Lack of Competition Results in Efficient Packaging of Heterologous Murine Retroviral RNAs and Reticuloendotheliosis Virus Encapsidation-Minus RNAs by the Reticuloendotheliosis Virus Helper Cell Line

JANET E. EMBRETSON AND HOWARD M. TEMIN*

McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, Wisconsin 53706

Received 9 March 1987/Accepted 19 May 1987

We constructed recombinant reticuloendotheliosis virus (Rev)-derived and murine leukemia virus-derived vectors to characterize the specificity of packaging retroviral RNAs in Rev proteins. Using this approach, we further localized the Rev encapsidation sequence (E) to a 144-nucleotide region and determined that there are sequences in both the 5' and 3' halves of this region which are necessary in *cis* for viral replication. We found that the Rev E, like the murine leukemia virus E (psi), is position independent (R. Mann and D. Baltimore, J. Virol. 54:401-407, 1986). Also, a 156-nucleotide region of the Rev intron enhanced replication in a *cis*-acting fashion in the presence, but not in the absence, of helper virus. Finally, we showed that packaging of E⁻ and heterologous retroviral genomes occurred efficiently in the Rev helper cell in the absence of competing E-containing (E⁺) viral RNAs.

The viral sequences necessary in *cis* for encapsidation of retroviral RNAs (E) have been defined by deletion analyses for spleen necrosis virus (SNV), an avian reticuloendotheliosis virus (Rev) (41), for Moloney murine leukemia virus (Moloney MLV) (27), and for amphotropic MLV 4070A (36). For each of these viruses, E (designated psi for MLV) is located in a 200- to 350-nucleotide region near the 5' end of the genome. The selective packaging of genomic-length viral RNAs into virions appears to be the result of the absence of E in cellular RNAs and spliced viral RNAs. It has been estimated that the amount of a specific nonviral RNA (globin) within virions is 0.01 to 0.1% of total virion-associated RNA (15), while 5% of the RNA within virions is spliced viral RNA (17, 39). These observations indicate that even without E, some viral RNA is packaged more efficiently than nonviral RNAs. Furthermore, deletion of any of several regions throughout the genome of the avian leukosis and sarcoma viruses affects encapsidation (20, 31-33, 35), supporting the hypothesis that there are multiple recognition sequences for packaging.

We have been studying regions of the Rev genome involved in the specificity of packaging. To this end, we have constructed retrovirus vectors containing a dominant selectable marker and different Rev-specific sequences. We present evidence that (i) the action of the Rev E is position independent; (ii) the Rev E is contained in a 144-base region; (iii) a 156-base region of the Rev intron enhances replication in *cis*; and (iv) Rev RNAs without a Rev E and MLV RNAs (also Rev E⁻) are packaged efficiently by the Rev helper cell line.

MATERIALS AND METHODS

Nomenclature. The positions of sequences within a vector are given in base pairs or kilobase pairs relative to the 5' end

of the provirus. In viral RNA, these sequences are 391 bases closer to the 5' end.

All of the non-MLV-based vectors used in this study were derived from SNV. The helper virus for SNV-based vectors was Rev strain A (Rev-A). Since SNV and Rev-A have 98% nucleotide sequence homology, the term Rev will be used in the text to refer to SNV, Rev-A, and recombinant viruses derived from their molecular clones.

neo, the bacterial aminoglycoside 3' phosphotransferase gene from Tn5 (16), confers resistance to G418 when expressed in eucaryotic cells (38). NEO TU (neomycin transforming units) is the number of cells transformed to neomycin resistance (Neo^r) per milliliter of virus. To determine the NEO TU titer or helper-free (HF; virus derived from the Rev helper cell line) NEO TU titer of a stock of virus, D17 (canine) cells were infected with 10-fold dilutions of *neo*-containing virus. Selection began the day after infection, and approximately 2 weeks after infection the number of Neo^r colonies per milliliter of virus inoculum was determined. *hyg* is the hygromycin B phosphotransferase gene (11); when *hyg* is expressed in mammalian cells it confers resistance to hygromycin B (11). The HYG TU titer of a virus stock was determined in a manner identical to the NEO TU titer, except that hygromycin B was added as the selective agent.

The promoter from the herpes simplex virus type 1 thymidine kinase gene (*tk*) is contained on a 0.9-kbp *Bam*HI-to-*Bgl*II fragment (41). This fragment contains all the elements necessary for expression of a gene linked 3' to the *Bgl*II site.

The letter p designates plasmid DNA (e.g., pJE129) and distinguishes plasmids from viruses (e.g., JE129) derived from those plasmids. An exception to this nomenclature is *cistoneo*, which was constructed by Sorge et al. (36).

E refers to sequences previously described for Rev (41) and Moloney MLV and amphotropic 4070A MLV (27, 36) as necessary for efficient packaging of the viral genome into virions.

Plasmid constructions. All plasmid constructions were made by standard techniques (25). pJD215 (pJD214neo in

* Corresponding author.

reference 5) (see Fig. 1, 4, 5, and 6) is a Rev-based vector containing the *neo* gene expressed from the promoter in the 5' long terminal repeat (LTR). pJD215 contains Rev sequences up to the *Sall* site at 0.861 kbp from the 5' end of the provirus and approximately 600 bp from the 3' end of the provirus including the 3' LTR and approximately 30 bp of pBR322-derived sequences just 5' to the 3' LTR. A polylinker derived from pUC12 was inserted into the unique *Sall* site located at 0.861 kbp on the Rev genome.

pJE129 (see Fig. 1, 4, and 6) was derived from pJD215 by digestion with *KpnI* (0.676 kbp) and *XbaI* (the *XbaI* site lies within the polylinker), treatment with T4 DNA polymerase, and ligation. Thus the Rev E was deleted from pJE129.

pJE129 5' and pJE129 3' (see Fig. 1) were derived from pJE129 by digestion with *Sall* (the *Sall* site is in the polylinker), treatment with T4 DNA polymerase, and ligation with the T4 DNA polymerase-treated *KpnI*-to-*SstI* fragment (5', 0.676 to 0.765 kbp) or *SstI*-to-*Sall* fragment (3', 0.765 to 0.861 kbp) of the Rev E.

pJD215 6-9 (see Fig. 1) was derived from pJD215 by exonuclease digestion from the *Sall* site within the polylinker. DNA sequence analysis indicated that the deletion extended 41 bp 5' of the *Sall* site into Rev sequences (data not shown). The 5' endpoint of the deletion was at 0.820 kbp.

