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Pseudotyped Retroviral Vectors Reveal Restrictions to Reticuloendotheliosis Virus Replication in Rat Cells

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Reticuloendotheliosis viruses (Rev) replicate in chicken and dog cells, but not in rat cells. Amphotropic murine leukemia viruses (Am-MLV) replicate in chicken, dog, and rat cells. Transcription from the Rev long terminal repeat, determined by the chloramphenicol acetyltransferase assay, was not significantly different from transcription from the MLV long terminal repeat in rat cells. To determine further the step(s) in the retroviral life cycle that is blocked for Rev replication in rat cells, we took advantage of the wide host range of Am-MLV (S. Rasheed, M. B. Gardner, and E. Chan, J. Virol. 19:13–18, 1976) and the ability to form Rev-Am-MLV pseudotypes. Data from these pseudotypes indicate that the block to Rev replication in rat cells is posttranscriptional.

Reticuloendotheliosis virus strain A (Rev-A) is a replication-competent avian retrovirus. Chicken and dog cells are permissive for Rev-A infection and replication. Rat cells are semipermissive.

Blocks to retrovirus replication can be at any step(s) in viral replication. For example, blocks to replication could be at viral entrance, reverse transcription, integration, transcription, RNA processing, translation, posttranslational processing, packaging, or virus maturation. Reports of blocks to retroviral replication include host restrictions (i) that are at viral entrance for both murine and avian leukemia viruses (5, 16, 19, 26, 40, 45), (ii) that are encoded by the *Fv-1* gene which blocks replication between viral entrance and integration of murine leukemia viruses in mouse cells (19, 20–22, 26, 38) (iii) that prevent proper posttranslational processing of the *gag* precursor in Rous sarcoma virus-infected rodent cells (7), and (iv) that are due to chromatin structure surrounding a provirus in Rous sarcoma virus-infected rat cells (12). The most frequent restriction appears to be at the level of viral entrance.

Initially, we analyzed the relative levels of Rev-A-specific transcription by quantification of Rev-A RNA levels in infected rat and chicken cells and by the chloramphenicol acetyltransferase (CAT) assay to determine whether viral RNA levels limit Rev-A virus production in rat cells. To determine whether pretranscriptional events were limiting, we prepared Rev-amphotropic murine leukemia virus pseudotypes. These pseudotypes contain defective retroviral genomes which include all of the *cis*-acting elements needed for replication and a dominant selectable gene. The titers of these pseudotypes on dog and rat cells and the efficiency of the Rev-A long terminal repeat (LTR) in these cells indicate that the block to Rev-A replication in rat cells is posttranscriptional.

MATERIALS AND METHODS

Nomenclature and abbreviations. Spleen necrosis virus and reticuloendotheliosis virus strain A (Rev-A) have 98% nucleotide sequence homology. In the following experiments, the exact derivation (spleen necrosis virus versus Rev-A) of the Rev genomes and proteins will not be identified, but instead all these viruses will be referred to as Rev. MLV is used in the text (i) to mean murine leukemia viruses or (ii) to designate Moloney MLV and Harvey murine sarcoma virus (Ha-MSV)-derived sequences. Amphotropic MLV 4070A is designated Am-MLV (17). The *neo* gene is the bacterial aminoglycoside 3′ phosphotransferase gene from Tn*5* which, when expressed in mammalian cells, confers resistance to the neomycin analog G418 (23). Neo^r and TK^+ refer to a phenotype, while *neo* and *tk* refer, respectively, to the genes for bacterial aminoglycoside 3′ phosphotransferase and herpes simplex thymidine kinase. NEO TU is the quantification of the neomycin transforming units (titer) of a stock of virus. This titer is derived by determining the number of Neo^r colonies formed per milliliter of virus stock. Plasmids are designated by p before their name (e.g., pMLV110), while virus stocks made from the plasmids do not contain the p (e.g., MLV110). Virus stocks are referred to by the genome and, in parentheses, the virion proteins [e.g., REV111(Am-MLV) contains a REV111 genome packaged in Am-MLV proteins]. Cell clones containing a selectable provirus are named by the cell line from which they were derived, the provirus which they contain, and an identifying letter or number (e.g., D17-MLV110.A is clone A of D17-derived cell clones containing a MLV110 provirus).

att refers to those sequences which are necessary in *cis* for integration of retrovirus DNA (31). E refers to those sequences which are necessary in *cis* for encapsidation of retrovirus RNA (28, 35, 42). CEF and BRL mean chicken embryo fibroblasts and Buffalo rat liver cells, respectively. Chloramphenicol acetyltransferase is designated as CAT, and simian virus 40 is SV40. LTR means long terminal repeat, and kbp is kilobase pairs.

