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Avian Retroviral RNA Encapsidation: Reexamination of Functional 5′ RNA Sequences and the Role of Nucleocapsid Cys-His Motifs

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RNA packaging signals (ψ) from the 5′ ends of murine and avian retroviral genomes have previously been shown to direct encapsidation of heterologous mRNA into the retroviral virion. The avian 5′ packaging region has now been further characterized, and we have defined a 270-nucleotide sequence, *A*ψ, which is sufficient to direct packaging of heterologous RNA. Identification of the *A*ψ sequence suggests that several retroviral *cis*-acting sequences contained in ψ+ (the primer binding site, the putative dimer linkage sequence, and the splice donor site) are dispensable for specific RNA encapsidation. Subgenomic *env* mRNA is not efficiently encapsidated into particles, even though the *A*ψ sequence is present in this RNA. In contrast, spliced heterologous ψ-containing RNA is packaged into virions as efficiently as unspliced species; thus splicing per se is not responsible for the failure of *env* mRNA to be encapsidated. We also found that an avian retroviral mutant deleted for both nucleocapsid Cys-His boxes retains the capacity to encapsidate RNA containing ψ sequences, although this RNA is unstable and is thus difficult to detect in mature particles. Electron microscopy reveals that virions produced by this mutant lack a condensed core, which may allow the RNA to be accessible to nucleases.

Packaging signals are the *cis*-acting retroviral sequences required for specific encapsidation of genomic RNA into the virion. These signals were first identified by analysis of a quail cell line containing a spontaneous Rous sarcoma virus (RSV) mutant provirus, *SE*21Q1b (29), which has 179 bp deleted from the 5′ leader of the recombinant proviral genome (3, 42). Analyses of this mutant and of other avian and mammalian retroviral mutants have localized RNA packaging signals (ψ) to the 5′ ends of retroviral genomes (25, 26, 32, 48). Assays have also been developed to define sequences which are required for efficient packaging of nonviral RNAs. Such assays have shown that about 800 nucleotides (nt) of 5′ sequences containing part of the *gag* gene (called ψ+) can direct packaging of heterologous RNA, equivalent to that of genomic RNA, into murine leukemia virus (MLV) particles (1, 5). Sequences from the 5′ end of the avian retroviral genome (avian leukosis virus [ALV]) have also been tested for the ability to induce specific packaging of nonviral RNA (4). A 683-base ψ+ sequence has been found to direct efficient encapsidation of a heterologous mRNA into avian retroviral virions. However, unlike the case of MLV, this packaging was reduced about 10-fold relative to that of RNA encoded by a retroviral genome or complete retroviral vectors (4).

Subgenomic *env* mRNAs are not efficiently encapsidated by wild-type avian or murine retroviruses. The ψ sequences in MLV and spleen necrosis virus are located between the splice donor and acceptor sites; thus, ψ is not contained in the subgenomic *env* mRNA. In contrast, the defined 5′ ψ signal in avian viruses is upstream of the splice donor and thus is included in both the genomic and the spliced mRNAs. Nevertheless, *env* RNA is inefficiently encapsidated into ALV virions. Another *cis*-acting packaging signal, the 115-nt direct repeat (DR) sequence, originally found flanking the *src* gene of RSV, has been implicated in the encapsidation of avian retroviral RNA into virions (43). However, because the DR is present in both spliced subgenomic and unspliced genomic RNAs, it cannot explain the difference in packaging between these RNAs.

The retroviral genome encodes three polyprotein precursors, Gag, Pol, and Env. In the case of avian retroviruses, Gag is proteolytically cleaved to yield five polypeptides: matrix (MA), a protein of unknown function (p10), capsid (CA), nucleocapsid (NC), and protease (PR). Only the *gag* gene product is required for production of retroviral particles containing RNA. Viruses with mutations in the PR active site in which no processing of Gag occurs form particles which contain wild-type amounts of RNA (13, 44). These and other data (38) support the idea that the full-length Gag precursor is both required and sufficient for RNA packaging. After proteolysis of Gag, the NC polypeptide binds RNA in vitro with high affinity but with no apparent specificity (17). Proteolytic cleavage also coincides with the formation of mature C-type virions containing a highly condensed electron-dense core (24).

Analyses of virions produced by mutated proviruses have indicated that a 14-amino-acid Cys-His motif, CX_2CX_3 (G)HX_4C (where X represents any amino acid) found within the NC domain of Gag, is required for production of particles containing genomic RNA (34, 35). Cys-His boxes have generally been thought to form metal binding domains somewhat similar to zinc finger metal binding domains. It was reported that retroviruses do not contain zinc (21), but more recent work has detected zinc in association with Gag proteins (for example, see reference 7). Although zinc fingers are generally associated with DNA binding activity, transcription factor IIIA binds to RNA as well as DNA and contains multiple zinc fingers (6).

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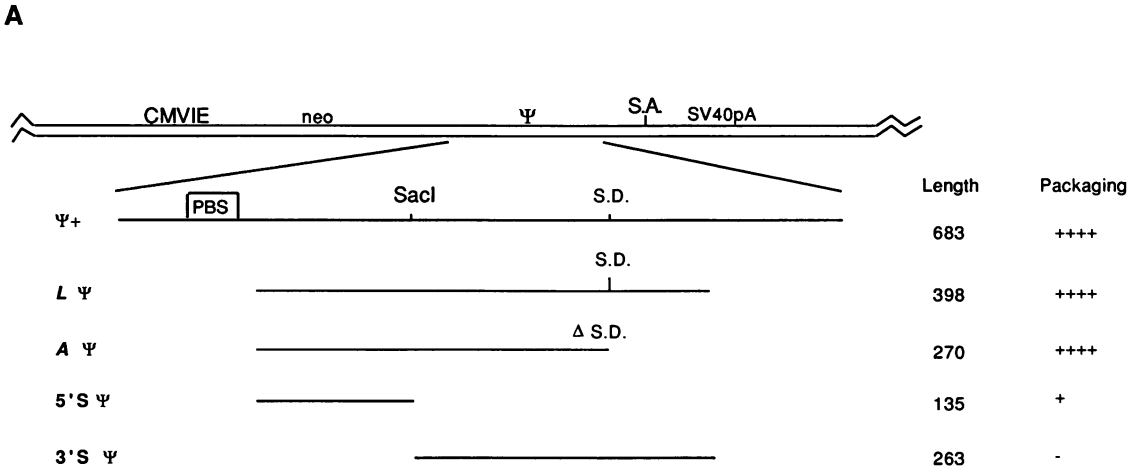
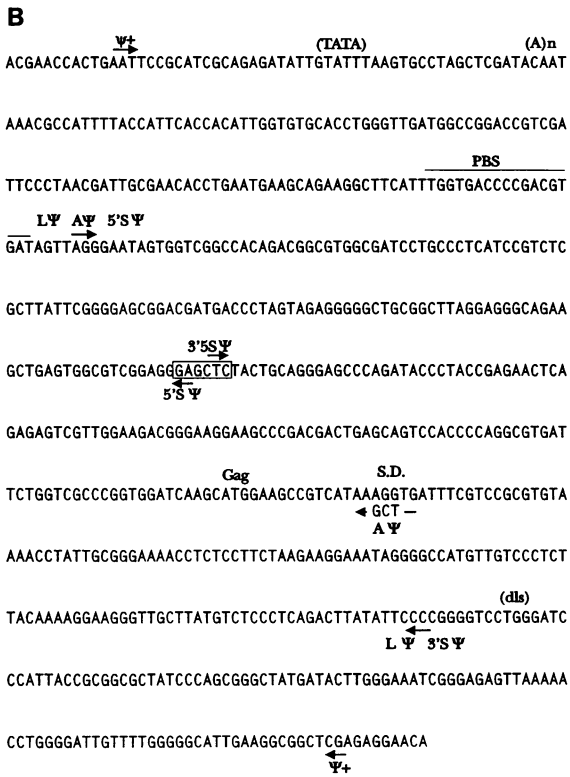