pJE129 5'3' and pJE129 3'5' (see Fig. 4) were derived from pJE129 by digestion with *Sall*, subsequent treatment with T4 DNA polymerase, and ligation with the T4 DNA polymerase-treated *KpnI*-to-*Sall* fragment (Rev E, 0.676 to 0.861 kbp) of Rev. pJE129 5'3' contained the *KpnI*-to-*Sall* fragment in the sense orientation, while pJE129 3'5' contained the same fragment in the opposite orientation.

pJE158 was derived from pJE129 by digestion with *Clal* (located 3' to the *neo* gene; see Fig. 4), treatment with T4 DNA polymerase, and ligation with the T4 DNA polymerase-treated *KpnI*-to-*Sall* fragment (Rev E).

pJE155 (see Fig. 5) was made from pJD215 by replacing the 1.3-kbp *HindIII* fragment containing *neo* with the 1.9-kbp *HindIII* fragment containing 0.3 kbp of pBR322 from the *HindIII* site to the *BamHI* site and the *neo* gene under the control of the herpes simplex virus *tk* promoter.

pJE156 (see Fig. 5) is like pJE155, except that the Rev E was deleted. pJE153 (see Fig. 5), relative to pJE155, contained an additional 156 bp (*Sall* at 0.861 kbp to *XbaI* at 1.017 kbp) of Rev sequences just 3' to the *Sall* site. pJE154 (see Fig. 5) is similar to pJE153, except that it has a deletion of the Rev E. pJE159 was derived from pJE153 by the deletion of Rev sequences at the 3' end of the genome (see Fig. 5). These additional sequences are located from 6.626 to 7.691 kbp on the Rev genome. *cistoneo* (see Fig. 6) contains all the amphotropic MLV *cis*-acting sequences necessary for viral replication and the *neo* gene (36; kindly provided by J. Sorge). pME136 (see Fig. 6) was derived from AFVXM (kindly provided by M. Kriegler). AFVXM was derived from EVX, a Moloney MLV vector (21). AFVXM was digested with *BglII* and *HindIII*, and the *neo* gene (*BglII*-to-*HindIII* fragment) was inserted. pJD214HY+ (5; see Table 1) is analogous to pJD215, except that the *neo* gene was replaced by the *hyg* gene (11).

Cells. Chicken embryo fibroblasts (CEF), D17, and D17-C3 canine cells were grown as previously described (8, 41, 42). D17 is a canine cell line derived from an osteosarcoma. D17-C3 is a Rev helper cell line derived from D17 cells which provides Rev proteins in *trans* (42). Selection for G418-resistant D17 cells was done in the presence of 400 μ g of G418 (GIBCO Laboratories) per ml. Selection for hygro-

mycin-resistant D17 and D17-C3 cells was done in the presence of 80 μ g of hygromycin B (Calbiochem-Behring) per ml.

Viruses. Viruses were recovered from cells by three methods.

(i) **Cotransfection of CEF.** CEF were transfected with replication-competent helper virus DNA and vector (Rev or MLV) DNA in a ratio of 0.5 μ g of helper virus DNA to 10 μ g of vector DNA per 60-mm dish. Virus was collected 4 to 5 days after transfection.

(ii) **Transient transfection of D17-C3 cells with vector DNA.** D17-C3 cells (Rev helper cells) were transfected with 10 μ g of vector DNA per 60-mm dish. At 5 days after transfection, virus was harvested and subjected to three freeze-thaw cycles before being used to infect fresh cells.

(iii) **Stable transfection of D17-C3 cells with *neo*- and *hyg*-containing vector DNAs.** D17-C3 cells were cotransfected with 10 μ g of *neo*-containing vector DNA and 1 to 5 μ g of *hyg*-containing vector DNA or 1 to 5 μ g of *hyg*-containing vector DNA alone. Virus was harvested from cultures of Hyg^r cells approximately 3 weeks after transfection. Virus stocks were subjected to three freeze-thaw cycle before being used.

All transfections were done by the dimethyl sulfoxide-Polybrene method (18).

Nucleic acid analyses. Genomic DNA (12, 14) and unintegrated viral DNA (13) were isolated as previously described. Samples were run on 1.0 to 1.2% agarose gels, blotted by the method of Southern (37), and hybridized with probes labeled by the method of Feinberg and Vogelstein (9). Densitometric scanning was used to quantify relative amounts of unintegrated viral DNA as previously described (7).

S1 nuclease analysis was done as previously described (30). The probe was made by digesting pJE129 with *BamHI* and *PstI* and 3' end labeling the *BamHI* site with the Klenow fragment of DNA polymerase I. The labeled fragment was gel purified before being hybridized with 10 to 30 μ g of RNA from uninfected or SNV-infected CEF. Hybridization at 37 to 50°C for 16 h yielded similar results. The S1 digestion was done at 37°C for 0.5 to 2 h. The S1-resistant fragments were separated by electrophoresis on a 6% polyacrylamide-8.3 M urea gel and visualized by autoradiography with an intensifying screen.

The primer extension analysis was done as follows (method kindly provided by Peter Good, University of Wisconsin). The primer was an oligonucleotide (Pr1; University of Wisconsin Biotechnology Center) homologous to sequences from 20 to 40 nucleotides downstream of the 3' splice site of SNV. Pr1 was 5' end labeled with T4 polynucleotide kinase. Pr1 (10⁶ cpm) and RNA from uninfected or SNV-infected CEF (10 to 30 μ g) were mixed to a final volume of 10 μ l in annealing buffer (0.3 M NaCl, 10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) and heated for 5 min at 80°C. The mixture was then incubated at 42°C for 30 min. After 30 min, 40 μ l of reverse transcriptase buffer (29) containing 5 U of avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories, Inc.) and 0.5 μ l of RNasin (Promega Biotech) was added, and the mixture was incubated at 42°C for 60 min. The mixture was extracted once with phenol and once with chloroform-isoamyl alcohol, and the extension products were precipitated with ethanol.

