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Specificity of Retroviral RNA Packaging

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Encapsidation of retroviral RNA has been shown to be dependent on specific *cis*-acting signals, in particular, the packaging region (ψ) located near the 5′ end of the retroviral genome. In this report, we show that a 683-base avian extended packaging sequence (ψ^+) derived from Rous sarcoma virus will direct packaging of heterologous hygromycin mRNA into avian virions when present at the 3′ end of the transcript in the sense orientation. However, this packaging is not as efficient as the packaging of RNA encoded by a standard avian retroviral vector. A quail cell line containing a Rous sarcoma virus mutant, *SE*21Q1b, produces virions which will package endogenous cellular mRNAs randomly, roughly in proportion to their intracellular concentrations. We found that viral particles from *SE*21Q1b retain the capacity to specifically encapsidate hygromycin mRNAs containing the avian ψ^+. To determine whether packaging of cellular mRNA would occur in other retroviral packaging lines, we assayed virion RNA isolated from the retroviral particles produced by avian and murine packaging lines for the presence of endogenous cellular mRNAs. Endogenous cellular mRNAs were not found randomly packaged into virions produced by any of the packaging lines examined except *SE*21Q1b. Some specific sequences, however, were found packaged into avian virions. Endogenous retrovirus-related mink cell focus-inducing murine leukemia virus RNAs and 30S viruslike RNAs were found to be efficiently packaged into murine virions even in the presence of RNAs containing all *cis*-acting retroviral sequences.

Replication-defective retroviral vectors are widely used to introduce genes of interest into either tissue culture cells or organisms. These vectors are usually transfected into retroviral packaging lines, which provide the *trans*-acting functions for production of infectious virions from their own defective proviral sequences. Virions produced by the packaging line, carrying the gene of interest in the context of the retroviral *cis*-acting sequences (i.e., long terminal repeats), are then used to infect target cells. The development of helper virus-free viral packaging cell lines has been a focus of many investigations (28, 30, 41, 44). These cell lines are designed to minimize the chance of recombination leading to the production of replication-competent helper viruses. Such recombinants are undesirable not only because the spread of replication-competent virions could complicate interpretation of data but also because viral interference mediated by these particles could block the entry or integration of the viral vector being studied. Since some of these packaging lines have been proposed as efficient vector systems for human gene therapy (29), concerns exist about packaging and transfer of sequences other than those under study.

Packaging signals (ψ) are the *cis*-acting sequences that are required for the packaging of viral RNA into the retroviral virion. These signals were first identified by the analysis of a quail cell line containing a spontaneous Rous sarcoma virus (RSV) mutant, *SE*21Q1b (24), which has deleted 180 bp from the 5′ end of its proviral genome (2a, 37). Virions produced by this cell line do not contain wild-type (wt) levels of genomic RNA. Analyses of this and other avian and mammalian retroviral mutants and of heterologous vectors containing various amounts of viral sequence have localized packaging signals primarily to the 5′ end of the viral genome (17, 19, 28, 43). The murine leukemia virus (MLV) and spleen necrosis virus (SNV) ψ is located between splice sites and is therefore not contained in the subgenomic *env* mRNA (Fig. 1A). However, in avian leukosis viruses, this 5′ signal is included in both the genomic and the spliced *env* mRNAs. Other signals, in particular the 115-nucleotide direct repeats (DR) bordering the *src* gene of RSV, have been implicated in the encapsidation of avian retroviral RNA into virions (40). However, because these DRs are present in both spliced subgenomic and unspliced genomic RNAs, other signals may be important for the distinction between these two RNAs in avian retroviral packaging. Sequences within the *gag* gene have also been shown to enhance packaging of RNA into MLV virions (3). The extended packaging signal containing these sequences is termed ψ^+. Some of the *cis*-acting sequences identified in the retroviral genome are diagrammed in Fig. 1A.

It has been shown that the MLV ψ sequence is orientation dependent but relatively position independent (27). An ~800-base MLV ψ^+ has been shown to be sufficient for efficient packaging of heterologous *neo* transcripts when placed at the 3′ end in the correct orientation. Adam and Miller (1) showed that this packaging occurs at a rate equivalent to the packaging of both wt MLV and an MLV-based retroviral vector (pLNL6) containing all defined murine retroviral *cis*-acting sequences. In the present study, we show that an avian extended packaging signal from the 5′ end of the avian retroviral genome can also direct efficient encapsidation of a heterologous mRNA into virions but that such packaging is not equivalent to that of RNA encoded by a standard avian leukosis virus-derived retroviral vector.

One retrovirus mutant is known to efficiently package RNA lacking packaging signals. Viral particles produced by the quail cell line *SE*21Q1b contain cellular mRNAs roughly in proportion to their intracellular concentrations (reference 12 and unpublished observations), a phenomenon we term random packaging. In addition to the 5′ 180-bp deletion in *SE*21Q1b's RSV provirus, a number of nonconservative changes in the deduced amino acid sequence of several *SE*21Q1b *gag* proteins have been found (2a). Although the

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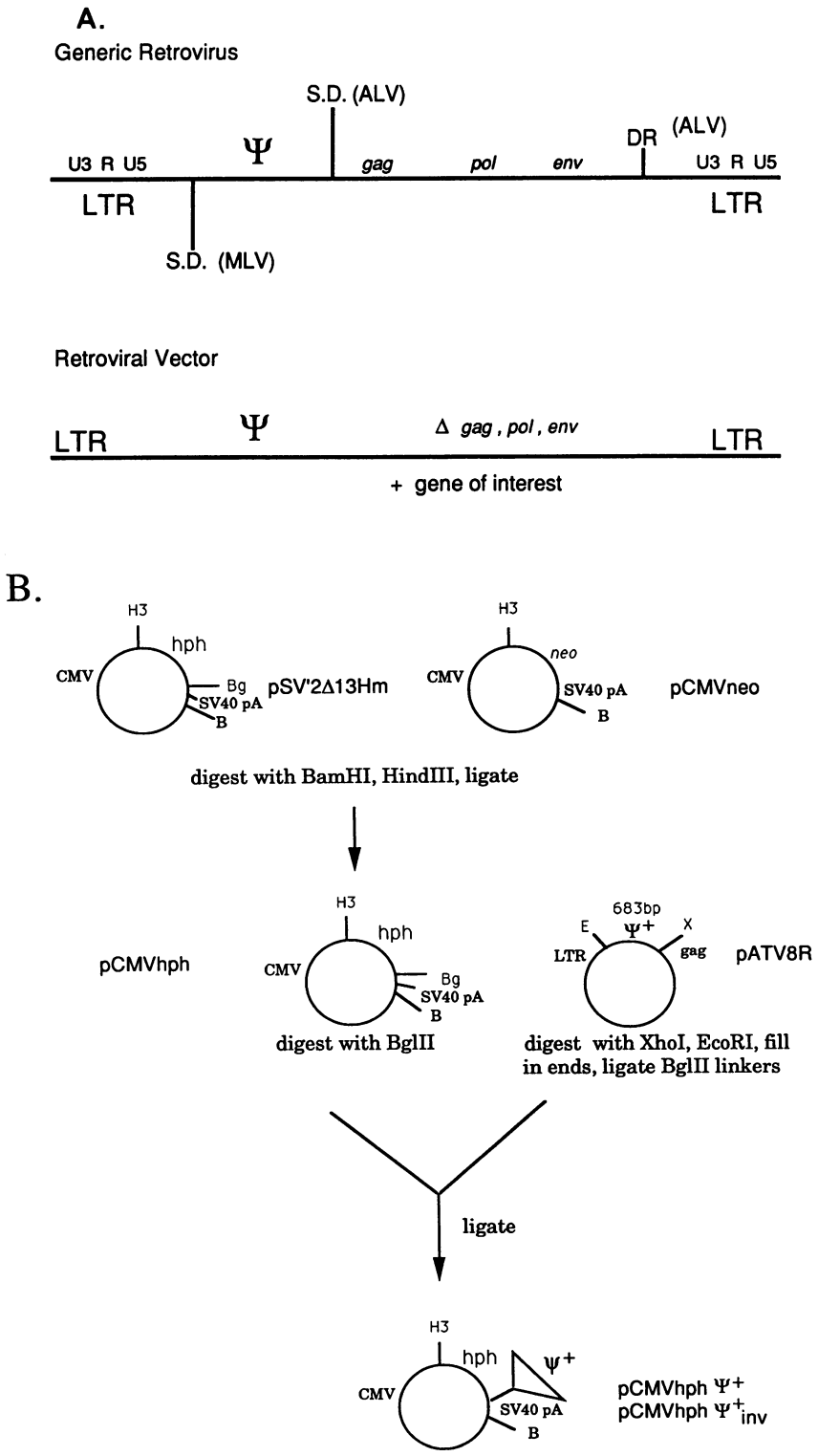