Plasmid constructions. All DNA constructions were made by standard techniques (27). pREV111 (see Fig. 3) has been described previously as pME111 (9). pREV111 is a Rev-derived vector which contains all Rev *cis*-acting sequences, where the *neo* gene is expressed from the 5′ LTR, and where the *tk* gene is expressed from its own promoter. pMLV110 (see Fig. 3) was constructed in the following manner: a permuted clone of Ha-MSV containing one LTR, H-1 (8), was digested with *Eco*RI and *Bam*HI, and the 3.3-kbp fragment containing the LTR was subcloned into pUC12 giving pMLV109. pSV2*neo* (37) was digested with *Hin*dIII and *Sma*I, *Hin*dIII linkers were added, and the 1.3-kbp

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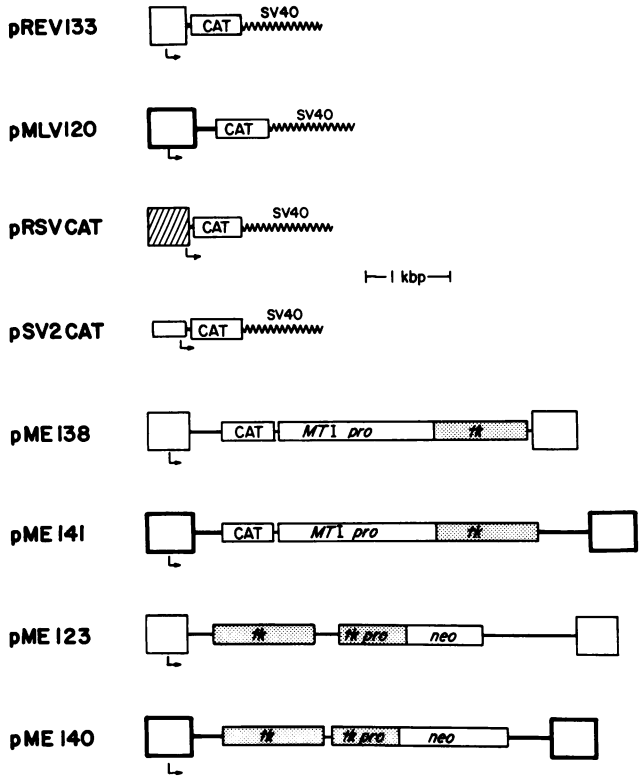


FIG. 1. Schematic representation of vectors used in the CAT and thymidine kinase assays. Large open boxes in pREV133, pME123, and pME138 represent Rev LTRs, while large open boxes with heavy lines in pMLV120, pME140, and pME141 represent MLV LTRs. The large open box with diagonal lines for pRSVCAT (13) represents the U3 region and part of R of a Rous sarcoma virus proviral LTR and 5′ flanking sequences. The small open box in pSV2CAT (14) represents the early transcriptional elements from SV40. Arrows underneath these boxes represent the transcriptional start sites. The boxes marked CAT represent the CAT gene. The wavy lines represent SV40 polyadenylation sequences. In pMLV120 the straight line represents sequences from Ha-MSV, and in pME140 and pME141 the straight lines represent sequences from MLV (see the text); in pRSVCAT, pREV133, and pSV2CAT, they represent SV40 sequences, and in pME123 and pME138 they represent Rev sequences. The box marked *MT*I pro represents sequences from the mouse metallothionein I gene promoter region. The dotted boxes represent the coding sequences for the herpes simplex virus thymidine kinase gene (*tk*). The dotted boxes marked *tk* pro represent the promoter sequences for the *tk* gene. The boxes marked *neo* represent the bacterial aminoglycoside 3′ phosphotransferase gene (23).