RESULTS

Further characterization of the Rev E. (i) Both the 5' and 3' halves of the Rev E have *cis*-acting functions. Previously,

Watanabe and Temin (41) demonstrated that the Rev E was located in a region between 0.676 and 0.861 kbp from the 5' end of the Rev provirus. The Rev E was defined experimentally by deletion analyses of recombinant Rev-derived vectors carrying a dominant selectable marker, the herpes simplex virus *tk* gene. CEF were cotransfected with each vector DNA and with Rev helper virus DNA, and the supernatants were assayed for the number of TK⁺ colonies produced after infection of TK⁻ cells. Viruses with a deletion of Rev sequences between 0.676 to 0.861 kbp (SW251) produced no TK⁺ colonies ($<10^{-4}$) relative to viruses with this sequence (SW227). Virion RNA isolated from supernatants of CEF cotransfected with pSW250 (deleted from 0.676 to 1.017 kbp) or pSW227 and helper virus DNA showed that the amount of *tk*-containing RNA in SW250 virions was less than 1% that of SW227 virions.

To determine whether the *cis*-acting function(s) in the Rev E lies exclusively in the 5' or 3' half of this 185-bp region, the 5' half (from the *Kpn*I site at 0.676 kbp to the *Sst*I site at 0.765 kbp, JE129 5'; Fig. 1) or the 3' half (from the *Sst*I site at 0.765 kbp to the *Sal*I site at 0.861 kbp, JE129 3'; Fig. 1) of the Rev E was inserted in the sense orientation into a Rev⁻ vector (JE129; Fig. 1). Virus stocks were derived from pJE129 5', pJE129 3', pJD215 (Rev E⁺), and pJE129 (Rev E⁻) by cotransfection of CEF with each vector DNA and Rev helper virus DNA. The amount of *neo*-containing virus in each stock was quantified by determining the NEO TU titer on canine cells (D17) and analyzing the amount of *neo*-specific unintegrated linear viral DNA in infected CEF.

FIG. 1. Role of the 5' and 3' halves of the Rev E. Schematic diagrams of the DNA form of each virus are shown. The large open boxes represent the LTRs. Straight lines and boxes not marked neo represent viral sequences. The solid boxes represent Rev sequences from the *Kpn*I site at 0.676 kbp to the *Sst*I site at 0.765 kbp (relative to the 5' end of a provirus). The hatched boxes represent Rev sequences from the *Sst*I site at 0.765 kbp to the *Sal*I site at 0.861 kbp. The solid and hatched regions together constitute the Rev E (41). The rectangular box marked neo represents the bacterial 3' aminoglycoside phosphotransferase gene from Tn5 (16), which confers resistance to the drug G418 in eucaryotic cells (38). pJE129 5' contains the 5' half of the Rev E (*Kpn*I to *Sst*I, 0.676 to 0.765 kbp) and pJE129 3' contains the 3' half (*Sst*I to *Sal*I, 0.765 to 0.861 kbp) of the Rev E. pJE129 lacks the Rev E. pJD215 6-9 has a 41-bp deletion at the 3' end of the Rev E (from 0.820 to 0.861 kbp). The relative NEO TU is the NEO TU titer of each virus relative to JD215. Viruses derived by cotransfection of CEF were harvested 5 days after transfection. The titers of JD215 stocks derived in this manner were 0.5×10^6 to 1.0×10^6 NEO TU/ml. The relative NEO TU titers were each determined from at least three separate transfections. Some relevant restriction enzyme cleavage sites and their locations are noted.

The NEO TU titer of JE129 was less than the NEO TU titer of JD215 by a factor of 50 (Fig. 1). Both JE129 5' and JE129 3' had higher NEO TU titers than did JE129, but their titers were not as high as that of JD215. Analysis of the amount of *neo* unintegrated viral DNA produced by these virus stocks showed that the amount of JE129 viral DNA was less than 1% that of JD215, that the amount of unintegrated linear viral DNA for JE129 5' was 1 to 10% that of JD215, and that the amount of JE129 3' was less than 1% that of JD215 (data not shown). These results confirmed the presence of a *cis*-acting function in the 185-bp region between the *Kpn*I and *Sal*I sites of Rev and indicated that there were functions in both the 5' and 3' halves of the Rev E such that neither half had as much activity as the intact region. The reduced levels of unintegrated linear viral DNA in JE129 5'- and JE129 3'-infected CEF indicated that both subregions of the Rev E were involved in viral replication, not in *neo* expression.

pJD215 6-9 has a deletion that removes 41 bp from the 3' end of the Rev E (Fig. 1). The NEO TU titer of JD215 6-9 was only twofold less than that of JD215, indicating that the *cis*-acting function(s) present in the 3' half of the Rev E lies between 0.767 and 0.820 kbp. This result was puzzling, since it placed the Rev E 5' to the previously reported splice donor of Rev (42). To resolve this conflict, we used S1 nuclease analysis and primer extension analysis to remap the splice donor.

RNA from SNV-infected or uninfected CEF was hybridized with a 3' end-labeled probe that contained viral sequences from 0.534 to 0.676 kbp and 0.013 kbp of plasmid sequence (Fig. 2). Two S1-resistant fragments smaller than the probe were detected. The 137-base fragment corresponded to the expected size for protection of genomic-length viral RNA. The 117-base S1-resistant band corresponded to a subgenomic viral RNA with a splice site at 260 bases kbp.

To confirm this result, we used primer extension (Fig. 3). The primer was homologous to 20 bases of viral sequence from 5.718 to 5.738 kbp just downstream of the 3' splice acceptor (at 5.698 kbp). The 5'-end-labeled primer was hybridized to RNA from SNV-infected or uninfected CEF and incubated with avian myeloblastosis virus reverse transcriptase. The extension products were separated on a denaturing polyacrylamide gel. The length of the larger extension product varied from experiment to experiment. The large extension product probably represented a cDNA of genomic viral RNA. The 300-base extension product was the size expected for spliced viral RNA if the splice donor was at 260 bases. Thus remapping of the splice donor by S1 nuclease analysis (Fig. 2) and primer extension analysis (Fig. 3) showed the splice donor to lie at 0.651 kbp, 5' of the *Kpn*I site.

(ii) **The Rev E is position independent.** The *cis*-acting defect in JE129 could be the result of the lack of a relatively autonomous *cis*-acting region, the deletion of sequences just at the termini of the deletion (the ends were treated with an exonuclease to produce blunt ends prior to ligation) or a requirement for spacing between specific viral sequences. To differentiate among these possibilities, the following experiments were done.