FIG. 1. (A) Generic retrovirus representation, showing splice donor sites (S.D.) relative to the MLV and avian leukosis virus (ALV) 5′ packaging signals (Ψ), and the avian DR (40) located at the 3′ end of the avian viral genome. Long terminal repeats (LTR) flank both the wt retroviral proviral DNA and standard retroviral vectors. These consist of U5, U3, and repeated (R) elements and are formed during reverse transcription of the RNA genome of the retrovirus. The *gag*, *pol*, and *env* structural and catalytic genes of the retroviral genome are generally deleted (Δ) in retroviral vectors and replaced with a gene of interest. (B) Construction of heterologous selectable constructs containing the avian ψ^+. Packaging signals were cloned at the 3′ end of the gene encoding hph resistance, upstream of polyadenylation (pA) signals. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H3, *Hin*dII; X, *Xho*I; CMV, cytomegalovirus immediate-early promoter, which is used to drive the expression of the heterologous constructs; SV40, simian virus 40.

phenotype of random packaging was found to be *trans* dominant in superinfection experiments with helper virus, such that cellular mRNA was found packaged even in the presence of wt particles and genomes (24), we were interested to see whether the defect would preclude recognition of a packaging signal in the absence of replication-competent helper virus. In this study, we found that this mutant does retain the capacity to recognize packaging signals and encapsidates heterologous mRNAs containing ψ at a rate at least 10-fold greater than would be expected for random packaging.

Encapsidation and stable transfer of some heterologous RNAs by retroviral particles has been documented. Endogenous viruslike 30S RNAs (VL30s) from mouse and rat cells have been packaged and transduced to cells infected by several type C retroviruses (34–36, 38). Efficient packaging of genomic MLV RNA into virions produced by a reticuloendotheliosis virus helper line in the absence of the competing reticuloendotheliosis virus genome has also been demonstrated (11). Abundant RNAs, like the 7S L RNA, a component of the signal recognition particle, have been found to be packaged and reverse transcribed in avian retrovirus particles (5). Packaging of nonretroviral endogenous mRNA sequences into retroviral particles, however, has been closely analyzed in only one case. Ikawa et al. showed that globin mRNA could be found associated with the 60S viral RNA of Friend leukemia virus (15). This cellular mRNA was found to constitute 0.001 to 0.002% of the total viral RNA, or about one globin mRNA per 1,000 60S viral genomes. The levels of globin mRNA in the cells utilized for this study are not stated, but globin message is likely to be a major component of the intracellular mRNA of both cell lines utilized. Hence, the amount of this message packaged into the Friend leukemia virus virions is unlikely to be proportionate to its intracellular abundance. In addition, Dornburg and Temin reported on the efficiency of packaging of heterologous hygromycin (hph) mRNA in an SNV-based packaging cell line (7). In this study, packaging of hph RNA was shown to be reduced by a factor of at least 10^4 relative to the packaging of hph RNA encoded by an SNV-derived retroviral vector. However, the intracellular abundance of RNA encoded by this retroviral vector was shown to be 1 order of magnitude higher than that of the other packaging cell lines expressing the heterologous constructs. Thus, hph RNA lacking SNV encapsidation signals was packaged at least 3 orders of magnitude less efficiently relative to its intracellular abundance. These studies show that packaging of RNA into the retroviral virion generally exhibits high specificity.

Since the contributions of the *gag* mutations and the 5′ packaging region deletion to the *SE*21Q1b random-packaging phenotype have not yet been dissected, we wished to determine whether a 5′ ψ deletion alone could lead to packaging of cellular mRNA. Thus, we have examined RNA encapsidation into virions produced by other avian and murine retroviral packaging cell lines in the presence and absence of RNAs containing defined retroviral *cis*-acting sequences. Here we present evidence that random packaging of cellular mRNAs is a feature of *SE*21Q1b that does not occur in any of the packaging lines examined.

MATERIALS AND METHODS

Cell culture. Mammalian cell lines ψ2 (28), PA317 (30), and NIH 3T3 or NIH 3T3 infected with wt Moloney MLV were grown in DMEG (Dulbecco modified Eagle medium with glucose [4.5 g/liter] and 10% calf serum). Avian cell lines *SE*21Q1b (24), Q2bn-4D (41), and QT35 (32) were grown in GM+D+CK (Ham's F10 medium supplemented with 10% tryptose phosphate broth [Difco], 5% calf serum, 1% heat-inactivated chick serum, and 1% dimethyl sulfoxide). Cells were grown at 37°C in a 6% CO_2 atmosphere. Transfections were performed by the calcium phosphate method of Chen and Okayama (4). All cells were seeded in Dulbecco modified Eagle medium with 10% calf serum prior to the addition of plasmid DNA. Media used for selection of drug resistance were as follows: for avian cell lines, CM (Ham's F10 medium containing 10% tryptose phosphate broth, 12% calf serum, 4% chick serum, and 1% nonessential vitamins); for mammalian cell lines, DMEG. Concentrations of drugs (Sigma Chemical Co., St. Louis, Mo.) were as follows: for avian cell lines, hph was at 0.1 mg /ml and G418 was at 0.25 mg/ml; for mammalian cell lines, hph was at 0.1 mg/ml and G418 was at 1.0 mg/ml.