FIG. 1. (A) Constructs used to assay 5′ regions of the genome for ψ function. Portions of the 5′ end of the RSV genome to be tested were cloned into the plasmid pCMVneo as described in Materials and Methods. S.D., splice donor site in *gag*; ΔS.D., splice donor site mutated in construct; S.A., splice acceptor site in SV40 sequences. (B) Sequences of ψ+, *L*ψ, *A*ψ, and *S*ψ. Arrows denote the limits of the 683-base ψ+, 398-base *L*ψ, 270-base *A*ψ, 135-base 5′*S*ψ, and 270-base 3′*S*ψ sequences. The PBS is denoted by a line above the sequence. The promoter element (TATA), the polyadenylation [(A)n] signal, the start of Gag synthesis, the major 5′ splice donor site (S.D.), and the putative dimer linkage sequence (dls) are indicated above the sequence. The location of the *Sac*I restriction endonuclease site (GAGCTC) is indicated by a box. The splice site is mutated in *A*ψ to a *Hin*dIII restriction site as indicated by GCT at the arrowhead.



In this report, we demonstrate that a sequence of 270 nt, *A*ψ, can direct heterologous *neo* RNA into ALV. We also show that in contrast to spliced *env* mRNA in the retroviral context, both spliced and unspliced heterologous mRNAs containing ψ are efficiently packaged. In addition, reexamination of the role of the ALV Cys-His motifs in RNA packaging suggests that these are dispensable for initial interactions of Gag with ψ. An RSV Cys-His deletion mutant produces particles containing RNA, but this RNA is not intact and thus is more difficult to detect in mutant than in wild-type virion RNA preparations.

MATERIALS AND METHODS

Cell culture and transfections. The quail cell lines *SE*21Q1b (29), Q2bn-4D (46), and QT35 (36) and QT35 containing the del1,2 provirus (35) were grown in GM+D+CK (Ham's F10 medium containing 10% tryptose phosphate broth [Difco], 5% calf serum, 1% heat-inactivated chick serum, and 1% dimethyl sulfoxide). The del1,2 producer cell line was derived by transfecting QT35 cells with the RSV del1,2 proviral plasmid (see below) and the pCMVneo*L*ψ plasmid at a ratio of 10:1. Cells were grown at 37°C in a 6% CO_2 atmosphere.

Transfections were performed by the modified calcium phosphate method of Chen and Okayama (10). All cells were seeded in Dulbecco modified Eagle medium with 10% calf serum prior to the addition of plasmid DNA. Cells were selected for drug resistance in CM (Ham's F10 medium containing 10% tryptose phosphate broth, 12% calf serum, 4% chick serum, and 1% nonessential vitamins [GIBCO]). Hygromycin (Sigma) was used at 0.1 mg/ml and G418 (Sigma) was used at 0.25 mg/ml. Mass cultures of drug-resistant cells were obtained after 1 to 3 weeks of selection. Individual drug-resistant cell clones were picked with finely pulled out Pasteur pipets into fresh selective media.

Plasmids. Plasmids were constructed by standard techniques (31). pCMVneo and pCMVhphψ$^+$ have been previously described (4, 27). ψ test sequences (*A*ψ, *L*ψ, 5′*S*ψ, and 3′*S*ψ) were inserted into the *Sma*I site of pCMVneo in either the sense or antisense (ψ_T) orientation. Figure 1A compares the *A*ψ, *L*ψ, and *S*ψ sequences relative to the previously described ψ+ sequence (4). To construct *L*ψ, the 5′-most *Bst*EII-*Xho*I fragment of pATV8R was cloned into vector pSL1180 (9), creating pSL1180ψ. This was the template for amplification with the following oligonucleotides: 5′ primer (5′→3′), CGAGTCGACGGGAATAGTGGTC, and 3′ primer, CGATCATGAGCCTTCAATGCC. The amplified product was isolated, digested with *Hin*cII and *Bam*HI, and

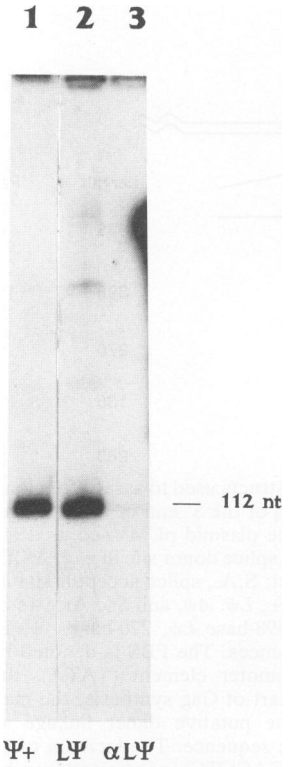


FIG. 2. Primer extension of analysis of virion RNA to determine ψ+- versus *L*ψ-directed encapsidation. Q2bn-4D cells were transfected with pCMVneoψ+ (ψ+), pCMVneo*L*ψ (sense) (*L*ψ), or pCMVneo*L*ψ (antisense) (α*L*ψ), and pools of G418-resistant clones were selected. Equivalent amounts of viral supernatants were pelleted, and virions were extracted. RNA was hybridized to a 22-base *neo*-specific antisense oligonucleotide, and primer extension assays were performed.