To test whether the Rev E was a relatively autonomous *cis*-acting sequence (i.e., position independent), the entire Rev E (*Kpn*I-to-*Sal*I fragment) was inserted into pJE129 at the 3' end of the virus (*Clal* site, JE158; Fig. 4) in the sense orientation. Virus stocks were derived by cotransfection of CEF with each of the recombinant viral DNAs and helper

FIG. 2. Determination of the 5' splice site of SNV by S1 nuclease analysis. Symbols: $\square$, LTR; —, viral sequences internal to the LTRs. The probe was derived from pJE129 by digestion with *Bam*HI and *Pst*I and was 3' end labeled (see Materials and Methods). The probe contained 137 bases of viral sequences and 13 bases of pUC12-derived sequences. The 3'-end-labeled probe was hybridized at 45 or 50°C to 30 μ g of RNA from SNV-infected CEF for approximately 16 h. After digestion with S1 nuclease, S1-resistant fragments and 5'-end-labeled *Msp*I-digested pBR322 fragments were separated on a 6% polyacrylamide–8.3 M urea gel.

virus DNA. Virus stocks were assayed for their NEO TU titer on D17 cells. The NEO TU titer of JE158 was only threefold less than that of JD215, indicating that the Rev E is position independent in a retroviral genome. The MLV E (psi) has similar properties (26).

To determine whether sequences at the exonuclease-treated ends of the Rev E were important for efficient packaging or whether the spacing between sequences on either side of E were important for packaging, the Rev E was inserted into pJE129 at the *Sal*I site in both orientations by blunt-end ligation (pJE129 5'3' and pJE129 3'5'; Fig. 4). pJE129 5'3' contained the Rev E in the sense orientation, while pJE129 3'5' contained the Rev E in the antisense orientation (Fig. 4). Virus stocks were derived by cotransfection of CEF, and the NEO TU titers were determined on D17 cells. The NEO TU titer of JE129 5'3' was the same as that of JD215 (relative titer, 1.0), while the titer of JE129 3'5'

was the same as that of JE129 (relative titer, 0.02). Therefore, neither the sequences at the ends of the Rev E nor the spacing of two or more regions of the virus were essential for packaging, but the orientation of the Rev E was important.

Sequences immediately 3' to the Rev E enhance viral replication. Although the defect in JE129 was *cis*-active, the decrease in titer of JE129 (Rev E[−]) relative to JD215 (Rev E⁺) was only a factor of 50. This factor of 50 was in contrast to the factor of ten thousand decrease in titer which originally defined the Rev E (41). To test whether the presence of additional Rev-derived sequences which were present in the Rev constructs used to define E increased the specificity of encapsidation, we constructed several other vectors. These three regions were (i) the noncoding region of the intron (*Sal*I to *Xba*I, 0.861 to 1.017 kbp), (ii) *gag* coding sequences (*Xba*I to *Bgl*II, 1.017 to 1.960 kbp; schematic diagram not shown), and (iii) *env* coding sequences (*Bgl*II to *Avr*II, 6.626 to 7.691 kbp) (Fig. 5). These vectors expressed *neo* from an internal promoter, the herpes simplex virus *tk* promoter.

FIG. 3. Mapping of the 5' splice site of SNV by primer extension analysis. Symbols: $\square$, LTRs; —, viral sequences internal to the LTRs. The primer was a 20-mer whose sequences were homologous to 20 bases of viral sequences 20 bases downstream of the 3' splice site. The primer was 5' end labeled and was hybridized to 10 to 30 μ g of RNA from uninfected or SNV-infected CEF. See Materials and Methods for reaction conditions. The size of the extension product expected (Fig. 2) is shown.

FIG. 4. Position independence of the Rev E. pJD215, pJE158, pJE129 5'3', and pJE129 3'5' contain the entire Rev E. pJD215 and pJE129 5'3' have the Rev E in the sense orientation, while pJE129 3'5' has the Rev E in the antisense orientation. pJE129 lacks the Rev E. pJE158 contains the Rev E in the sense orientation inserted at the *ClaI* site that lies between the 3' end of the *neo* gene and the 5' end of the 3' LTR. Descriptions of symbols and relative titers are the same as in the legend to Fig. 1. The relative NEO TU titers were each determined from at least three separate transfections. The range of NEO TU per milliliter for JD215 was described in the legend to Fig. 1.

Therefore differences in the length and content of the 5' untranslated sequences should not affect *neo* expression. CEF were cotransfected with these vectors and helper virus DNA, and the resulting viruses were assayed on D17 cells (Fig. 5).

pJE155 contained the same Rev-derived sequences as did pJD215. However, in a JE155 provirus the *neo* gene was expressed from an internal promoter. The NEO TU titers of JD215 and JE155 were the same (approximately 1.0×10^6 NEO TU/ml), indicating that their ability to replicate was the same. The NEO TU titer of JE156 (Rev E⁻) was 0.001 times that of JE155. The presence of the *SalI*-to-*XbaI* fragment in

addition to E (0.861 to 1.017 kbp, JE153) increased the NEO TU titer fourfold (compare JE153 with JE155, Fig. 5). The E⁻ analog of JE153, JE154, had a NEO TU titer approximately a factor of one hundred less than JE153. The relative amounts of unintegrated viral DNA of each E-containing virus correlated with their NEO TU titer (data not shown). There was no detectable *neo*-containing unintegrated viral DNA in cells infected with any of the E⁻ viruses.

To determine whether additional *gag* or *env* sequences could also enhance replication, a set of vectors was made that contained an additional 0.943 kbp of *gag* sequence (*XbaI* to *BglII*, 1.017 to 1.960 kbp on the Rev genome; schematic diagram not shown) and additional *env* sequences from the 1.065-kbp *BglII*-to-*AvrII* region (pJE159, 6.626 to 7.691 kbp on the Rev genome; Fig. 5) of Rev. The presence of these additional *gag* and *env* sequences did not change the ability of these viruses to replicate relative to their parental viruses or affect the efficiency of encapsidation (data not shown).

We concluded that (i) the addition of sequences from the leader region (pre-*gag*) of Rev (0.861 to 1.017 kbp, *SalI*-to-*XbaI* fragment) enhanced the replication of Rev-derived vectors; (ii) deletion of the Rev E from Rev-derived vectors did not absolutely prevent virus production; and (iii) *gag* sequences from 1.017 to 1.960 kbp and *env* sequences from 6.626 to 7.691 kbp did not enhance replication, and, by implication, did not affect the specificity of packaging.