Plasmids. The construct pCMVneo has been previously described (21). The plasmid pCMVhph was constructed by substituting the *Bam*HI-*Hin*dIII hph-coding region and simian virus 40 pA signal from pSV′2Δ13HM from the lab of P. Berg (Stanford University) for the *Bam*HI-*Hin*dIII *neo* region of pCMVneo (Fig. 1B). Constructs pCMVhphψ$^+$ and pCMVhphψ$^+_{inv}$ were created by digesting pCMVhph at its unique *Bgl*II site (at the 3′ end of the hph-coding region, just 5′ of the polyadenylation signals) and inserting in both orientations a 683-bp *Eco*RI-*Xho*I fragment from the RSV proviral clone pATV8R. Prior to ligation, the ends of this fragment were blunt ended with the Klenow fragment of DNA polymerase I, ligated with *Bgl*II linkers, and digested with *Bgl*II. The murine retroviral vector pLNL6 (3) was provided by A. D. Miller (Fred Hutchinson Cancer Research Center). The hph-containing avian retroviral vector pRCAS-hph was obtained as RCAS-*Efs* from the lab of H. Varmus (University of California—San Francisco). It is derived from the replication-competent RSV vector, pRCAS (14) containing one copy of the 115-bp 3′ DR and a unique *Cla*I cloning site. Virus encoded by this construct is envelope deficient, since 180 bp of epidermal growth factor was inserted in the *Eco*RI site of the *env* gene for fusion protein studies unrelated to this work. The total amount of virus produced from Q2bn-4D cells after transfection with pRCAS-hph is within twofold of the amount obtained from Q2bn-4D alone, as measured by Bradford protein analysis. The plasmid construct utilized for generating riboprobes for RNase protection assays, pBSt-HYG, was from the lab of H. Eisen (Fred Hutchinson Cancer Research Center). pRCΔDR-hph is a permuted clone reconstructed from clones obtained from S. Hughes (National Cancer Institute Frederick Cancer Research Facility), p779 Nc TAQ and p1063-215neo, with the addition of the gene encoding hph resistance at the *Cla*I site.

RNA preparation. Viral supernatants were collected 2 to 4 weeks posttransfection from stable drug-resistant mass cultures. The 12- or 24-h supernatants were harvested from subconfluent or barely confluent monolayer cultures and clarified by low-speed centrifugation. Virions were pelleted through a 2-ml 20% sucrose cushion in isotonic buffer (SB) (0.1 M NaCl, 1 mM EDTA, 10 mM Tris hydrochloride) at 23,000 rpm for 2 h in an SW28 Beckman rotor. Viral pellets were resuspended in 100 μl of SB. Bradford protein analysis was performed on resuspended pellets by the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, Calif.). Virions were lysed in 0.5% sodium dodecyl sulfate (SDS), and RNA was purified by repeated phenol-chloroform ex-

tractions followed by chloroform extraction and ethanol precipitation. RNA pellets were resuspended so that 1 ml of RNA was equivalent to 1 ml of original viral supernatant, and optical densities at 260 nm were obtained by UV spectrophotometry (Beckman). Total cellular RNA was extracted by the acid guanidinium-thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (6). RNA integrity was monitored by ethidium bromide staining of rRNAs from extracted cellular RNA in 0.8% agarose minigels. All solutions were made in baked glassware with diethylpyrocarbonate-treated distilled, deionized H_2O.

RNA analysis. Methods for Northern RNA blot and primer extension analyses have been previously described (23). Sequences for oligonucleotide primers were taken from sites near the 5′ ends of specific mRNA sequences obtained from the GenBank data base in antisense orientation and are as follows (5′ to 3′): β-tubulin 24-mer, CTGGATGTGCACGAT TTCCCTCAT; murine sarcoma virus (MSV)/MLV unique 5′ (U5) 22-mer, CTCAGAGGAGACCCTCCCAAGG; ubiquitin 20-mer, CACAAAGATCTGCATCGTCA; and VL30 25-mer, CATGAAAGCACACAGAGCTCTGGGG. Northern blot hybridization probes were as follows: *gag*, a 1.4-kb *Bam*HI fragment from the RSV clone pATV8R (16); mink cell focus-forming sequence (MCF), a 9-kb *Eco*RI fragment from the MCF-13 MuLV clone (46); β-actin, a 2-kb *Pst*I fragment of pA1 (18); amphotropic envelope sequence, a 1.2-kb *Cla*I-*Eco*RI fragment of pPAM (31); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 1.2-kb *Pst*I fragment from pGAD-28 (9). Transcription reactions for RNase protection experiments were performed with T7, T3, or SP6 polymerase and the protocol recommended by Promega Corp. (Madison, Wis.). ^{32}P-labeled antisense riboprobes were gel purified by electrophoresis through 8% acrylamide gels and eluted in 0.5 M ammonium acetate–0.1 mM EDTA–0.1% SDS at 37°C. About 2.5×10^4 cpm of probe (determined by Cerenkov counts) were combined with the test RNA and total yeast RNA (to 10 μg total) in a 10-μl reaction mixture containing 0.4 M NaCl, 0.1 M morpholinepropanesulfonic acid (MOPS), and 10 mM EDTA, heated at 90 to 100°C for 1 min, and hybridized at 65°C for more than 3 h. An RNase cocktail (90 μl) (0.3 M NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA, 1 μg of RNase A per ml, and 20 U of RNase T_1 [Pharmacia, Uppsala, Sweden] per ml) was added to the reaction mixture and incubated for 30 min at room temperature. After a 15-min proteinase K (0.2 mg/ml) (Sigma) treatment at 37°C followed by phenol-chloroform extraction and precipitation with ethanol, the samples were electrophoresed through 6% acrylamide gels containing 50% urea. Reaction mixtures with 0, 1, 2, 5, 10, 20, and 50 ng of cold sense RNA (transcribed in vitro) were utilized to determine a standard curve for densitometric quantitation.

Quantitation. RNase protection reactions were performed with extracted input virion RNA equivalent to 2 to 10 ml of viral supernatant. Typically, 1 ml of supernatant is equivalent to ~5 μg of pelleted protein, as determined by Bradford protein assay, and yields ~0.1 μg of virion RNA. Whole-band analysis by the Bioimages-Visage system was utilized to quantitate the standard curve of hph-specific RNA protected by increasing amounts of cold sense RNA. Intensity of the protected radioactivity was linear up to 20 ng of input sense RNA. Values for each protected band obtained from reactions with each virion RNA preparation were determined by whole-band analysis and converted to nanograms with the graph of the standard curve.

