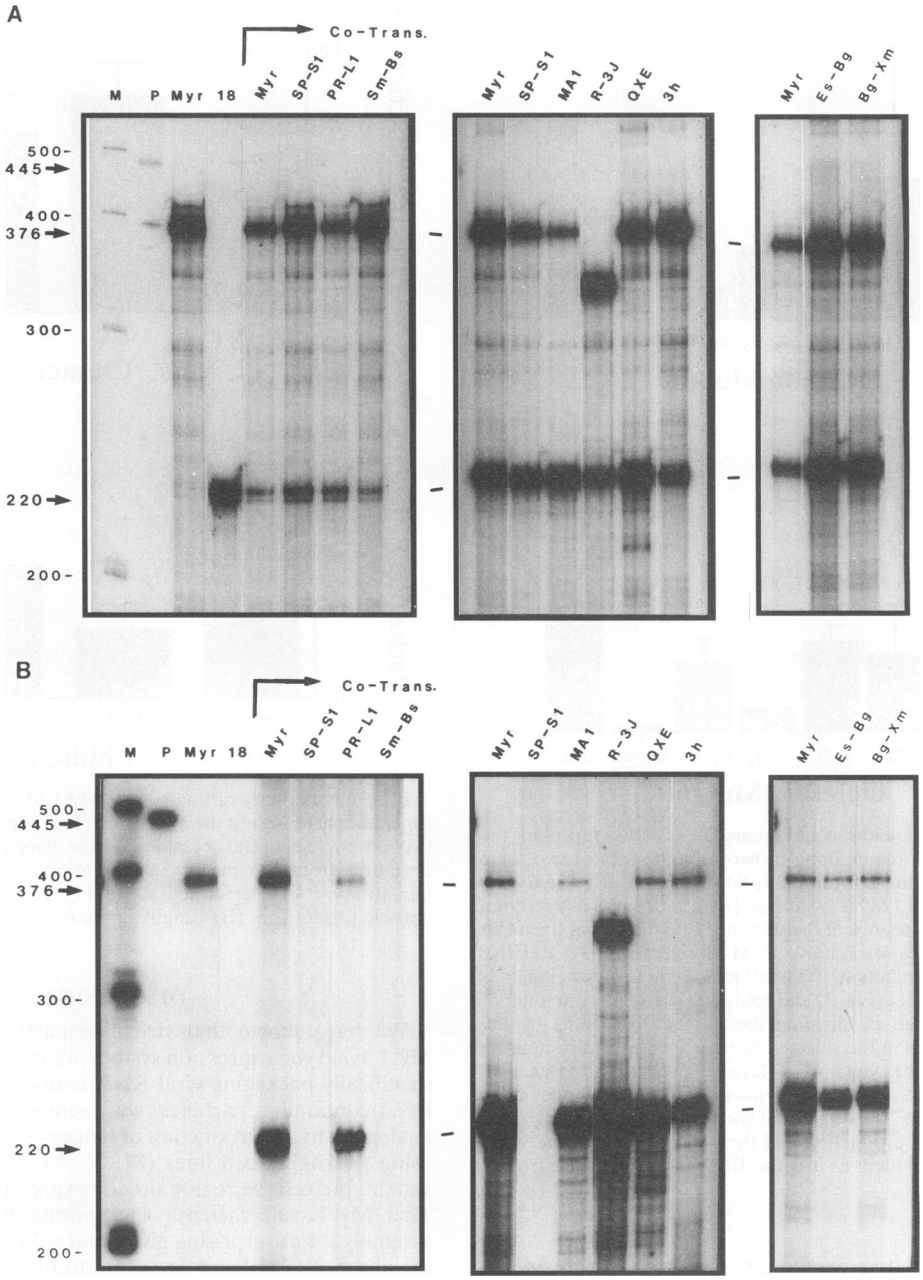

FIG. 7. RNase protection using the *gag* antisense probe. Assays were performed as for Fig. 2. Lanes M are molecular size standards, lanes Myr are single transfections with pSV.Myr1A, and lanes 18 are single transfections with pSV.P18A. Lanes shown by arrows as cotransfections were of pSV.P18A with the *gag*-expressing construct as indicated. Numbers denote the sizes, in bases, of molecular size standards. Numbered arrows indicate the sizes, in bases, of the probe and protected fragments. (A) Analysis of total cell RNA. Each lane represents protection of one-fifth of total extracted RNA from each transfected plate of cells. (B) Analysis of RNA in virus-like particles. Each lane represents protection of one-half of total RNA from particles produced by each transfected plate of cells. Exposure times are approximately five times that for panel A.

molecules. Other than measurements of the total composition of virus, in which RNA was found to compose ~1% by weight (52), the quantity of RNA per particle has not been carefully addressed in published studies. However, the size limit for RNA has been studied and for avian viruses is reported to be about 11 kb (18). To our knowledge, the smallest RNA to be specifically packaged is the parental version of the tester RNA used in our studies (47). How many genomic RNAs might a retrovirus be expected to package? The genome of ASLV is approximately 7.5 kb in length, and each virion contains between 2,000 and 4,000 Gag precursors (51). Given a binding site size of five bases per NC protein (20), each virion contains approximately enough NC molecules to coat the genomic RNA dimer. If this stoichiometry determines the amount of RNA incorporated in a virus particle, then more than two Ψ^+ RNAs might be packaged if the RNAs are small. Although our

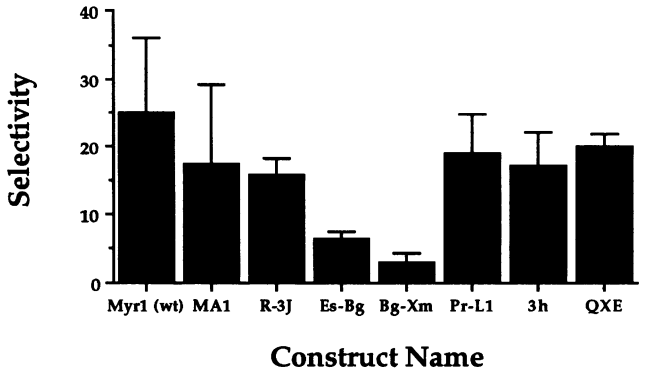