Specificity of packaging in the Rev helper cell. (i) **The Rev E is not an absolute requirement for packaging.** To determine whether the packaging of JE129 was above the background packaging of heterologous RNAs, we cotransfected CEF with pJD215, pJE129, and two MLV-derived vectors (cistron and pME136) and Rev helper DNA. The NEO TU titers of the pseudotyped MLV-derived viruses were very low, less than 0.03 to 0.2% that of JD215 (Fig. 6) and only 1% that of JE129. Thus in the presence of helper virus RNA, heterologous MLV-derived RNAs were packaged inefficiently. Assuming that this efficiency of packaging of MLV genomes represents a background level of packaging random

FIG. 5. Enhancement of viral replication by a 156-bp region 3' to the Rev E. Symbols: , herpes simplex virus *tk* promoter; , pBR322 sequences from the *HindIII* to the *BamHI* site. See below and the legends to Fig. 1 and 4 for a description of other symbols. pJE155 is similar to pJD215 in its content of Rev sequences; however, the *neo* gene is expressed from the *tk* promoter. pJE153 contains an additional 156 bp of Rev sequences ( ) (*SalI* to *XbaI*, 0.861 to 1.017 kbp). pJE154 has the Rev E deleted, but it contains the additional 156 bp of Rev sequences present in pJE153. pJE156 is similar to pJE129, except that, as in pJE153, pJE154, and pJE155, the *neo* gene is expressed from the *tk* promoter. pJE159 contains additional sequences (relative to pJE153) from the Rev *env* gene (*BglII* site at 6.626 kbp to *AvrII* site at 7.691 kbp). The titer of stocks of JD215 and JE155 were 0.5×10^6 to 1.0×10^6 NEO TU/ml. The relative NEO TU was determined as described in the legend to Fig. 1. The relative NEO TU is an average of the relative NEO TU from at least three separate transfections.

FIG. 6. Heterologous genomes are packaged efficiently in the Rev helper cell. cistoneo is derived from a molecular clone of amphotropic MLV 4070A (36). pME136 is a MLV hybrid vector in which the LTR of the resulting provirus consists of Moloney MLV-derived sequences up to the *Sma*I site in R (21, 34) and Harvey murine sarcoma virus-derived sequences in the rest of the LTR. The thin lines in cistoneo represent sequences from amphotropic MLV, while those in pME136 represent sequences from Moloney MLV. The heavy straight line in pME136 represents VL30 sequences from Harvey murine sarcoma virus. A description of symbols not mentioned here can be found in the legends to Fig. 1 and 4. The relative transient HF NEO TU is the NEO TU titer of virus collected 5 days after transfection of the Rev helper cell line relative to JD215. The titers of stocks of JD215 were 0.5×10^6 to 1.0×10^6 NEO TU/ml. The titer of HF stocks of JD215 was 1×10^3 to 3×10^3 HF NEO TU/ml. The relative HF NEO TU were determined from at least three separate transfections.

RNAs, the packaging of JE129 genomes was above background by factors of 5 to 30. Thus Rev-derived E⁻ genomes (JE129) contain additional *cis*-acting signals for packaging.

However, in all of the experiments mentioned above, virus stocks were prepared by cotransfection of Rev-derived vector DNA and helper virus DNA. One explanation for the high titers seen in virus derived from pJE129-transfected CEF was a high level of recombination with the helper virus DNA. To determine whether JE129 could be packaged efficiently in a background with lower levels of recombination and whether MLV genomes could be efficiently packaged in the absence of competition with wild-type Rev, we transfected the Rev helper cell line with pJD215, pJE129, cistoneo, and pME136 and assayed virus harvested 5 days after transfection (Fig. 6). The HF NEO TU titer of JE129 (Rev E⁻) was only a factor of 5 less than that of its Rev E⁺ containing counterpart, JD215. The HF NEO TU titer of cistoneo was the same as that of JE129, while the titer of ME136 was intermediate between the titers of JE129 and JD215 (Fig. 6). Thus the packaging of Rev E⁻ genomes, both MLV and Rev derived, in the Rev helper cell was much more efficient than expected.

To determine whether the Neo^r colonies produced by infection of D17 cells with HF stocks of JE129 contained the expected provirus, the genomic DNAs of four Neo^r JE129 cell clones derived by infection at limiting dilution were analyzed by Southern blotting (data not shown). The proviruses of three of four cell clones analyzed had no gross rearrangements, insertions, or deletions (at a resolution of approximately 50 bp). The JE129 provirus of one cell clone had a small insertion. Rehybridization of the filter with a Rev E probe showed that none of the JE129 proviruses had sequences with homology to the Rev E (data not shown).

These results indicate that the specificity of packaging viral RNAs after transfection of the Rev helper cell was not great, since retroviral genomes without the Rev E (Rev derived or MLV derived) were packaged with high efficiencies.

(ii) Competition of heterologous and E⁻ genomes with

TABLE 1. Competition in the Rev helper cell^a

Vectors cotransfected	Relative HF NEO TU	Relative HF HYG TU	NEO/HYG ^b
pJD215-pJD214HY ⁺	1.0	1.0	0.8
pJE129-pJD214HY ⁺	0.03	1.4	0.007
pME136-pJD214HY ⁺	0.01	1.0	0.008
cistoneo-pJD214HY ⁺	0.008	1.0	0.004
pJE153-pJD214HY ⁺	1.2	1.0	0.9
pJE154-pJD214HY ⁺	0.005	6.0	0.003
pJE155-pJD214HY ⁺	0.75	0.5	1.0
pJE156-pJD214HY ⁺	0.006	3.0	0.001
pJD214HY ⁺	0	4.0	0
pJE137	0	0.4	0

^a See Fig. 1, 5, and 6 for schematic diagrams of plasmid clones.

^b Average of at least three separate transfections (ratio of HF NEO TU per milliliter to HF HYG TU per milliliter from the same virus stock).

homologous genomes for Rev proteins in the Rev helper cell.

The competition for Rev proteins in the cotransfection experiments mentioned above was between wild-type Rev and Rev- or MLV-derived recombinant genomes. To determine how efficiently MLV derived genomes or Rev-derived genomes containing various amounts of Rev sequences competed for Rev proteins in the presence of a Rev-derived genome containing the Rev E (one recombinant genome competing with a different recombinant genome), we cotransfected the Rev helper cell with pJD214HY⁺ and different *neo* vectors and selected for Hyg^r transfectants. pJD214HY⁺ is Rev E⁺ and is the hygromycin-containing analog of pJD215. The supernatant media from Hyg^r cultures were assayed on D17 cells for both their HF NEO TU and their HF HYG TU (Table 1).