The percentage of RNA protecting the probe relative to the total input virion RNA (as determined from the optical density at 260 nm) is reported in Table 1, as are the values normalized to that of packaged ψ^+ RNA. These percentages are not corrected for the amount of hph sequence detected by the probe (350 bases) to simplify the comparison; thus, the apparent percentages are low. For instance, in the case of the retroviral vector pRCAS-hph, there are two hph mRNAs, a full-length ~9-kb species (3.8% of which can be detected by the probe) and a spliced ~3-kb species (11.6% of which can be detected), both of which are packaged at a ratio of about 3:1. If corrected for percent of protected RNA, the actual percentage of RCAS-hph RNA relative to the total input RNA would be ~35% rather than the reported 1.64%. In the case of pCMVhphψ^+, since only 11% of the 3.2-kb CMVhphψ^+ RNA was detected by this probe, at least 3% of the input RNA encodes hph RNA.

Table 2 utilizes the values in Table 1 (input virion RNA column) to determine the efficiency of packaging of hph RNA relative to its intracellular concentration. Expressed as a calculation, the efficiency of hph packaging equals (percent hph-specific virion RNA)/(percent hph-specific intracellular RNA) × 0.05. The 0.05 correction is due to our assumption that 5% of the total cellular RNA is polyadenylated.

TABLE 1. Quantitation of hph RNA packaged into avian virions by RNase protection assays[a]

Cell line and virus	% hph RNA packaged relative to:	
	Virion RNA[b]	pCMVhphψ^+
*SE*21Q1b		
pCMVhph	0.029	0.029
pCMVhphψ^+	1.0	1.0
pCMVhphψ^+_{inv}	0.027	0.027
Q2bn-4D		
pCMVhph	<0.002	<0.006
pCMVhphψ^+	0.32	1.0
pCMVhphψ^+_{inv}	<0.002	<0.006
pRCAS-hph	1.64	5.33

[a] For details of analysis, see "Quantitation" in Materials and Methods.
[b] These percentages are based solely on the amount of virion-associated RNA detected by the antisense hph probe. Thus, the apparent percentages are low, as the probe represents a small fraction of the encapsidated RNAs, as further discussed in Materials and Methods.

TABLE 2. Packaging of hph RNA into virions relative to the steady-state cellular mRNA level

Cell line and virus	% hph RNA relative to input total cell RNA[a]	Efficiency of hph RNA packaging normalized to cellular hph mRNA levels[b]
*SE*21Q1b		
pCMVhph	0.0015	1.0
pCMVhphψ^+	0.0045	11.0
pCMVhphψ^+_{inv}	0.0012	1.1
Q2bn-4D		
pCMVhphψ^+	0.0045	3.6
pCMVhphψ^+_{inv}	0.0012	<0.08
pRCAS-hph	0.0013	63.1

[a] The percentage of input total cellular RNA protecting the hph probe was determined without correcting for the percentage of each message detected by the probe; for details, see Materials and Methods.
[b] Derived from the percentage of hph in virion RNA in Table 1 and data in this table.

RESULTS

Packaging of nonretroviral RNAs containing ψ^+ into avian virions. It has been previously shown that a ca. 800-base portion from the 5′ end of the MLV retroviral genome is sufficient to direct packaging of heterologous *neo* RNAs into murine retroviral particles at levels equivalent to the level of packaging of an RNA encoded by a murine retroviral vector or wt MLV (1). We were interested in determining whether a similar situation exists for avian retroviruses, since other *cis* sequences in addition to ψ, such as the 3′ DRs of RSV, have been implicated in avian retroviral packaging (40). We were also interested in whether the RSV packaging mutant *SE*21Q1b retains the capacity to recognize a retroviral packaging signal, since earlier data from superinfection experiments had indicated that it might lack this ability (24). To this end, we inserted a 683-bp *Eco*RI-*Xho*I fragment from the RSV clone pATV8R (16) into the heterologous pCMVhph plasmid and assayed the ability of this sequence to target hph RNAs into avian virions. This 683-bp region (putative avian ψ^+) encompasses 53 bp of U3 and all of the R and U5 LTR elements as well as the entire RSV leader sequence and 250 bp of *gag*. The fragment was inserted in the same orientation relative to transcription of the viral genome in the construct pCMVhphψ^+ and in the opposite orientation in the construct pCMVhphψ^+_{inv}. These constructs and the retroviral vector construct pRCAS-hph (14) were transfected into the Rous-associated virus-1 (RAV-1)-derived packaging line Q2bn-4D (41) as well as into *SE*21Q1b, and RNA was extracted from virions obtained from mass hph-resistant cultures. Virus production from all of these transfected packaging lines was within the same order of magnitude, as measured by Bradford protein assays of resuspended viral pellets (data not shown).

RNase protection assays were used to quantitate the effect of avian ψ^+ sequences on packaging of hph RNA (Fig. 2). The 400-base hph antisense riboprobe and the expected 350-base fragment protected from RNase degradation by hph mRNA are diagrammed in Fig. 2A. RNase protection of virion RNAs and one representative total cellular RNA are shown in Fig. 2B. No protected product was found in virions from Q2bn-4D cells in the absence of a transfected vector construct (Fig. 2B, lane 3). An intense band of protected RNA is evident when pCMVhphψ^+ is present in the RAV-1-derived packaging line, Q2bn-4D (Fig. 2B, lane 7). However, when pCMVhphψ^+_{inv} was present in the packaging line (Fig. 2B, lane 8), the amount of protection returned to background levels (compare lanes 8 and 3). Thus, this 683-base avian ψ^+ can direct specific encapsidation of heterologous hph RNA into avian virions when present in the correct orientation. When the avian retroviral vector pRCAS-hph was transfected into the Q2bn-4D cell line, however, hph packaging was clearly much more efficient (Fig. 2B, lane 4). The inclusion of the full complement of avian retroviral *cis*-acting sequences thus leads to optimal packaging of this hph RNA by the RAV-1-derived packaging line, in contrast to the result observed in the murine system (1).

When virion RNA obtained from *SE*21Q1b cells transfected with the same plasmid constructs was analyzed, hph RNA was also found packaged, but this packaging was not strictly dependent on the presence of the avian ψ^+. Protection of the antisense hph RNA probe by RNA obtained from *SE*21Q1b virions occurred in the absence of ψ^+ (pCMVhph [Fig. 2B, lane 2]) and in the presence of an inverted ψ^+ (pCMVhphψ^+_{inv} [Fig. 2B, lane 6]). Similar results showing