inserted into vector pSL1180 DNA previously digested with *Bam*HI and *Stu*I. The resultant subclone (pSL1180*L*ψ) was then digested with *Pvu*II and *Sma*I and ligated into *Sma*I-linearized pCMVneo. The *A*ψ fragment was amplified from pSL1180*L*ψ by polymerase chain reaction with the 5′*A*ψ oligonucleotide primer, CGAGTCGACGGGAATAGTGG TC, and the 3′*A*ψ primer, ATCAAGCTTATGACGGCT TCC. The 3′*A*ψ primer introduces a mutation in the retroviral splice donor site that creates a *Hin*dIII restriction endonuclease site (underlined). The amplified fragment was isolated and digested with *Hin*dIII and *Hin*cII; the ends were repaired with Klenow polymerase and ligated into pCMVneo linearized with *Sma*I. pCMVneo5′*S*ψ and pCMVneo3′*S*ψ plasmids were constructed by digesting the *L*ψ fragment with Ecl136II (an isoschizomer of *Sac*I) and ligating the 5′ or 3′ fragment into the *Sma*I site of pCMVneo. pVZ*L*ψ was constructed for generation of RNase protection probes by inserting the *Nsi*I-*Bam*HI fragment from pSL1180*L*ψ into the *Pst*I-*Bam*HI-digested pVZ1 (pBluescribe [Stratagene, La Jolla, Calif.] containing additional restriction enzyme sites in the polylinker [20]). When pVZ*L*ψ is digested with *Eco*RI and transcribed by T3 polymerase, a sense *L*ψ RNA is generated; when pVZ*L*ψ is digested with *Hin*dIII and transcribed by T7 polymerase, an antisense RNA is generated. The RSV PR active site mutant (p*gag.neo*.D37N) and deletion (p*gag.neo*.ΔPR) constructs were kindly provided by L. Stewart and V. Vogt (45). D37N also contains a second mutation in PR (D→Y at amino acid 41 [47a]). These plasmids express only the appropriately mutated retroviral *gag* gene (not *pol* or *env*) and a *neo* gene (that is expressed from a spliced mRNA). The RSV NC mutant proviral construct, pAPrCdel1,2, deleted for both Cys-His boxes, was kindly provided by P.-F. Spahr (35; the detailed construction is given in this reference).

RNA preparations. Viral supernatants were collected from virus-producing cell line cultures every 12 h and clarified of cell debris by low-speed centrifugation (2,500 rpm for 10 min) in an American Scientific Products Omnifuge. Virions were pelleted through a 2-ml 20% sucrose cushion in isotonic buffer (SB) (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) by centrifugation at 23,000 rpm for 2 h in a Beckman SW28 rotor. Viral pellets were resuspended in 100 μl of SB. The protein concentration was determined by Bradford analysis of resuspended pellets (Bio-Rad, Richmond, Calif.). Virions were lysed in 0.5% sodium dodecyl sulfate (SDS), and RNA was extracted by using two or three phenol-chloroform extractions followed by a chloroform extraction and ethanol precipitation. In some experiments, virion preparations were incubated in the presence of 0.5% SDS and 200 μg of proteinase K (VWR Scientific) per ml for 30 min at 37°C prior to the phenol-chloroform extractions. Viral RNA pellets were resuspended so that each microliter of RNA was equivalent to 1 ml of original viral supernatant. Total cellular RNA was extracted by the guanidinium isothiocyanate method of Chomczynski and Sacchi (11) in 0.5 ml of solution D per 10-cm^2 plate of tissue culture cells. RNA integrity was monitored by ensuring the presence of distinct rRNA bands after ethidium bromide staining of total cellular RNA electrophoresed in 0.8% agarose minigels.

RNA analysis. Methods for primer extension and RNase protection have been previously described (4, 28). The sequence of the *neo*-specific oligonucleotide (*neo* 22-mer) primer is (5′-3′) GCAGCCCTTGCGCCCTGAGTGC. Samples were resuspended in loading dye and electrophoresed through 8% (primer extensions) or 6% (RNase protections) polyacrylamide gels containing 8 M urea.

Protein analysis. SDS-polyacrylamide gel electrophoresis and techniques for cell labelling and immunoprecipitation have been previously described (41). After gel electrophoresis, proteins were electroblotted onto nitrocellulose or Immobilon P (Millipore) with a Semi-phor apparatus (Hoefer Scientific Instruments). Western immunoblots were performed with one of the following primary antibodies (Abs); rabbit αRSV Pr-C (used at a dilution of 1:500) and monoclonal Abs αCA and αMA (used at a dilution of 1:1,000) obtained from V. Vogt (Cornell University). To prevent nonspecific Ab binding, filters were incubated for 2 h in phosphate-buffered saline–0.5% Tween–5% dried milk. The filters were then incubated with the primary Ab diluted in phosphate-buffered saline–0.5% Tween–5% dried milk for 1 h at room temperature with shaking and then washed for 10 min three times with the same buffer. Secondary Abs (Amersham, Arlington Heights, Ill.), either horseradish peroxidase-conjugated donkey α-rabbit Abs (1:6,000) for rabbit-derived antisera or horseradish peroxidase-conjugated α-mouse Abs (1:2,000) for reaction with monoclonal Abs, were incubated with the filter in phosphate-buffered saline–0.5% Tween–5% dried milk for 1 h at room temperature. Filters were washed as described above, and signal was detected by using the enhanced chemiluminescence system (Amersham).

Electron microscopy. Culture supernatants were clarified of cell debris by low-speed centrifugation, and viruses were