In general, the HF HYG TU titers were the same for all transfectants. However, the titer of JD214HY⁺ was often higher when it was cotransfected with a E⁻ genome. For example, the HF HYG TU titer of supernatant media from pJE156-pJD214HY⁺-transfected Rev helper cells was 6×10^4 , while the HF HYG TU titer of pJE155-pJD214HY⁺-transfected Rev helper cells was 1×10^4 . Moreover, the HF HYG TU of virus from cells transfected with pJE137, the E⁻ hygromycin-containing analog of pJE129, was only a factor of 10 less than that of virus from pJD214HY⁺-transfected helper cells.

Genomes which lacked a Rev E (JE129, cistoneo, ME136, JE154, and JE156) were packaged 0.1 to 0.8% as efficiently as genomes which contained a Rev E after cotransfection. For example, the NEO/HYG TU ratio of supernatant media from pME136-pJD214HY⁺-transfected Rev helper cells was 0.008. The NEO/HYG TU ratio of virus

TABLE 2. E⁻ RNAs can compete in the Rev helper cell^a

Vectors cotransfected	Relative HF NEO TU	Relative HF HYG TU	NEO/HYG
pJD215-pJD214HY ⁺	1.0	1.0	1.0
pJE129-pJD214HY ⁺	0.03	0.5	0.02
pJE129-pJE137	1.0	2.0	1.0
cistoneo-pJE137	0.5	1.0	0.5
pJD215-pJE137	10	0.1	35
pJE155-pJE137	6	0.1	10
pJE156-pJE137	0.01	1.0	0.005
pJD214HY ⁺	0	4.0	0
pJE137	0	1.0	0

^a For protocol used to obtain and assay virus, see the text. See Fig. 1, 5, and 6 for schematic diagrams of plasmid clones.

from cultures transfected with two Rev vectors, both of which contained the Rev E, was approximately unity (e.g., pJD215-pJD214HY⁺), indicating that viral genomes with the same Rev-derived sequences were packaged with equal efficiencies. The region of the Rev genome that enhanced replication in the presence of helper virus (*Sall* to *XbaI*, pJE153; Fig. 5) did not enhance replication when virus was derived from the Rev helper cell line (pJE153-pJD214HY⁺).

To determine whether a Rev E⁻ genome could compete in this assay, we cotransfected the Rev helper cell with pJE137 (the Rev E⁻ counterpart of pJD214HY⁺) and different *neo*-containing vectors and selected for Hyg^r transfectants. The supernatant media from Hyg^r cultures were assayed on D17 cells for both their HF NEO TU and their HF HYG TU (Table 2). In two cases in which the two vectors were E⁻ (pJE129-pJE137, cistron-*neo*-pJE137), the HF NEO TU was approximately equal to the HF HYG TU, indicating that these E⁻ genomes were packaged with similar efficiencies. In the other case (pJE156-pJE137), the E⁻ *neo*-containing genome (JE156) competed poorly with JE137. Perhaps the larger size of the JE156 genome accounts for the inability of JE156 to compete with JE137 (see Discussion), since the viral sequences present in JE156 are identical to those in JE129 and JE137. As expected, E⁻ genomes were packaged much less efficiently when an E-containing genome was present (pJD215-pJE137, pJE155-pJE137). Thus these results confirm our observations of competition in the Rev helper cell (Table 1).

In summary, results from stably transfected helper cells in which there was competition with genomes that contained the Rev E indicated that (i) competition occurred, implying that some factor was limiting for virus production (for example, the HF NEO TU and HF HYG TU titers from pJD215-pJD214HY⁺ were 2×10^4 NEO TU/ml and 2×10^4 HYG TU/ml, respectively, while the HF NEO TU and HF HYG TU titers of pJE129-pJD214HY⁺ were 7×10^2 NEO TU/ml and 3×10^4 HYG TU/ml, respectively); (ii) the replication-enhancing region of the Rev genome (*Sall* to *XbaI*) did not enhance replication at the level of packaging; and (iii) even with competition, E⁻ and heterologous RNAs were still packaged, although at a lower efficiency (compare Fig. 6 and Table 1).

DISCUSSION

We have shown that (i) the Rev E was position independent, similar to the MLV E (psi); (ii) a noncoding region of the Rev genome 3' to the Rev E enhanced replication in the presence of, but not the absence of, helper virus; (iii) there was competition for some limiting factor(s) present in Rev helper cells; and (iv) the packaging of viral RNAs that did not contain the Rev E was efficient in the Rev helper cell line in the absence of competing Rev E-containing RNAs.

Properties of the Rev E. Using in vitro-constructed deletion mutants, we further localized the Rev E to a 144-base region of the Rev genome between the *KpnI* site at 0.676 kbp and 0.820 kbp relative to the 5' end of the provirus (JD215 6-9; Fig. 1). Furthermore, we determined that the Rev splice donor maps upstream of the *KpnI* site (Fig. 2 and 3), confirming that E lies within the intron. The previously mapped 5' splice site (42) was in error.

Other deletion mutants (JE129 5' and JE129 3'; Fig. 1) showed that functions existed in both the 5' and 3' halves of the Rev E that were necessary for viral replication and that these two regions were different as seen by the amounts of unintegrated viral DNA produced by each virus. We also

showed that the orientation of the Rev E was important for its function (Fig. 4) and that, like the MLV E (psi) (26), the Rev E was position independent (Fig. 4), implying that this region (E) acts as a relatively autonomous signal within a retroviral genome.

cis-Acting sequences involved in replication. To determine whether there were other viral sequences that affected the specificity of packaging, we constructed a series of vectors with different lengths of 5' and 3' Rev-specific sequence (Fig. 5). Regions of *env* (pJE159; Fig. 5) between 6.626 and 7.691 kbp and of *gag* between 1.017 and 1.960 kbp did not alter the replicative ability or change the packaging efficiency of Rev E⁻ viruses. However, a region of the Rev genome between 0.861 and 1.017 kbp did enhance viral replication fourfold. These results confirm previous observations by Watanabe and Temin (41) and by ourselves (J. E. Embretson, unpublished observations). This region was not necessary for the replication of defective viruses (JE155 compared with JE153; Fig. 5), since viruses which did not contain this sequence replicated in the presence of helper virus and in the Rev helper cell line. Also, this region did not appear to be involved in packaging efficiency, since it did not increase NEO TU titers when the virus was derived from the helper cell line (JE153 versus JD215 and JE155; Table 1) and it did not increase the NEO TU titers of Rev E⁻ viruses (Table 1; data not shown). This replication-enhancing region consists primarily of noncoding sequences, but it contains the *gag* ATG and 24 bp of *gag* coding sequences. A similar replication enhancing region that lies within a retroviral intron has been reported for MLV-derived viruses (6, 19) and for avian leukemia and sarcoma viruses (2).