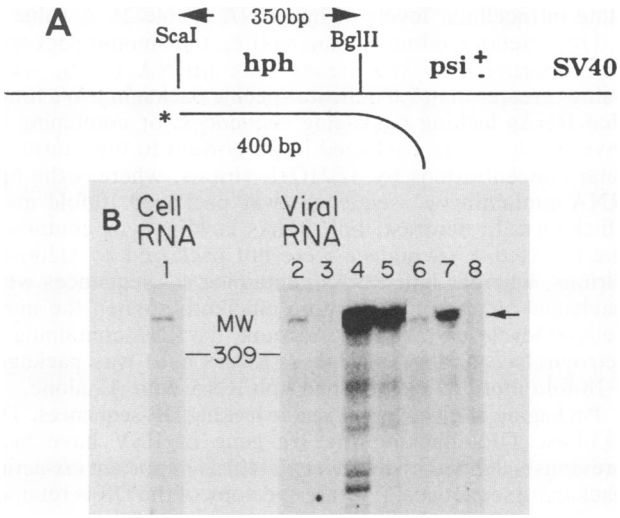

FIG. 2. (A) Diagrammatic representation of the expected RNase protection result. The 400-base in vitro-transcribed ^{32}P-labeled antisense hph RNA probe anneals with hph RNA to protect 350 bases from RNase A and RNase T_1 degradation, which is subsequently detected by autoradiography of a 6% acrylamide sequencing gel. SV40, Simian virus 40 polyadenylation signal. (B) RNase protection assay to quantitate packaging of heterologous hph vectors into avian virions. Lane 1, 10 μg of total cell RNA from *SE*21Q1b transfected with pCMVhphψ^+; lane 2, *SE*21Q1b plus pCMVhph; lane 3, Q2bn-4D; lane 4, Q2bn-4D plus retroviral vector pRCAS-hph; lane 5, *SE*21Q1b plus pCMVhphψ^+; lane 6, *SE*21Q1b plus pCMVhphψ^+_{inv}; lane 7, Q2bn plus pCMVhphψ^+; lane 8, Q2bn plus pCMVhphψ^+_{inv}. MW, Molecular weight (in nucleotides).

packaging of *neo* mRNA lacking ψ and of the *gag* RNA encoded by *SE*21Q1b's ψ-deleted provirus have also been obtained (data not shown). Thus, packaging of these RNAs into *SE*21Q1b virions occurred in the absence of ψ, unlike the result with the Q2bn-4D virions described above. However, *SE*21Q1b cells containing pCMVhphψ^+ (Fig.2B, lane 5) exhibited a marked increase in packaging efficiency relative to the packaging efficiency observed with RNA encoded by pCMVhph or pCMVhphψ^+_{inv} (Fig. 2B, lane 6). Thus, this RSV mutant recognizes the avian extended packaging signal as a specific RNA encapsidation signal.

Using a standard curve obtained from in vitro-transcribed cold hph sense RNA, we calculated the percentage of hph-specific mRNA relative to the total input RNA (Table 1). In the case of *SE*21Q1b cells, when analyzed in relation to the amount of input viral RNA, the packaging of ψ^+-containing RNA was found to be ~35-fold more efficient than random packaging of hph RNA in the absence of ψ. *SE*21Q1b virions thus appear to be capable of recognizing sequences at the 5′ end of the RSV genome as a specific RNA encapsidation sequence. In contrast, ψ^+-directed packaging is at least 150-fold more efficient in Q2bn-4D virions, since no hph RNA was detected in Q2bn-4D virions in either the absence of ψ or the presence of an inverted ψ. Interestingly, RNA encoded by the hph-containing avian retroviral vector pRCAS-hph was packaged fivefold more efficiently than the ψ^+ construct, in contrast to the result seen in the murine system (1), in which RNA encoded by the murine ψ^+-containing heterologous *neo* construct was packaged at least as well as either the full murine retroviral vector or wt MLV.

We also analyzed RNA packaging relative to the steady-

state intracellular levels of hph RNA (Table 2). A value of 1.0 represents random packaging (i.e., the amount packaged is proportional to the steady-state mRNA level), while values greater than 1.0 indicate specific packaging. We found that RNAs lacking packaging sequences, or containing an inverted ψ^+, were packaged in proportion to their intracellular concentrations by *SE*21Q1b virions, whereas the hph RNA containing ψ^+ sequences was packaged 10-fold more efficiently. In contrast, hph RNAs lacking ψ or containing the inverted ψ^+ sequence were not packaged by Q2bn-4D virions, whereas hph RNAs containing ψ^+ sequences were packaged at least 45-fold more efficiently. When the intracellular levels are taken into account, the RNA containing all retroviral *cis*-acting sequences (RCAS-hph) was packaged ~18-fold more efficiently than hph RNA with ψ^+ alone.

Packaging of a retroviral vector lacking DR sequences. The 115-base DRs flanking the *src* gene of RSV have been previously defined by Sorge et al. (40) as important *cis*-acting packaging sequences. At least one copy of the DR is retained in all avian leukosis viruses. The presence of this sequence in the full-length retroviral vector pRCAS-hph may account for its more efficient packaging relative to that of hph RNA containing only the extended 5′ packaging region of RSV. To test this hypothesis, we constructed a retroviral vector in which the single DR was deleted (14) and inserted the gene encoding hph at the unique *Cla*I site (pRCΔDR-hph). Because this is a permuted clone that must be digested and ligated prior to transfection for correct transcription, the transfection efficiency of cells to hph resistance by this construct is much lower than with other nonpermuted plasmid constructs. Thus, it was necessary to screen single hph-resistant clones rather than mass cultures to assay for packaging of the RCΔDR-hph RNA.

RNase protection assays were performed on viral RNAs obtained from three different Q2bn-4D clonal lines containing pRCΔDR-hph (Fig. 3, lanes 6 to 8) as well as viral RNAs obtained from mass cultures of Q2bn-4D transfected with pCMVhphψ^+, pCMVhphψ^+_{inv}, and pRCAS-hph (Fig. 3, lanes 3 to 5, respectively). Relative intensities of the protected bands were determined by densitometry and normalized to the amount of probe protected in viral RNA obtained from pCMVhphψ^+ transfection of Q2bn-4D cells. As described above, the ψ-containing RNAs were packaged efficiently, while the full retroviral vector resulted in optimal packaging. The more than 25-fold difference in packaging of the RCAS-hph RNA in this experiment can be accounted for by a somewhat higher intracellular level of RCAS-hph RNA in this mass culture than in the experiment described above (data not shown). RCΔDR-hph RNA was not packaged as well as the full-length retroviral vector (Fig. 3, compare lanes 6 to 8 with lane 5). Packaging of RCΔDR-hph RNA is approximately 10-fold less efficient if one averages the relative band intensities of the three clones. Thus, it is possible that the absence of the DR led to the relative inefficiency of CMVhphψ^+ RNA.