FIG. 8. Selectivity of packaging. Data from protection experiments (Fig. 7) to detect both specific (Ψ^+) and nonspecific (*gag*) RNAs are summarized. Values represent the averages of four experiments done as sets of two on two separate occasions. Error bars represent the standard deviation from the mean. Note that the scale is absolute. wt, wild type.

results are qualitatively consistent with this hypothesis, one might have expected 10 molecules of the tester RNA per particle rather than four as found. This discrepancy could be explained by the presence of variable amounts of cellular RNAs in particles. Alternatively, the minimum requirement for RNA in particle assembly may be less than the theoretical capacity of the particle, with inefficient incorporation of extra molecules beyond that minimum threshold. A careful study of total RNA in retrovirus particles, assembled in cells expressing Ψ^- RNA or Ψ^+ RNA of different sizes, would need to be carried out to address this hypothesis directly.

Having established that Myr1 can serve as a wild-type control, we examined the packaging ability of the Myr1-based *gag* deletion mutants. Some deletions had little effect. Because the avian MA protein can bind to RNA in vitro, albeit nonspecifically (48), we expected that a deletion within this domain might interfere with packaging. In fact, deletion of the N-terminal half of MA had only a modest effect. In contrast to avian MA, BLV MA (p15) has been reported to bind viral RNA specifically in vitro (21). This protein is processed further into two smaller proteins, p10 and p4, plus a seven-amino-acid peptide. Only the full MA (p15) species of BLV could bind RNA specifically (22). In ASLV, a similar pattern of processing yields MA (p19), p10 protein, and a p2 peptide (Fig. 1B). Thus, by analogy with BLV, a determinant for specific RNA recognition might lie within the p2-plus-p10 region in ASLV. If such a specific RNA recognition domain exists in ASLV, it must be close to or within the assembly domain between the deletions in MA1 and R-3J.