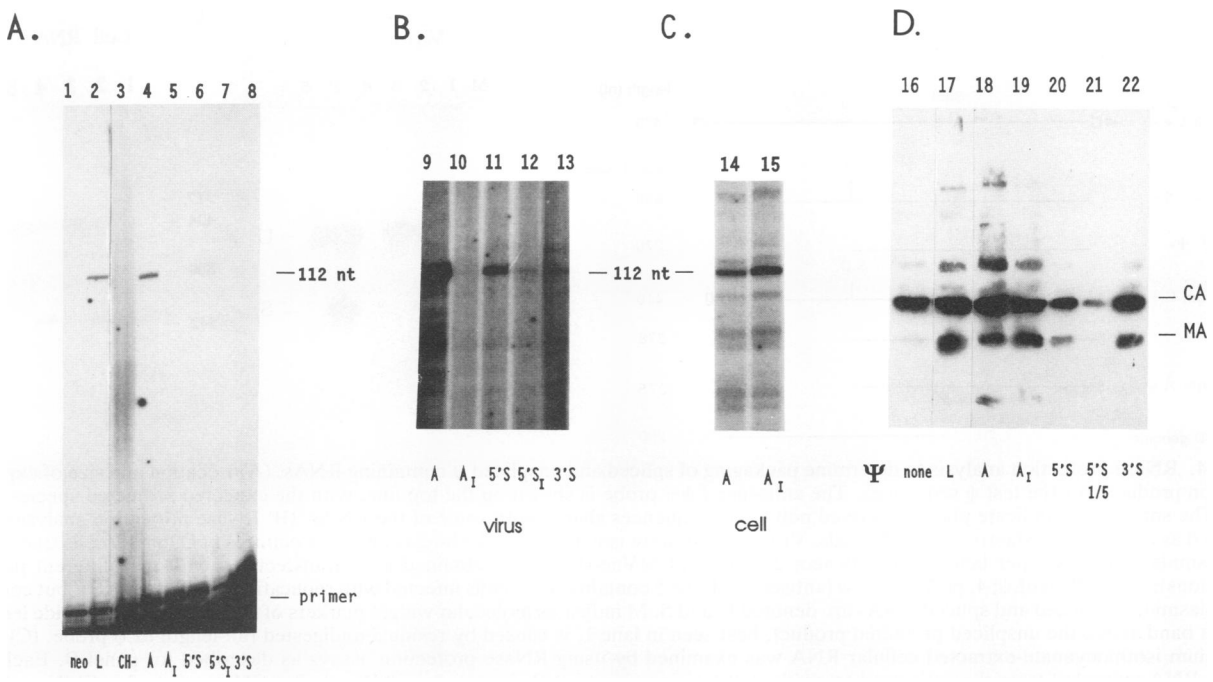


FIG. 3. Primer extension analysis of viral RNAs containing neoψ sequences. (A) RNAs were extracted from virion RNAs, and primer extension analysis with the *neo* 22-mer oligonucleotide was performed as described in Materials and Methods. Equivalent amounts of viral supernatants were loaded in each lane. Q2bn-4D cells were transfected with pCMVneo with the indicated test ψ sequence. Lanes: 1, none (neo); 2, *L*ψ; 3, NC mutant del1,2; 4, *A*ψ; 5, *A*ψ$_I$ (antisense or inverted); 6, 5′*S*ψ; 7, 5′*S*$_I$; 8, 3′*S*ψ. The gel was exposed for 18 h with two intensifying screens. (B) Exposure of the gel containing lanes 4 to 8 (panel A) for 3 days with intensifying screens; samples were as indicated for panel A. (C) Cellular RNA extracted from pCMVneoαAψ constructs with *A*ψ in the sense (lane 14) or antisense (lane 15) orientation. (D) Western blot analysis of virions produced by G418-resistant mass cultures. Test ψ sequences transfected into each Q2bn-4D line are listed below each lane. The bands corresponding to the Gag proteins CA (27 kDa) and MA (19 kDa) are indicated. Lane 21 contains one-fifth the amount of sample that lane 20 contains.

pelleted through a 20% sucrose cushion. The viral pellets were fixed in a 1:1 dilution of SB and half-strength Karnovsky's fixative (22) for 4 to 6 h and then dehydrated in a graded series of ethanols. The pellet was then infiltrated with a mixture of propylene oxide and Luft's Epon 812 (30), with gradual increasing of the proportion of Epon 812 to 100%. After being infiltrated overnight, the pellet was embedded in Epon 812 and polymerized in a 60°C oven for 48 h. Sections were cut at a thickness of 70 to 80 nm, stained with uranyl acetate and lead tartrate, and examined with a JEOL 100SX transmission electron microscope at 80 kV.

RESULTS

Packaging of heterologous RNA containing avian retroviral ψ sequences. We previously found that a 683-base sequence called ψ+ can direct specific encapsidation of hygromycin (*hph*) RNA into the ALV virion when inserted 3′ of the coding sequences (4). In order to further define functional ψ domains, sequences contained within ψ+ were tested in a similar heterologous system. These ψ test fragments were inserted at the 3′ end of pCMVneo in sense and antisense (inverted) orientations (Fig. 1A). The sequences of the *A*ψ, *L*ψ, 5′*S*ψ, and 3′*S*ψ test fragments relative to that of the previously defined ψ+ are shown in Fig. 1B. *L*ψ lacks two retroviral *cis*-acting elements, the primer binding site (PBS) and a sequence postulated to be involved in genomic RNA dimerization, the dimer linkage sequence (dls) (8, 12). The *A*ψ sequence also lacks the PBS but ends at the major 5′ splice site, which was mutated during the cloning procedure so that it is no longer functional (Fig. 1B). Deletion analysis had previously shown the region surrounding the *Sac*I site in the avian leader region to be important for RNA packaging (25). Cleavage of *L*ψ at the *Sac*I site creates the 5′*S*ψ and 3′*S*ψ test fragments (Fig. 1B). The pCMVneo vectors containing each of these ψ test sequences were transfected into the Rous-associated virus type 1-derived packaging cell line Q2bn-4D (46). Virions produced by the cell lines were then assayed for the presence of heterologous *neo* RNA.

We first directly compared the packaging mediated by ψ+ to that directed by the longest test fragment, *L*ψ. As shown in Fig. 2, packaging directed by *L*ψ in the sense orientation (lane 2) is equivalent to that directed by ψ+ (lane 1), whereas in the antisense orientation (α*L*ψ; lane 3), this packaging is decreased by more than 100-fold. Because there is no difference between *L*ψ- and ψ+-directed packaging, we conclude that neither the PBS nor the dls contributes to specific encapsidation.

We next examined the ability of all of the test fragments to direct RNA packaging by using primer extension analyses (Fig. 3A). Both *L*ψ and *A*ψ sequences direct equivalent levels of packaging of the *neo* RNA (compare lanes 2 and 4). The 5′*S*ψ retains some ability to induce packaging (lane 6). This is more clearly seen in a longer exposure of this gel (Fig. 3B, lane 11). The 3′*S*ψ sequence does not direct specific encapsidation of the heterologous mRNA (Fig. 3A, lanes 8 and 13), nor do any of the inverted ψ sequences ([denoted by subscript I] lanes 5, 7, 10, and 12) relative to *A*ψ (lane 9). *neo* mRNA is present in all cell cultures at similar levels (Fig. 3C,