We constructed numerous Rev-derived vectors with and without the Rev E. The results with these vectors also indicated that the titer of a Rev E⁻ virus was not always a factor of ten thousand lower than that of its Rev E⁺ counterpart (41). The E⁻ viruses we tested had decreases in titer of factors of 50 to 10^4 relative to the analogous E⁺ viruses. We also constructed a MLV vector that lacked the MLV E (psi⁻, JE151; data not shown). This vector was packaged in the presence of amphotropic MLV helper virus in both murine and canine cells at an efficiency of 0.1 to 0.3% relative to cistron-*neo* (data not shown). Thus the phenomenon of packaging E⁻ RNAs was not specific to Rev-derived viruses.

Competition for Rev proteins in the Rev helper cell. Rev-derived E⁻ and murine retroviral RNAs were packaged very efficiently in transiently transfected Rev helper cells (Fig. 6). The packaging of these RNAs occurred at a much lower efficiency when helper virus or a genome with the Rev E was present (Fig. 6; Tables 1 and 2). The packaging of Rev E⁻ RNAs was not the result of the acquisition of Rev E sequences, since none of the four JE129 HF cell clones tested contained sequences with homology to the Rev E (data not shown). Our results support the finding of others that E⁻ RNAs, subgenomic retroviral RNAs (23, 24, 28, 40), other viral RNAs (43), and cellular RNAs (1, 3, 10, 15, 22), are packaged into virions. Our results also indicate that the frequency of these events is quite high.

What factors account for packaging specificity of retroviral RNA? The primary determinant for packaging was the presence of a *cis*-acting packaging signal (E) which enhanced packaging efficiency approximately 100-fold over genomes that did not contain this signal. If other viral sequences act in *cis* for packaging specificity, they were not present in *env* (6.626 to 7.691 kbp), *gag* (1.017 to 1.960 kbp), or a region just 3' to E (0.861 to 1.017 kbp). It has been proposed that

regions within the LTR are involved in forming a secondary structure necessary for packaging RNA into virions (4). However, deletion of most of the Rev U3 does not result in decreased titers relative to a Rev-derived vector containing these sequences, indicating that U3 of Rev does not affect packaging (5a). The observation that JE129 was packaged more efficiently than MLV-derived genomes in the presence of helper virus (Fig. 5) indicated that there are other signals for packaging remaining in this Rev E⁻ genome.

It should be noted that all Rev E⁻ genomes did not behave in an identical manner. JE154 and JE156 were packaged much less efficiently than JE129 both in transient and stable transfections of the Rev helper cell (Tables 1 and 2 and data not shown). However, JE129 was not the only Rev E⁻ genome which was packaged efficiently. JE137 (the *hyg* analog of JE129) was also packaged efficiently in both transient (data not shown) and stable transfections of the Rev helper cell relative to JD214HY⁺ (Tables 1 and 2).

The size of the Rev E⁻ genome did play some role in packaging efficiency. In the presence of helper virus there was an inverse linear relationship between the size of the genome (genome sizes in the range of 1.8 to 3.1 kb) and the efficiency with which an E⁻ genome was packaged relative to its E⁺ counterpart (data not shown). In contrast, Rev E⁺ genomes were packaged with equal efficiencies in the same size range (2.0 to 3.4 kb) when they contained the same amount of viral sequences (JD215 and JE155; Fig. 5).

Finally, a factor(s) produced in infected cells, presumably a viral protein(s), also influenced the RNAs that were packaged. In the absence of competition, Rev E⁻ RNAs were packaged efficiently (Fig. 6; Tables 1 and 2). When two Rev-derived E⁺ genomes were present in the same cell, each genome was packaged with a similar efficiency (Table 1). However, when one of the two genomes lacked a Rev E, the factors preferentially packaged the genome with E.

ACKNOWLEDGMENTS

We thank S. Coe, J. Couch, S. Hellenbrand, S. Hines, and J. Rein for technical assistance. We thank Ralph Dornberg, Joe Dougherty, Celine Gelinias, Tom Gilmore, Cindy Miller, Rex Risser, and Bill Sugden for comments on the manuscript. Special thanks to Joe Dougherty and Cindy Miller for advice on S1 analysis and Peter Good for advice on the primer extension analysis.

This work was supported by Public Health Service research grants CA-22443 and CA-07175 from the National Institutes of Health. J.E.E. is supported by Public Health Service predoctoral training grant CA-09135 from the National Institutes of Health. H.M.T. is an American Cancer Research Professor.