Packaging of endogenous cellular mRNAs. The experiments described above reveal that virions from *SE*21Q1b cells will package heterologous mRNAs in proportion to their intracellular concentrations within the pool of mRNA, although the virions still retain the capacity to recognize ψ^+. Thus, we were interested in determining whether packaging of cellular mRNAs is a function of the 5′ packaging-region deletion of the *SE*21Q1b provirus. Although virions obtained from the RAV-1-derived Q2bn-4D packaging line did not exhibit such ψ-independent random packaging, we were interested in determining whether other packaging lines,

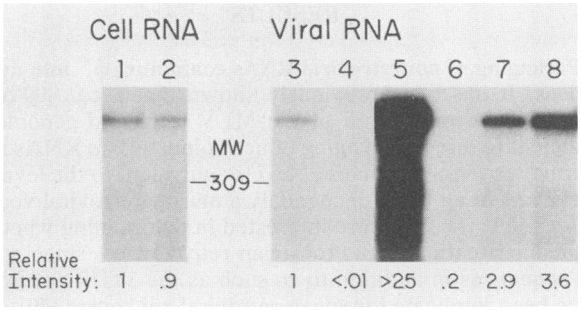

FIG. 3. RNase protection assay to determine the contribution of the DR to efficiency of packaging of the full-length avian retroviral vector, pRCAS-hph. A permuted retroviral vector lacking DR (pRCΔDR-hph) was reconstructed as described in Materials and Methods and transfected into the Q2bn-4D packaging line. Lanes 1 and 2, 10 μg of total cell RNA from hph-resistant mass cultures of Q2bn-4D transfected with, respectively, pCMVhphψ^+ and pCMVhph ψ^+_{inv}; lanes 3 to 5, viral RNAs obtained from hph-resistant mass cultures of Q2bn-4D plus pCMVhphψ^+, Q2bn-4D plus pCMVhphψ^+_{inv}, and Q2bn-4D plus pRCAS-hph, respectively; lanes 6 to 8, viral RNAs obtained from single clonal populations of hph-resistant Q2bn-4D transfected with the digested and ligated pRCΔDR-hph's RCΔDR cl-D, RCΔDR cl-E, and RCΔDR cl-F, respectively. Relative intensities were determined by whole-band analysis by the Bioimages Visage system, with values normalized to hph ψ^+ packaging (lane 3). MW, Molecular weight (in nucleotides).

with analogous 5′ ψ deletions, would contain detectable levels of endogenous cellular mRNAs in the absence of a packageable retroviral vector. In order to determine whether particles released by retroviral packaging cell lines or cells infected with wt virus contain cellular mRNAs, we utilized molecular probes specific for several different abundantly transcribed cellular genes. Virions were obtained from the murine packaging lines PA317 (30) and ψ2 (28), the avian RAV-1-derived packaging line Q2bn-4D (41), and the cell line harboring the RSV mutant *SE*21Q1b, as well as from NIH 3T3 cells infected with wild-type Moloney MLV and primary quail embryo fibroblast cells infected with the wild-type Prague A strain of RSV. Virions were also obtained from LNL6, a G418-resistant mass culture of the PA317 packaging line transfected with the murine retroviral vector pLNL6 (3). Virion production levels by all of these cell lines were within the same order of magnitude as those measured by Bradford protein assays (data not shown). Both extracted viral RNA and total cellular RNA were analyzed by primer extension and Northern blot hybridization (Fig. 4).

We analyzed virions produced by the murine packaging lines and MLV-infected cells for the presence of murine β-tubulin and ubiquitin mRNA by primer extension with ^{32}P-labeled antisense oligonucleotides specific for these sequences (Fig. 4A). While the correct extension products for these mRNAs were readily detectable in the murine total cellular RNA preparations (Fig. 4A, cell RNA lane), no corresponding extension products could be detected with RNA isolated from MLV or PA317 virions. While multiple primer extension products were faintly detectable in the ψ2 virion RNA lane with the β-tubulin probe, none corresponded to the expected size for the bona fide β-tubulin mRNA. With a month-long exposure, ubiquitin extension products of the expected size were barely detectable on the autoradiogram of the ψ2 virion RNA lane. However, we

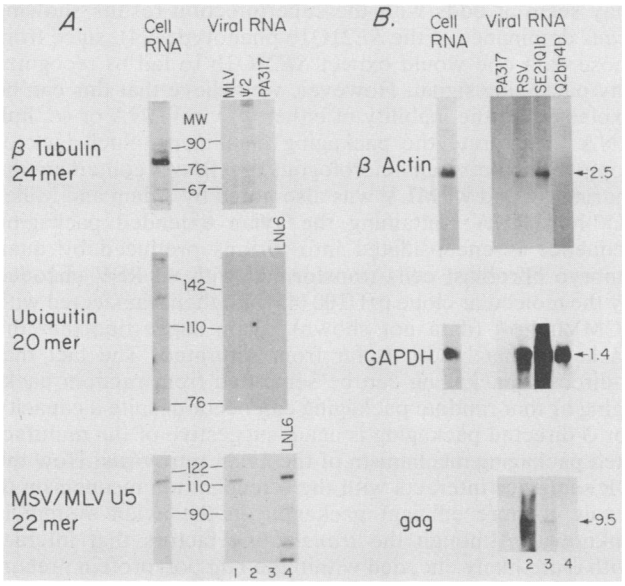

FIG. 4. Endogenous mRNAs. (A) Primer extension assays with murine-specific oligonucleotides. Viral supernatants from NIH 3T3 cells infected with wt Moloney MLV (MLV) and from murine packaging lines ψ2 (28) and PA317 (30) were collected, pelleted over 20% sucrose, and extracted as described in Materials and Methods. Viral RNA equivalent to 12.5 ml of viral supernatant was utilized in each primer extension reaction (except for the MLV and LNL6 reactions with the MSV/MLV U5 primer, which utilized RNA equivalent to 5 ml of viral supernatant), performed as previously described (23). One lane (Cell RNA) utilized 20 μg of total cellular RNA (for β-tubulin, from PA317 cells; for ubiquitin and MSV/MLV U5, from NIH 3T3 cells infected by wt MLV) and is included for each primer to show the expected primer extension products for each mRNA. Overnight exposures are shown, except for ubiquitin 20-mer, which was a 4-week exposure. LNL6 is a *neo*-resistant mass culture of the PA317 packaging line transfected with the murine retroviral vector pLNL6 (3). MW, Molecular weight (in nucleotides). (B) Northern blot probed with β-actin, GAPDH, and *gag* (avian) sequences. The cell RNA lane contains 20 μg of total cellular RNA obtained from the PA317 packaging line (30). Lanes 1 to 4 contain viral RNAs equivalent to 9 ml of viral supernatants obtained from virus-producing cell lines as follows: lane 1, PA317; lane 2, wt PR-A strain of RSV; lane 3, *SE*21Q1b; lane 4, Q2bn-4D. Hybridizations were at 42°C in 50% formamide, and washes were at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS. Arrows, Molecular weight (in thousands).

cannot rule out the possibility that these extension products result from RNAs that are associated with, rather than packaged into, these virions, since we did not pretreat the virions with RNase prior to extraction. We have found that such nonspecific association of nucleic acids with pelleted virions can be a problem with very sensitive techniques. For instance, both RNase A and DNase I treatments (singly or concurrently) of resuspended viral pellets prior to viral RNA extraction will result in marked differences in the results of polymerase chain reaction experiments on cDNAs made from the isolated viral RNA (unpublished observations). We obtained a similar result in primer extension analyses with an antisense oligonucleotide specific for the translation factor EF-1α; again, none of this RNA could be detected in the murine virions (data not shown).