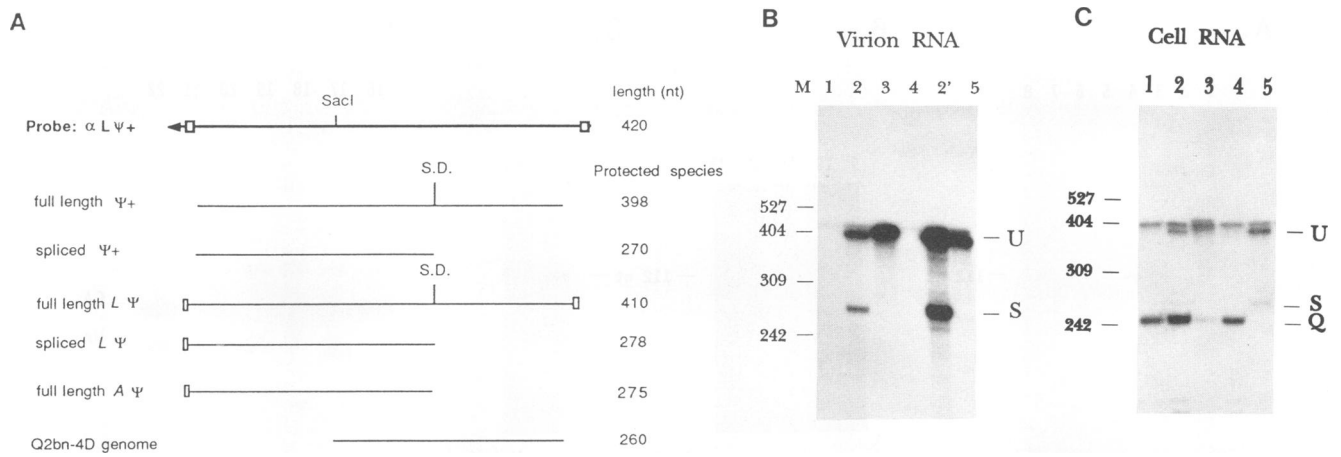


FIG. 4. RNase protection analysis to determine packaging of spliced and unspliced ψ-containing RNAs. (A) Location and size of expected protection products for the test ψ sequences. The antisense *L*ψ+ probe is shown on the top line, with the expected protected species listed below. The small boxes indicate plasmid-derived polylinker sequences shared with some of the RNAs. (B) RNase protection analyses were performed as described in Materials and Methods. Virion RNAs were isolated from G418-resistant mass cultures of Q2bn-4D cells transfected with plasmids as follows (per lane): 1, pCMVneo; 2 and 2′, pCMVneoψ+ (virus obtained after transfections with two different plasmid preparations); 3, pCMVneo*L*ψ; 4, pCMVneo*L*ψ (antisense). Lane 5 contains QT35 cells infected with replication-competent ALV but contains no *neo* plasmid. Unspliced and spliced RNAs are denoted U and S. M indicates molecular weight markers of the indicated nucleotide lengths. The faint band above the unspliced protected product, best seen in lane 1, is caused by residual undigested full-length α*L*ψ probe. (C) Total guanidinium isothiocyanate-extracted cellular RNA was examined by using RNase protection assays as described for panel B. Each lane contains RNA extracted from the cells used to produce the virions in panel B. Lanes: 1, pCMVneo; 2, pCMVneoψ+; 3, pCMVneo*L*ψ; 4, pCMVneo*L*ψ (antisense); 5, QT35 cells infected with ALV. The location of the Q2bn-4D packaging vector band is denoted (Q). There is no sequence mismatch between the probe and this vector.

lanes 14 and 15 for *A*ψ and *A*ψ$_I$, and data not shown), regardless of the orientation of the test ψ insert. As a measure of viral particle production, the level of viral proteins was examined by Western blot analysis. Similar levels of particles (within threefold) are produced by each Q2bn-4D culture (Fig. 3D). When the primer extension experiment shown in Fig. 3 was quantitated by densitometry, after correction for the amount of RNA loaded, it was found that *A*ψ directs encapsidation that is greater than 100-fold-more efficient than the level seen with no *A*ψ sequence or with *A*ψ$_I$. Although 5′*S*ψ directs some encapsidation of *neo* RNA, the level is increased only by about 5- to 10-fold above background (either *neo* alone or *neo* containing inverted ψ sequences).

Spliced RNA is efficiently packaged in the heterologous vector system. Although both genomic and spliced subgenomic avian retroviral RNAs contain ψ sequences, only genomic RNA is efficiently encapsidated. Because the *L*ψ and ψ+ sequences contain the retroviral 5′ splice site and the simian virus 40 (SV40) sequences included in the heterologous vector contain a 3′ splice site (Fig. 1A), the *neo* plasmids could be used to determine whether splicing affects packaging of heterologous RNAs. We previously detected several different ψ+ RNA bands by Northern (RNA) blot analysis, indicating that the splice sites are used in the pCMVneoψ+ construct (data not shown). In order to determine whether spliced heterologous RNA is packaged into virions, an antisense *L*ψ probe was used for RNase protection analysis with cell and virion RNA from Q2bn-4D cell lines transfected with *neo*ψ vectors.

Figure 4A depicts the RNase-protected products expected for each ψ-containing RNA species. The results of the RNase protection assays with virion RNAs (Fig. 4B) and cellular RNAs (Fig. 4C) are shown. Control cells were transfected with plasmid encoding ALV. As expected for wild-type virus, both unspliced and spliced ALV RNA is detected in cell RNA (Fig. 4C, lane 5) but only unspliced RNA can be readily detected in virion RNA (Fig. 4B, lane 5). We found that the retroviral splice site is used in the case of the pCMVneoψ+ construct but not the *L*ψ construct (Fig. 4C, lanes 2 and 3). Both the spliced and unspliced ψ+-containing pCMVneoψ+ RNA species are efficiently encapsidated into virions (Fig. 4B, lanes 2 and 2′). Since the identical splice sites are not used in transcripts encoded by the pCMVneo*L*ψ construct, no spliced product is detected in virions (Fig. 4B, lane 3). In the case of the *L*ψ protected species, the unspliced RNA was difficult to separate from the free probe on this gel, but these could be distinguished by using gradient gels (data not shown). The RNA encoded by the Q2bn-4D packaging cell vector can clearly be seen in all of the Q2bn cell RNAs (band Q in Fig. 4C, lanes 1 to 4), but this ψ-deleted RNA is packaged very inefficiently (Fig. 4B, lanes 1 to 4).

A comparison of the ratio of unspliced to spliced (U/S) RNAs in the cell and virion RNAs provides an estimate of the efficiency of encapsidation of each RNA. In the case of pCMVneoψ+, the total intracellular U/S ratio is 8.3 (Fig. 4C, lane 2), while in two different virion RNA preparations, the U/S ratios are 2.9 and 1.4 (Fig. 4B, lanes 2 and 2′). These numbers are not corrected for the difference in the number of radioactive residues protected by each RNA species and thus overrepresent unspliced RNA. We conclude that spliced *neo* RNAs are not excluded from the virion, unlike the case for the spliced ALV *env* mRNA (which is the same size as the spliced vector products; Fig. 4B and C, lanes 5). The ratio of U/S ALV RNAs in the cell is 9.8; however, in the virus it is more than 65 (at the limit of quantitation of this gel). As the 270-base *A*ψ lacking a splice donor site is packaged, these results suggest that sequences which are 3′

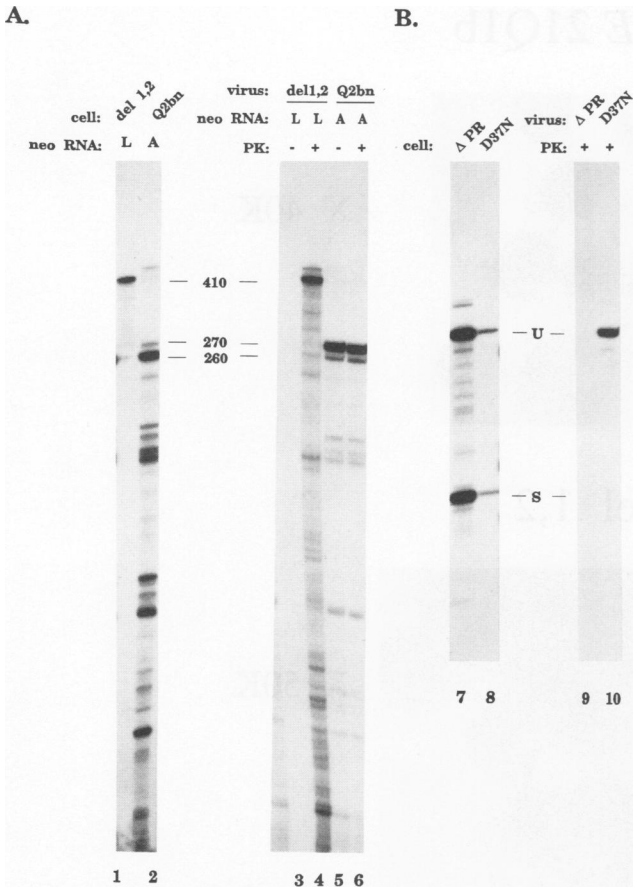


FIG. 5. Effect of proteinase K treatment on virion RNAs detected by using RNase protection. (A) RNA was extracted as described in Materials and Methods from cells containing the del1,2 provirus and transfected with pCMVneo$L\psi$ (lane 1) or from Q2bn-4D cells transfected with pCMVneo$A\psi$ (lane 2). Virus was isolated from these cultures, and RNA was extracted either after digestion (lanes 4 and 6) with 200 μg of proteinase K per ml or with no addition of proteinase K (lanes 3 and 5). Virus was from del1,2 cells (lanes 3 and 4) and Q2bn-4D cells (lanes 5 and 6). RNA extracted from equivalent volumes of viral supernatant were loaded in each lane. (B) RNA was extracted from cells containing the ΔPR provirus (lane 7) or the PR active site point mutant provirus D37N (lane 8). Virion RNA was extracted after proteinase K treatment (lanes 9 and 10).

of the splice site and the splicing process itself are dispensable for specific RNA encapsidation.