With the exception of PR-L1, mutants less efficient at particle production were in general less efficient at encapsidating RNA (compare Fig. 5A and B). For example, deletion Sm-Bs is the most deficient in particle production (except for SP-S1, which is completely defective) and also the most deficient in packaging. Its particles contained essentially no RNA because it was deleted of most of NC. The proximity of this deletion to the third assembly domain (56) may explain its low particle production. Deletion Es-Bg was reduced approximately 50% in particle production but reduced 75% in packaging. The behavior of this mutant was unforeseen, since it is not deleted within the NC domain and lacks only a small region in CA. The phenotype of Es-Bg suggests that the major homology region (MHR), which is within the deleted region, may play some role in packaging, perhaps by assisting NC to obtain the optimum conformation. The MHR is the most conserved amino acid sequence in retrovirus *gag* genes (56). Deletion Bg-Xm was also low in particle production, as expected because of the deletion within NC, but it still incorporated RNA into particles efficiently. Together, the deletions in mutants Bg-Xm and Sm-Bs cover all of NC. Our results and data from studies of RSV-HIV chimeras (6) suggest that there are two functional copies of the third assembly domain in NC. In addition, it was recently demonstrated that this domain is not always necessary for particle production (54) but that it increases the density of shed particles, perhaps through interaction with RNA.

In direct contrast with the results of Oertle and Spahr (39) and also of Aronoff et al. (3), who found that virus particles formed in chicken cells by Gag deleted of PR (mutant CM5) do not contain viral RNA, we observed that the same Gag protein in the context of Myr1 can encapsidate RNA in COS cells. Even with the worst-case assumption that the PR-L1 precursor is underestimated by twofold, PR-L1 would still be efficient in RNA packaging (Fig. 5B). A possible explanation of this discrepancy is that the overexpression in the COS cell system somehow compensates for the defect seen in chicken cells when PR is deleted. Compensation for a deletion by overexpression has been seen in a study of Mason-Pfizer monkey virus assembly, in which case mutants deleted in p12 are assembled poorly in HeLa cells but efficiently in COS cells (46). It is also possible that some of the originally published data are in error. A more recent analysis by Bowles and Spahr shows that mutant CM5 does incorporate RNA but at a level only slightly less than the wild-type level (9). The earlier results from the same laboratory might be explained by the difficulty in quantitating the efficiency of particle formation by the ΔPR mutant.

The reduced packaging efficiency of mutant QXE, carrying a nearly total substitution of Myr1 by the SE21Q1b sequence, was expected since similar results have already been reported (4). We made the two chimeric mutants QXB and QBE, which represent substitutions of complementary halves of the same sequence, to try to localize the defect(s) within the SE21Q1b precursor that is responsible for its reduced efficiency and random RNA packaging behavior. The fact that each chimera displayed about half the deficient phenotype of QXE suggests that the phenotype of SE21Q1b results from the cumulative effect of the several amino acid substitutions throughout the precursor (2). In addition, our results confirm those of Aronoff and Linial (4) that SE21Q1b can specifically select Ψ^+ RNA (Fig. 8).