LITERATURE CITED

- Adkins, B., and T. Hunter. 1981. Identification of a packaged cellular mRNA in virions of Rous sarcoma virus. *J. Virol.* **39**: 471-480.
- Arrigo, S., M. Yun, and K. Beemon. 1987. *cis*-Acting regulatory elements within *gag* genes of avian retroviruses. *Mol. Cell. Biol.* **7**:388-397.
- Chen, P., A. Cywinski, and J. M. Taylor. 1985. Reverse transcription of 7S L RNA by an avian retrovirus. *J. Virol.* **54**: 278-284.
- Darlix, J. 1986. Control of Rous sarcoma virus RNA translation and packaging by the 5' and 3' untranslated sequences. *J. Mol. Biol.* **189**:421-434.
- Dougherty, J., and H. M. Temin. 1986. High mutation rate of a spleen necrosis virus-based retrovirus vector. *Mol. Cell. Biol.* **6**:4387-4395.
- 5a. Dougherty, J., and H. M. Temin. 1987. A promoterless retroviral vector indicates that there are sequences in U3 required for 3' RNA processing. *Proc. Natl. Acad. Sci. USA* **84**:1197-1201.
- Eglitis, M. A., P. Kantoff, E. Gilboa, and W. F. Anderson. 1985. Gene expression in mice after high efficiency retroviral-mediated gene transfer. *Science* **230**:1395-1398.
- Embretson, J. E., and H. M. Temin. 1986. Pseudotyped retroviral vectors reveal restrictions to reticuloendotheliosis virus replication in rat cells. *J. Virol.* **60**:662-668.
- Emerman, M., and H. M. Temin. 1984. Genes with promoters in retrovirus vectors can be independently suppressed by an epigenetic mechanism. *Cell* **39**:459-467.
- Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6-13.
- Gallis, B., M. Linial, and R. Eisenman. 1979. An avian oncovirus mutant deficient in genomic RNA: characterization of the packaged RNA as a cellular messenger RNA. *Virology* **94**: 146-161.
- Gritz, L., and J. Davies. 1983. Plasmid encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* **25**:179-188.
- Grunwald, D. J., B. Dale, J. Dudley, W. Lamph, B. Sugden, B. Ozanne, and R. Risser. 1982. Loss of viral gene expression and retention of tumorigenicity by Abelson lymphoma cells. *J. Virol.* **43**:92-103.
- Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.
- Horowitz, J. M., and R. Risser. 1982. A locus that enhances the induction of endogenous ecotropic murine leukemia viruses is distinct from genome-length ecotropic proviruses. *J. Virol.* **44**: 950-957.
- Ikawa, Y., J. Ross, and P. Leder. 1974. An association between globin messenger RNA and 60S RNA derived from Friend leukemia virus. *Proc. Natl. Acad. Sci. USA* **71**:1154-1158.
- Jorgensen, R. A., S. J. Rothstein, and W. J. Reznikoff. 1979. A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. *Mol. Gen. Genet.* **177**:65-72.
- Katz, R. A., R. W. Terry, and A. M. Skalka. 1986. A conserved *cis*-acting sequence in the 5' leader of avian sarcoma virus RNA is required for packaging. *J. Virol.* **59**:163-167.
- Kawai, S., and M. Nishizawa. 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. *Mol. Cell. Biol.* **4**:1172-1174.
- Keller, G., C. Paige, E. Gilboa, and E. F. Wagner. 1985. Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. *Nature (London)* **318**:149-154.
- Koyama, T., F. Harada, and S. Kawai. 1984. Characterization of a Rous sarcoma virus mutant defective in packaging its own RNA: biochemical properties of mutant TK15 and mutant-induced transformants. *J. Virol.* **51**:154-160.
- Kriegler, M., C. F. Perez, C. Hardy, and M. Botchan. 1984. Transformation mediated by the SV40 T antigens: separation of the overlapping SV40 early genes with a retroviral vector. *Cell* **38**:483-491.
- Linial, M., E. Medeiros, and W. S. Hayward. 1978. An avian oncovirus mutant (SE21Q1b) deficient in genomic RNA: biological and biochemical characterization. *Cell* **15**:1371-1381.
- Majors, J. E., and H. E. Varmus. 1980. Learning about the replication of retroviruses from a single cloned provirus of mouse mammary tumor virus. *UCLA Symp. Mol. Cell. Biol.* **18**:241-253.
- Majors, J. E., and H. E. Varmus. 1980. Nucleotide sequences at host-proviral junctions for mouse mammary tumor virus. *Nature (London)* **289**:253-258.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Mann, R., and D. Baltimore. 1985. Varying the position of a retrovirus packaging sequence results in the encapsidation of both unspliced and spliced RNAs. *J. Virol.* **54**:401-407.
- Mann, R., R. C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33**:153-159.

28. Martin, P., C. Henry, F. Ferre, C. Bechade, A. Begue, C. Calothy, B. Debuire, D. Stehelin, and S. Saule. 1986. Characterization of a *myc*-containing retrovirus generated by propagation of an MH2 viral subgenomic RNA. *J. Virol.* **57**:1191-1194.
29. McKnight, S. L., E. R. Gavis, R. Kingsbury, and R. Axel. 1981. Analysis of transcriptional regulatory signals of the HSV thymidine kinase gene: identification of an upstream control region. *Cell* **25**:385-398.
30. Miller, C. K., and H. M. Temin. 1986. Insertion of several different DNAs in reticuloendotheliosis virus strain T suppresses transformation by reducing the amount of subgenomic mRNA. *J. Virol.* **58**:75-80.
31. Nishizawa, M., T. Koyama, and S. Kawai. 1985. Unusual features of the leader sequence of Rous sarcoma virus packaging mutant TK15. *J. Virol.* **55**:881-885.
32. Pugatsch, T., and D. W. Stacey. 1983. Identification of a sequence likely to be required for avian retroviral packaging. *Virology* **128**:505-511.
33. Shank, P. R., and M. Linial. 1980. Avian oncovirus mutant (SE21Q1b) deficient in genomic RNA: characterization of a deletion in the provirus. *J. Virol.* **36**:450-456.
34. Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe. 1981. Nucleotide sequence of Moloney murine leukemia virus. *Nature (London)* **293**:543-548.
35. Sorge, J., W. Ricci, and S. H. Hughes. 1983. *cis*-Acting RNA packaging locus in the 115 nucleotide direct repeat of Rous sarcoma virus. *J. Virol.* **48**:667-675.
36. Sorge, J., D. Wright, V. D. Erdmann, and A. E. Cutting. 1984. Amphotropic retrovirus vector system for human gene transfer. *Mol. Cell. Biol.* **4**:1730-1737.
37. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
38. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* **1**:327-341.
39. Stacey, D. W. 1979. Messenger activity of virion RNA for avian leukemia virus envelope glycoprotein. *J. Virol.* **29**:949-956.
40. Svoboda, J., M. Dvorak, R. Guntaka, and J. Geryk. 1986. Transmission of (LTR-v-src-LTR) without recombination with a helper virus. *Virology* **153**:314-317.
41. Watanabe, S., and H. M. Temin. 1982. Encapsidation sequences for spleen necrosis virus, an avian retrovirus, are between the 5' long terminal repeat and the start of the gag gene. *Proc. Natl. Acad. Sci. USA* **79**:5986-5990.
42. Watanabe, S., and H. M. Temin. 1983. Construction of a helper cell line for avian reticuloendotheliosis virus cloning vectors. *Mol. Cell. Biol.* **3**:2241-2249.
43. Yakobson, E., and R. A. Weiss. 1981. Mutant retrovirus particles package vesicular stomatitis virus mRNA during mixed infection. *Virology* **109**:183-187.