Because the antisense $L\psi$ probe is also protected by the ψ-deleted RNA derived from the Q2bn-4D packaging line vector, it was possible to directly compare packaging of RNAs containing and lacking ψ in the same virion population. The ratio of the Q2bn-4D vector-derived RNA to unspliced *neo*ψ+ RNA in the cell is 6.4 (Fig. 4C, lane 2), while this ratio is 0.007 in the virion (Fig. 4B, lanes 2 and 2'; the Q2bn-4D-protected product of 260 nt is barely detectable at this exposure of the gel). Thus, packaging of the heterologous ψ-containing RNA is about 1,000-fold more efficient than that of the vector RNA lacking ψ.

NC Cys-His motifs are not required for RNA packaging. An intact Gag polyprotein precursor appears to be required for avian retroviral genomic RNA encapsidation (38), although processing of the precursor is not required (44). It has been suggested that the NC Cys-His motifs are an essential component of the packaging machinery, as deletion of both of the Cys-His motifs from the NC protein of RSV (del1,2) leads to production of virions in which RNA cannot be detected by Northern blot analysis (35). We confirmed this result by using denaturing Northern blots (data not shown). However, Northern blot analysis will detect only intact RNA molecules and is thus not a very sensitive assay. During the course of these studies, we obtained evidence that RNA was present in del1,2 viral particles, albeit in a degraded form. Primer extension analysis with virion RNA obtained from cells containing both a del1,2 provirus and pCMVneo$L\psi$ sometimes showed a smear of *neo* extension products, possibly indicative of degraded RNA (for example, see Fig. 3A, lane 3). In this experiment, only the del1,2 RNA yielded this result, although all virion RNAs were prepared at the same time and with the same reagents. In other experiments, we could sometimes detect a faint band of the expected size (data not shown).

This prompted us to reexamine whether ψ-containing RNA is packaged into the NC del1,2 virions by using RNase protection assays and a variation of our standard virion RNA extraction protocol. Intact virion RNA is readily obtained from wild-type virions by using phenol-chloroform extraction in the presence of 0.5% SDS. RNA can also be extracted from virion suspensions after digestion with proteinase K in the presence of 0.5% SDS (38, 44), although we have not found this treatment to affect RNA integrity when using wild-type virus. We reasoned, however, that if RNA in the del1,2 particles was more susceptible to RNase degradation, a proteinase K treatment might inhibit such nuclease activity. To examine whether proteinase K treatment would allow detection of del1,2 virion RNA, RNase protection assays were performed with the α$L\psi$ probe and RNA obtained from virions which had been extracted either by our usual method or after proteinase K treatment (Fig. 5). We compared cellular and virion RNAs from del1,2 cells transfected with pCMVneo$L\psi$ and Q2bn-4D cells transfected with pCMV neo$A\psi$. When total cellular RNA from del1,2 cells transfected with pCMVneo$L\psi$ was analyzed, the expected $L\psi$ band at 410 nt (Fig. 4A) is readily detectable (Fig. 5A, lane 1). A protected species of 270 nt is expected from hybridization of the α$L\psi$ probe to $A\psi$ RNA (Fig. 4A), and both the 270-base band and the Q2bn-4D ψ-deleted provirus band at 260 nt are evident in the Q2bn-4D cellular RNA (Fig. 5A, lane 2). $A\psi$ RNA, as expected, is enriched in virion RNA relative to the ψ-deleted packaging vector RNA, although the apparent ratio is lessened by the long exposure of the gel to film. Furthermore, proteinase K treatment has no effect on the amount of $A\psi$ or Q2bn-4D RNA detectable (lanes 5 and 6). In contrast, proteinase K treatment has a profound effect on the amount of RNA detectable in the del1,2 virions (lanes 3 and 4). In addition to the major protected species at 410 bases, there are many shorter protected species which are not found in the cellular RNA (compare lanes 1 and 4). In contrast, most of the shorter species found in Q2bn-4D virions are attributable to similar species in the cellular RNA (lanes 2, 5, and 6). These results are consistent with the presence of partially degraded RNA in the del1,2 virions. In this experiment, RNAs extracted from equal volumes of cell supernatant were loaded in each lane. The del1,2 QT35 culture produces about 40% of the number of particles produced by the Q2bn-4D cultures as detected by Western blot analysis (data not shown).

We also examined the PR mutant ΔPR and D37N virions

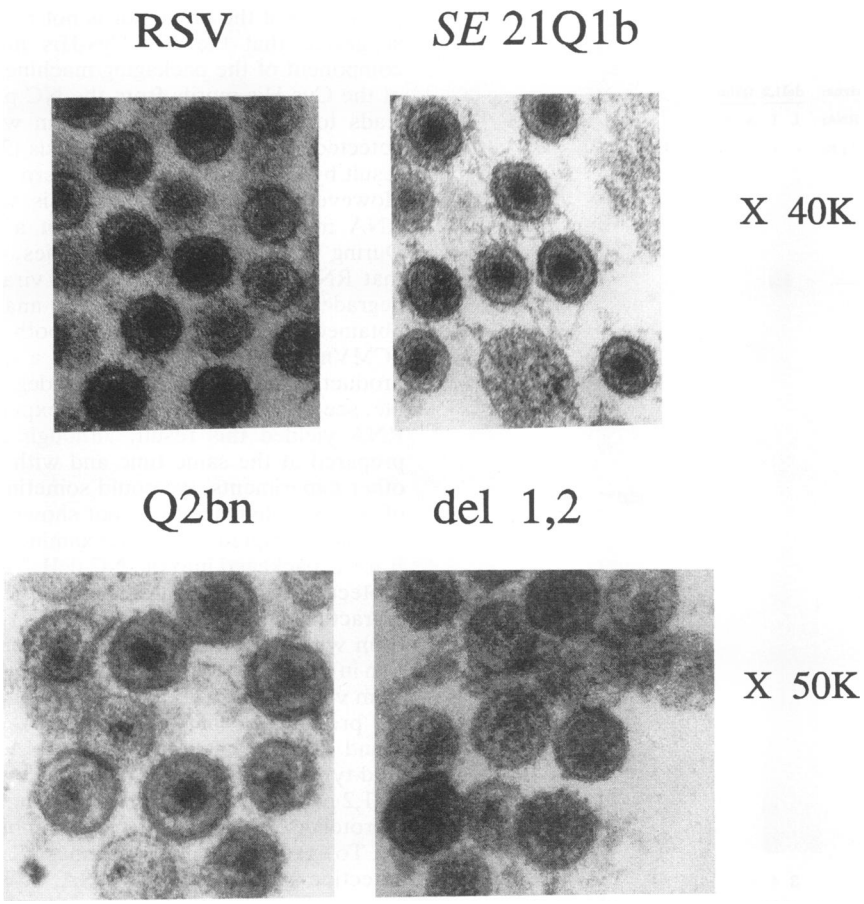


FIG. 6. Analysis of pelleted virions by electron microscopy. Preparation was as described in Materials and Methods. Magnifications: ×40,000 (top panels) and ×50,000 (bottom panels).