Only deletions within the center of the Gag precursor had a significant effect on specificity (Fig. 8). These two deletions, Bg-Xm and Es-Bg, either are partially within NC or contain a highly conserved region of CA that we hypothesize is important for the function of NC within the precursor. Bg-Xm, which lacks half of the proximal Cys-His motif of NC, is the most defective by measure of selectivity, in agreement with the studies of Dupraz et al. (13), in which point mutations in this same Cys-His motif produced a range of defective phenotypes, including the complete absence of RNA in particles. None of the Myr1 deletion mutants tested retained a fully wild-type level of specificity when its packaging efficiency was reduced. This result suggests that there is not a determinant within Gag, other than NC, that binds to RNA and confers specificity upon the interaction. We had hypothesized that such a second interaction domain might exist because mature ASLV NC does not bind RNA in a specific manner. If there is only a single RNA binding determinant in Gag, then why does the mature

NC itself not display this specificity in vitro? It may be that there are elements in Gag that assists NC in specific binding and that these elements themselves do not directly interact with RNA. As already mentioned, we hypothesize that the MHR may have such an auxiliary function.

Deletions at the ends of the precursor had only a modest effect on specificity but are nonetheless significant. PR-L1 and the similar C-terminal truncation 3h demonstrate that the PR domain is dispensable for specific as well as efficient packaging. Deletion MA1 retained the ability to select Ψ^+ RNA. However, in addition to revealing that the N-terminal half of MA is not involved in specificity, this mutant also reveals that the putative DL (deleted in this mutant) is not, in the absence of Ψ, a positive effector for RNA selection. If it were, then the selectivity value for MA1 should have been higher than for Myr1. Our results are consistent with those of Aronoff et al., who found that deletion of the DL in a heterologous RNA had no effect on the ability of Ψ to direct packaging (3).

Previous studies of packaging specificity had shown that an avian retrovirus is at least 150-fold more efficient at choosing a Ψ^+ RNA than a random RNA (4). In our assay, we found that the selectivity for Ψ^+ RNA over *gag* RNA deleted in Ψ was about 25. An explanation for this lower number might be that the *gag* RNA is not a completely neutral competitor because it is composed of viral sequences, some of which it shares with the Ψ^+ RNA. However, other than the DL, which was ruled out by analysis of mutant MA1, no obvious packaging signal is present in the *gag* RNA. We also attempted to measure the specificity of Ψ^+ RNA packaging by comparison with β-actin mRNA, perhaps the most abundant cellular mRNA. The analysis was performed in the identical manner as for the *gag* RNA, with a single probe consisting of antisense viral as well as antisense actin sequences. The probe recognized the Ψ^+ RNA as before and 141 bases of the COS cell β-actin mRNA. We found that the β-actin signal in particles was too low over background to make a determination of selectivity (data not shown). However, it was possible to estimate a lower bound on the selectivity of Myr1 for Ψ^+ RNA versus β-actin RNA. This selectivity value was at least as high as that determined for Ψ^+ RNA versus Gag RNA. What constitutes a neutral competitor for packaging is not understood. For example, the message for glyceraldehyde-3-phosphate dehydrogenase is efficiently encapsidated by avian retroviruses yet has little sequence homology with retroviral RNA (1, 4).

What is the nature of the packaging determinant within the Gag protein? It is noteworthy that those deletions near the middle of Gag that affect assembly also affect both the efficiency and the specificity of RNA packaging (Es-Bg, Bg-Xm, and Sm-Bs). In addition, by analogy with BLV, the region around p2 and the C terminus of MA might be involved in specific RNA binding; we could not test deletions in this region because it contains an essential assembly domain (56). It is not known if formation of retrovirus particles is dependent on RNA. A precedent for the role of RNA in assembly is provided by the alpha viruses, in which RNA acts as a scaffold for construction of the capsid (55). Clearly, retrovirus assembly cannot depend on a specific viral RNA, however, since budding and maturation are not compromised in packaging cell lines and numerous mutants lacking Ψ (24, 28, 31, 36, 50) or in cells treated with actinomycin D (19, 27). Nevertheless, given the properties of the Gag deletion mutants, we hypothesize that interaction of Gag with RNA, either viral or cellular, is required for assembly. Addressing this hypothesis directly will require an in vitro assembly system.

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