In addition, we examined murine virion RNA for the presence of RNAs encoding β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by Northern blot analysis, using avian probes which cross hybridize with the murine RNA transcripts. PA317 total cellular RNA is shown (Fig. 4B, cell RNA lane) to corroborate the extent of this cross hybridization. Only the results with PA317 virion RNA are included as the representative murine packaging cell line (Fig. 4B, lane 1), but there is no detectable β-actin or GAPDH in virion RNA obtained from any of the murine lines. To verify the integrity of the murine viral RNAs and the phenotype of the packaging lines, an MLV/MSV U5 antisense oligonucleotide primer specific for both genomic retroviral RNA and that encoded by the murine retroviral vector pLNL6 was utilized for primer extension (Fig. 4A). As expected, genomic viral RNA was found in wt MLV but not in the virions produced by the PA317 or ψ2 packaging lines. So, neither of these packaging lines detectably packaged the RNAs encoded by their 5′ ψ-deleted provirus. Packaging of the RNA encoded by the retroviral vector, on the other hand, was at least as efficient as packaging of wt MLV (compare lanes 1 and 4 of Fig. 4A at MLV/MSV U5).

Endogenous cellular mRNAs, in contrast, could be detected in the avian virion RNA. However, significantly less β-actin mRNA was found packaged into either wt RSV (Fig. 4B, lane 2) or Q2bn-4D virions (Fig. 4B, lane 4) relative to the amount packaged into virions produced by the RSV mutant *SE*21Q1b (Fig. 4B, lane 3), even though the intracellular abundance of this mRNA was similar in the three avian cell lines (data not shown). GAPDH mRNA was efficiently packaged into all avian virions (Fig. 4B, lanes 2 to 4) but was not detectable in the murine virions (Fig. 4B, lane 1), although the intracellular abundance of GAPDH mRNA was similar in both the murine and avian cell lines (data not shown). Interestingly, this mRNA was first detected in avian virions by in vitro translation experiments (2, 6a), indicative of its abundance in the avian virion RNA. Thus, avian virions do seem to be able to package some mRNAs, although only *SE*21Q1b packaged both β-actin and GAPDH efficiently. To examine the levels of retroviral sequence in these virions, an RSV *gag* probe was also hybridized to this blot. *gag*-specific RNA was found packaged into both wt RSV and *SE*21Q1b virions (Fig. 4B, lanes 2 and 3) but was not detectable in the virions produced by the 5′ ψ-deleted RAV-1-derived packaging line, Q2bn-4D (Fig. 4B, lane 4). Thus, specific exclusion of viral RNA lacking ψ occurred in the Q2bn-4D line but not in the mutant *SE*21Q1b.

The murine packaging lines did not appear to package the genomic RNA encoded by their 5′ ψ-deleted proviruses. Nor did they contain detectable levels of abundant mRNAs. Since recombination between endogenous and exogenous murine retroviral sequences has been documented (26, 39) and is likely to involve copackaging of RNA molecules (13), we were interested in directly determining whether murine virions contain endogenous retrovirus-related sequences. When the murine viral RNAs were annealed to the antisense oligonucleotide specific for the 5′ end of a member of the VL30 gene family, we found that virions produced by all of the murine lines, even by wt MLV, contained significant quantities of VL30-related RNA (Fig. 5A). Packaging of VL30 RNA into the virions seemed relatively efficient, since these RNAs were present in low abundance in the total cellular RNA, necessitating a longer exposure of the autoradiogram (Fig. 5A, cell RNA lane). Figure 5B (top panel) shows the Northern blot hybridized to a full-length MCF-defective endogenous viral genome. This analysis revealed that PA317 virions contain an abundant RNA of approximately 6 kb that hybridizes with the MCF probe (Fig. 5B,

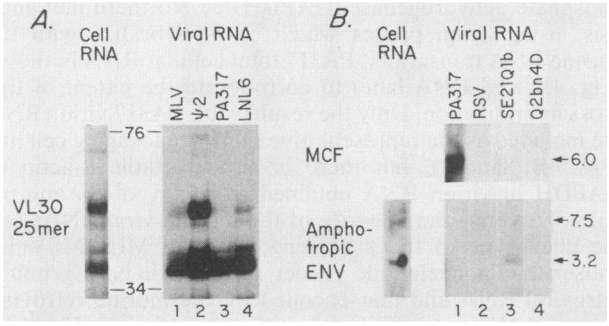

FIG. 5. Endogenous retrovirus-related sequences. (A) Primer extension reactions of more of the same RNAs as those utilized in Fig. 4A. The cell RNA lane (from ψ2 cells) was a 3-day exposure with two intensifying screens, while the viral RNA lanes were overnight exposures. (B) The same Northern blot as that in Fig. 4B, probed with MCF virus clone (46) and amphotropic envelope (ENV) sequences (31).

lane 1). In order to rule out cross hybridization of the MCF probe with the PA317 packaging vector sequences, an amphotropic envelope probe was hybridized to the blot. Although the two expected RNA species were found in the PA317 total cell RNA (Fig. 5B, cell RNA lane), no hybridization was detectable in the PA317 virion RNA (Fig. 5B, lane 1), confirming the MSV/MLV U5 primer extension result (Fig. 4A) and allowing the conclusion that virions obtained from PA317 package high-molecular-weight endogenous retroviral sequences specifically related to MCFs. Interestingly, virions obtained from *SE*21Q1b contained a ca. 3.2-kb RNA that hybridized with the amphotropic envelope probe (Fig. 5B, bottom panel, lane 3), while virions obtained from wt RSV and Q2bn-4D lacked this RNA (Fig. 5B, lanes 2 and 4). Thus, all murine retroviruses examined packaged endogenous retrovirus-related sequences. *SE*21Q1b, on the other hand, appears to be the only avian line whose virions will package any RNA, although we have not yet determined the identity of the RNA detected by the amphotropic envelope probe in these avian virions.

DISCUSSION

In this paper, we present data examining the specificity of RNA packaging into both avian and murine C-type retroviral particles. As in the murine system, an extended packaging signal obtained from the 5′ end of the RSV proviral genome can direct specific encapsidation of a heterologous transcript. This sequence is most likely recognized at the RNA level, since it no longer functions as a packaging signal in the inverse orientation. In the avian leukosis viruses examined, however, this signal is not sufficient for packaging equivalent to that of a standard retroviral vector. It seems most likely that the absence of the other avian *cis*-acting packaging locus, the 3′ DR, in the heterologous mRNA is the primary factor that leads to this relative inefficiency of packaging. Experiments to determine whether the DR alone could have inherent *cis*-acting packaging activity or inclusion of a DR in pCMVhphψ$^+$ will bring packaging efficiency up to the level of the full-length retroviral vector are currently in progress.