in the presence of proteinase K as controls. It has been shown by using assays other than RNase protection that ΔPR, which has a Gag polyprotein deleted for the PR domain, does not package RNA (38), while the PR active site point mutant D37N virions do contain wild-type levels of RNA (44). The expected results were obtained in both cases. The active site mutant D37N packages wild-type levels of unspliced genomic RNA but not spliced RNA (Fig. 5B, lanes 8 and 10). ΔPR virions have a severe packaging defect (Fig. 5B, lanes 7 and 9). The fact that no RNA was detectable by our techniques with the ΔPR virions further validates the fact that RNA is encapsidated into del1,2 particles by Gag lacking Cys-His domains. If this RNA was merely associated with the outside of the virions, it would have been detectable in all viral preparations.

We conclude from these studies that del1,2 virions retain the ability to encapsidate RNA. This does not contradict the previous conclusion that del1,2 virions do not contain intact virion RNA (35). However, it demonstrates that other domains of the Gag precursor, not the Cys-His boxes of the NC domain, are required for specific ψ recognition and RNA encapsidation.

Morphology of del1,2 and packaging cell line virions. Virions produced by packaging cell lines generally contain RNA, even in the absence of a packageable retroviral vector (4). Our studies with del1,2 led us to postulate that encapsidated RNA in this mutant is susceptible to degradation because mature NC binding is disrupted by the Cys-His box deletion. We wished to see whether this difference might be reflected in an altered del1,2 virion ultrastructural morphology. In addition, we wished to determine whether the presence of ψ-containing RNA might contribute to the formation of the well-characterized morphology of the mature type C virus (for example, see reference 39). We chose three viruses to analyze in order to address this second question. Wild-type RSV encapsidates abundant genomic RNA containing ψ sequences. Q2bn-4D virions contain little or no ψ-deleted RNA, derived from the packaging vector, and a subset of cellular RNAs such as glyceraldehyde 3-phosphate dehydrogenase (4). *SE*21Q1b is a packaging mutant which encapsidates cellular mRNAs, including the ψ-deleted mutant genome, randomly (3, 4, 42). Pelleted virions from wild-type RSV, Q2bn-4D, *SE*21Q1b, and del1,2 cells were analyzed by electron microscopy (Fig. 6). We found that virions released by the Q2bn-4D packaging cell line and cells containing the *SE*21Q1b mutant are indistinguishable from wild-type RSV and contain an electron-dense mature viral core. In contrast, the del1,2 virions have a diffuse central core, with a morphology resembling neither mature nor immature C-type particles. The virion proteins are processed in this mutant (35 and data not shown), so an immature morphology was not expected. However, the lack of a wild-type, hexagonal, and condensed mature core most likely reflects the inability

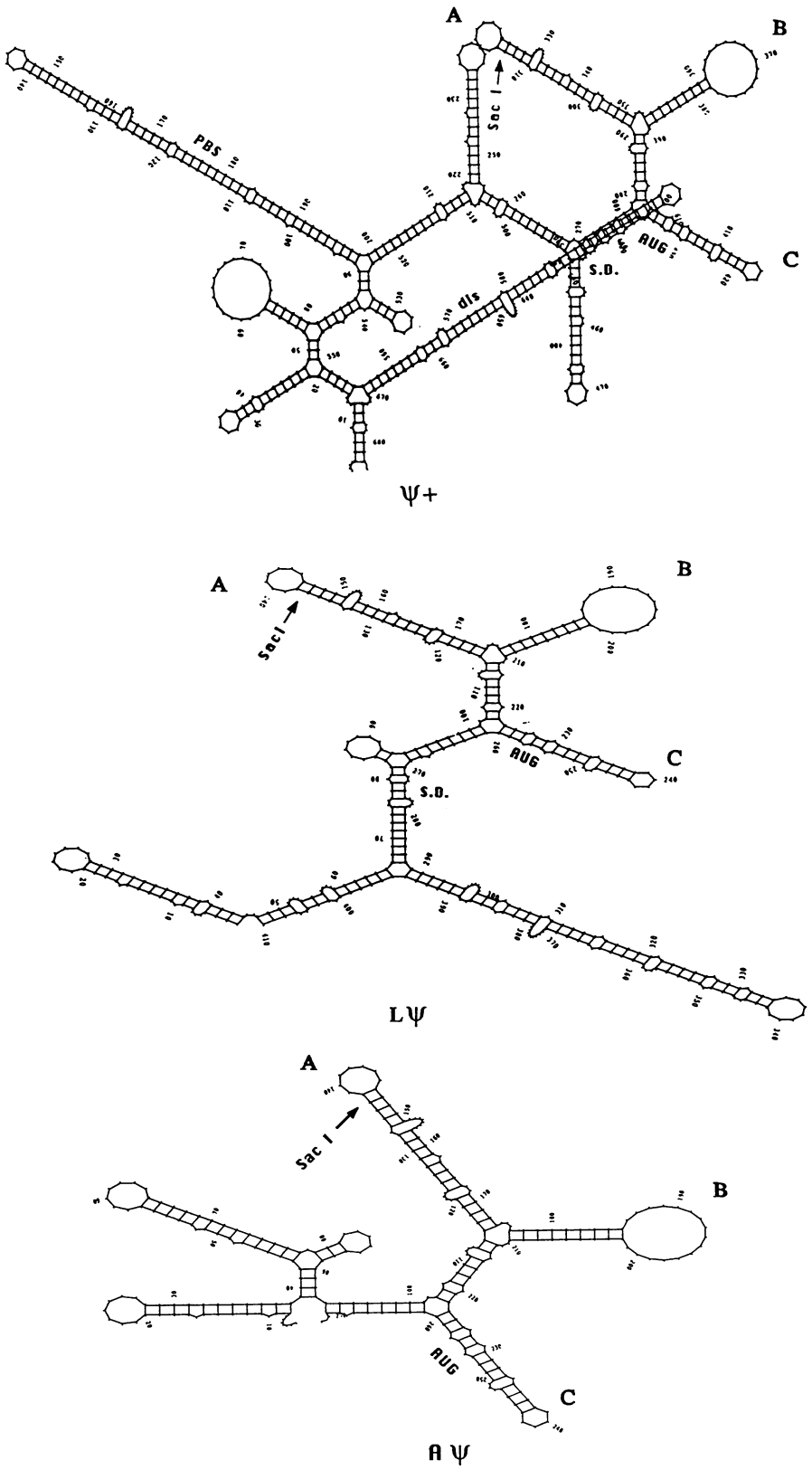


FIG. 7. Predicted secondary structures of ψ+, *L*ψ, and *A*ψ. The Genetics Computer Group (University of Wisconsin) (14) RNA folding program, FOLD, based on the computer algorithm of Zucker (49), and the graphics output program SQUIGGLES were used. Folding energies obtained (in tenths of kilocalories [1 cal = 4.184 J]) were as follows: for ψ+, −204.0; for *L*ψ, −134.3; and for *A*ψ, −90.9.

of del1,2 mutant NC protein to bind with high affinity to the packaged RNA.

DISCUSSION

In this study, we have examined the *cis*- and *trans*-acting determinants of RNA encapsidation into the avian retroviral virion. We show that the functional ALV 5′ ψ sequence is 270 nt or less in length, that spliced RNA can be encapsidated efficiently into retroviral particles, and that NC Cys-His boxes are not required for the initial Gag-ψ RNA interaction which brings specific ψ-linked RNA into the particle.

Regions of the *cis*-acting packaging domain, ψ, from the 5′ end of the retroviral genome were tested in a heterologous packaging assay. A 270-nt sequence, *A*ψ, was found to be sufficient to direct encapsidation of heterologous *neo* RNA into virions produced by an ALV-derived packaging cell line. Neither *A*ψ nor any of the other avian retroviral sequences tested can direct packaging that is as efficient as that of a complete retroviral vector RNA, possibly because of the lack of the 3′ DR sequence. However, we have not been able to show that the addition of DR sequences can either induce packaging when placed 3′ of a heterologous mRNA or augment packaging of heterologous ψ-containing RNA (4a). These data thus show that the ψ sequence is necessary for packaging in a retroviral context and sufficient for packaging in a heterologous context. However, it should be noted that ψ sequences defined as sufficient in a heterologous context may be different from those defined by deletions within genomic RNA, perhaps because of differences in RNA folding constraints. For example, although MLV sequences in U5 are not required for packaging of heterologous RNA (1), deletions in U5 can affect encapsidation of genomic RNA (37).

The *A*ψ sequence defined in the heterologous assay lacks the retroviral splice donor site, the putative dimer linkage sequence, and the PBS. Thus, none of these *cis*-acting retroviral elements is required for specific RNA encapsidation. In addition, the sequences present in the 398-base *L*ψ, which lacks both the PBS and the dls, are sufficient to allow detection of dimers and higher-order complexes in nondenaturing gel systems (4a). Thus, the dls is unlikely to be the sole determinant of RNA multimerization, an event which probably occurs only after proteolytic maturation of Gag (40), subsequent to the interactions determining specific RNA encapsidation.

What is required for sequences to have ψ function in a heterologous context? It is generally believed that RNA folds into a particular structural configuration to allow protein recognition (33). We have shown that ψ sequences can function when placed next to both *hph* and *neo*, and it is reasonable to assume that the functional ψ folds independently of the linked heterologous sequences. In order to analyze potential secondary structures that are conserved in the functional ψ sequences identified in this work, the Genetics Computer Group (University of Wisconsin) FOLD program (14), based on the Zucker algorithm (49), was used to fold sense, antisense, and scrambled ψ sequences. A variety of structures resulted, and the most stable structures obtained for sense ψ+, *L*ψ, and *A*ψ are shown in Fig. 7. Each contains three identical stem loops, denoted A, B, C, which could constitute a core packaging domain. Inverse and scrambled ψ sequences resulted in quite different predicted secondary structures which do not contain A, B, or C (data not shown). Similar computer modeling coupled with direct biochemical assays have defined potential ψ structures for human immunodeficiency virus and MLV (2, 19). However, it has not been determined whether the human immunodeficiency virus and MLV sequences analyzed actually function biologically in a heterologous context.

We have found that splicing does not affect the ability of a heterologous ψ RNA to be encapsidated. When the splice donor site is specifically mutated in *A*ψ, packaging is not affected. Furthermore, when *neo*ψ+ RNA is spliced to the SV40 splice acceptor, the level of packaging of this species is equivalent to that of the unspliced RNA. Swain and Coffin have also demonstrated efficient packaging of spliced ALV readthrough RNA (47). The packaged spliced mRNA was generated by using the *gag* 5′ splice site and the SV40 3′ splice site so that ψ was at the 5′ end of the RNA (47). In contrast, retroviral spliced subgenomic RNAs are not efficiently encapsidated. Together, these results show that the splice site is not required for packaging and that some feature other than an interaction with the splicing machinery distinguishes unspliced and spliced retroviral RNAs. The lack of packaging of viral *env* mRNA could be caused by a negative effect of juxtaposition of ψ and *env* sequences in spliced RNAs, or even long-distance interactions with DR or U3 regions which prevent proper folding. An analysis of packaging directed by heterologous constructs in which the 3′ splice site and *env* sequences of an avian retrovirus are inserted in place of the SV40 pA sequences of pCMVneoψ+ could address this issue. Alternatively, sequences within *gag* or *pol* could interact positively with 5′ ψ and 3′ DR sequences in genomic RNA so that, when these sequences are spliced from the transcript, efficient packaging no longer occurs. However, such positive elements have not been identified in avian viruses.

Suggestions have been made that Gag recognition of ψ involves the NC Cys-His domains (15, 17, 18, 35). Studies with chimeric viruses have reaffirmed the importance of the NC domain in ψ recognition (16). Our results with the RSV mutant lacking both NC Cys-His boxes strongly suggest that the primary RNA-protein interaction determining packaging specificity is genetically separable from the ability to form mature particles containing intact genomic RNA. By using RNase protection assays, we found that ψ-containing RNA is encapsidated into particles produced by cells containing the del1,2 provirus but that it is extremely susceptible to nucleolytic degradation. This is also reflected in the morphology of the del1,2 particles as seen by electron microscopy. The structure of the diffuse core seen in del1,2 particles is consistent with the idea that packaged RNA is readily accessible to nucleases and thus difficult to detect in virion RNA preparations. Nucleolytic degradation of encapsidated RNA could begin immediately after virion budding. Nevertheless, in the absence of Cys-His boxes the Gag polyprotein is capable of specifically recognizing and packaging ψ-containing RNA. The Cys-His boxes are required for binding of mature NC to RNA, an interaction important for complete virion maturation, protection of the encapsidated RNA from degradation, and subsequent infectivity. However, it is not known whether the protein domain encompassing the Cys-His boxes directly binds RNA or whether it mediates another interaction, perhaps protein multimerization, which is indirectly responsible for RNA binding and mature virion core condensation. Recent work with bovine leukosis virus suggests that the MA domain may play a role in specific RNA encapsidation (23). Combined with a requirement for the PR domain of ALV Gag, it is likely that the

interaction of Gag and ψ is complex and involves more than one protein domain.

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