Our finding that *SE*21Q1b, in spite of its ability to package cellular mRNAs randomly, is still able to recognize ψ$^+$ sequences for selective packaging implies that the avian retrovirus retains separable recognition processes for (i) packaging mRNA and (ii) packaging ψ$^+$ mRNA. These data may seem at odds with the superinfection results showing *trans* dominance of the *SE*21Q1b phenotype (24), since from those data one would expect *SE*21Q1b to fail to recognize any packaging signal. However, we believe that this can be explained by the inability of either wt viral RNA or ψ$^+$ hph RNA to saturate the packaging machinery. Such lack of competition between heterologous *neo* RNAs containing the murine ψ$^+$ and wt MLV was also noted by Adam and Miller (1). hph RNA containing the avian extended packaging sequence is encapsidated into virions produced by quail embryo fibroblast cells transformed with wt RSV encoded by the molecular clone pJD100 (45) and then transfected with pCMVhphψ$^+$ (data not shown), again suggesting that the packaging machinery is far from saturated. The fact that ψ-directed packaging can be separated from random packaging or that random packaging can occur despite a capacity for ψ-directed packaging is again suggestive of the multifaceted packaging mechanism of the avian retrovirus. How the DR sequence interacts with the ψ recognition mechanism to result in more efficient packaging in the avian system is unknown. Although the *trans*-acting factors that interact with ψ are likely encoded within the *gag* polyprotein precursor (25), even cellular proteins or RNAs could be involved in the recognition of ψ and DR elements for optimal packaging.

Our data indicate that cellular mRNAs are not randomly packaged into retroviral virions, even in the absence of RNAs containing *cis*-acting packaging signals. For the most part, cellular mRNAs could only rarely be detected in virions obtained from packaging cell lines and, if present, were not found packaged in proportion to their intracellular concentrations. Although we have carefully analyzed only the viral packaging relative to the intracellular concentration of RNAs for hph (Table 2) and *neo* and *gag* (data not shown) by quantitative RNase protection assays, there is clearly a marked difference between packaging of cellular mRNA exhibited by *SE*21Q1b and the rare packaging of such RNAs detectable in the other avian retroviruses. Preliminary data from experiments utilizing quantitative polymerase chain reactions with oligonucleotides specific for *gag* and for quail α-tropomyosin mRNA also support the hypothesis that packaging in *SE*21Q1b is indeed random within the pool of polyadenylated mRNAs. These data thus provide molecular confirmation of what had been shown previously by sucrose gradient centrifugation (24).

Our data suggest that the phenotype of the cell line containing the RSV mutant *SE*21Q1b is probably not caused solely by the deletion of the 5′ packaging signal, as nonspecific RNA packaging was not observed in virions produced by the packaging cell lines examined in this study despite the existence of a similar deletion in their proviral genomes. In the absence of RNAs with specific encapsidation sequences, murine virions do not encapsidate abundant cellular mRNAs but package viruslike RNAs (e.g., VL30s or MCF RNAs) (Fig. 5). These RNAs presumably contain some sort of packaging sequence or structure, since they specifically accumulate in virions in spite of their low intracellular abundance, even in the presence of the wt MLV genome. In light of this, the efficient packaging of MLV into virions produced by reticuloendotheliosis virus helper cell lines in the absence of competition (11) could be due to a common packaging structure in the MLV RNA which is recognized by reticuloendotheliosis virus. The high rate of recombination between exogenous and endogenous murine retroviruses (22) could be a reflection of this efficient packaging of endogenous retrovirus-related elements by exogenous retroviral particles. We should also note that significant levels of

RNA related to a characterized avian endogenous retrovirus-related family (10) were not found in RNA from either *SE*21Q1b or Q2bn-4D virions (unpublished observations). However, such sequences have not been characterized as well in the avian system as have their murine counterparts, so we cannot rule out the possibility that RNAs related to other endogenous families are efficiently packaged by avian virions.

Avian and murine retroviral virions seem to employ distinct strategies to obtain specificity in RNA packaging. The murine system (and the avian SNV) may rely on the specificity imparted by one *cis*-acting region, the 5′ ψ, since this sequence is spliced out of subgenomic RNAs (Fig. 1B) which are not encapsidated. The murine ψ^+ is also sufficient for packaging equivalent to that of a standard murine retroviral vector (1) when included in a heterologous RNA. Avian virions, on the other hand, have more complex *cis*-acting signals encoded in their genome, including both a 5′ ψ sequence and a 3′ DR sequence. However, packaging of RNA into the avian virions is not absolutely dependent on these particular sequences, as low levels of some mRNAs can also be detected in even wt avian virions. Perhaps this relaxed specificity (i.e., the capacity to package RNAs lacking ψ) is the reason the avian leukosis viruses require more complex genomic encapsidation signals. It is also interesting to note that this relaxation of RNA packaging specificity may not be equivalent for all RNAs. All of the avian retroviruses we tested packaged GAPDH mRNA, and preliminary experiments indicate that packaging of GAPDH into the avian virions, even into *SE*21Q1b virions, is more efficient than random.

While the basis for packaging of GAPDH by avian retroviral virions is unclear, it may reflect structural features in common between GAPDH RNA and the avian ψ. The avian ψ^+ utilized in these experiments, however, shares only 40% best alignment sequence identity with the GAPDH RNA. Potential RNA-folding models are currently being investigated with the Zucker algorithm (47), relative to the predicted model for the avian ψ^+ RNA folding, to see whether there is any basis for the hypothesis that the GAPDH mRNA contains an avian ψ-like structure. Alternatively, it is possible that the subcellular localization of the GAPDH mRNA, rather than its structure, promotes its packaging. Paradoxically, GAPDH has a huge pseudogene family in mice (>200 copies [33]), but no such GAPDH pseudogene family is found in chickens. Processed pseudogene formation, as has been suggested (42), could be mediated by endogenous retrovirus-related elements, for instance, murine intracisternal A particles, which are actively expressed in the developing mouse germ line. If packaging of RNA into endogenous retroviral particles occurs by mechanisms similar to those of the exogenous retroviruses, this leads us to postulate that the murine retroelements which could be mediating the formation of the large GAPDH repetitive pseudogene family would encapsidate RNAs via a mechanism more closely related to that of the avian exogenous retroviruses than to that of the murine exogenous retroviruses. It is possible that some non-retrovirus-related murine mRNAs are also packaged efficiently, since we assayed for only a few abundant messages. On the basis of these data, we predict that such RNAs would contain RNA sequences or structures which function as packaging signals. An experiment to address this could include making cDNA libraries from murine viral RNA, subtracting all of the VL30- and MCF-related sequences, and determining what the remaining, rarely packaged sequences encode.

The fact that heterologous mRNAs can be efficiently packaged with 683 bases from the retroviral ψ region added at the 3′ end of a given gene (~800 bases in the murine system [1]) in the correct orientation could be useful in certain systems in which minimal retroviral sequences are desired in the introduced construct. Although these mRNAs can be reverse transcribed and integrated into infected cells (8, 20; unpublished data), gene transfer via this system is not terribly efficient. An ordinary retroviral vector system is thus generally more preferable. The potential of this system for directing particular RNAs to the retroviral particle could however be exploited. For instance, many antisense RNA inhibition experiments are limited because they require high levels of antisense RNA relative to levels of the target RNA. If the retroviral genome is the target of such inhibition, inclusion of ψ in the antisense RNA should target the RNA to the particle efficiently, even in the absence of high levels of ψ^+ RNA transcription, and allow potential inhibition of specific viral functions, such as reverse transcription.

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