*Hin*dIII fragment was subcloned into pUC12 giving pME110. pME110 was digested with *Hin*dIII, and the *neo*-containing fragment was inserted into the *Hin*dIII site of pMLV109 giving pMLV105. A 3′ MLV LTR was added to pMLV105 by partial digestion of pMLV105 with *Hin*dIII and insertion of a 2.3-kbp *Hin*dIII fragment from pMLV109 which contains the Ha-MSV LTR, giving pMLV110. Cistorneo has been previously described (35). Cistorneo is an Am-MLV-derived vector which contains all Am-MLV *cis*-acting sequences and where the *trans*-acting sequences have been replaced with the *neo* gene.

pMLV120 (Fig. 1) was derived from pMLV109 by digesting pMLV109 with *Hin*dIII and inserting the MLV LTR-containing *Hin*dIII fragment into a subclone of pRSVCAT containing the CAT gene coding sequences without a promoter and SV40 polyadenylation sequences (pJE119, *Hin*dIII-to-*Bam*HI site of pRSVCAT in pUC12). pREV133 (Fig. 1) was derived from pSV2CAT by digestion with *Acc*I and *Hin*dIII to remove the SV40 early gene transcriptional elements, filling in the ends with DNA polymerase I (Klenow fragment), and ligating this with a Rev LTR. The Rev LTR was derived from pJD215 (J. Dougherty, personal communication) by digesting with *Eco*RI and *Bam*HI and filling in the ends with the Klenow fragment of DNA polymerase I. pJD215 is a Rev-derived vector which contains all Rev *cis*-acting sequences and where the *trans*-acting sequences have been replaced with the *neo* gene. The *Eco*RI site in pJD215 replaces the *Sac*I site at 0.014 kbp from the 5′ end of the Rev LTR. The *Bam*HI site is 0.037 kbp 5′ of the end of U5.

pME138 (M. Emerman, personal communication) was derived from pJD215. An *Eco*RI-*Bam*HI fragment containing the promoter from the mouse metallothionein I gene driving the expression of the *tk* gene was inserted into the unique *Cla*I site of pJD215 by using the Klenow fragment of DNA polymerase I, giving pME133. pME133 was digested with *Hin*dIII to remove the *neo* gene, and a *Hin*dIII fragment from pYEJ001 (P-L Biochemicals, Inc.) containing the CAT gene was inserted to give pME138. pME123 has been previously described (10). pME123 is a Rev-derived vector which contains all Rev *cis*-acting sequences, where the *tk* gene is expressed from the 5′ LTR, and where the *neo* gene is expressed from the *tk* promoter.

pME141 (M. Emerman, personal communication) was derived from AFVXM (kindly provided by M. Kriegler, personal communication). AFVXM, a derivative of a MLV-derived vector, EVX (25), was digested with *Bgl*II, and the *Bam*HI fragment from pME138 containing the CAT gene, metallothionein promoter, and *tk* gene was inserted to give pME141. pME140 (M. Emerman, personal communication) was derived from AFVXM by digestion with *Bgl*II and inserting the coding region of *tk*, resulting in pME137. The *Hin*dIII fragment of pME122 containing the *tk* promoter and *neo* gene was inserted into pME137 to give pME140.

Cells. CEF, Rat-1 (Rat), BRL TK$^-$, D17, and D17-C3 dog cells were grown as previously described (10, 42). D17-C3 is a D17-derived Rev helper cell line (42). Selection for G418-resistant colonies was done in the presence of 400 μg of G418 (GIBCO Laboratories) per ml, which was added 24 h post-infection. Selection for TK$^+$ cells was done as previously described (10). D17-derived cell clones were isolated by infection of D17 cells with a helper virus-free stock of MLV110(Rev) or REV111(Rev) and subsequent G418 selection.

Nucleic acid analyses. The protocols for isolation and analysis of unintegrated viral DNA (18) as well as Southern transfer and hybridization have been previously described (27, 33, 36). Quantification of the relative amounts of defective and replication-competent virus present in virus stocks was done by the method of Emerman and Temin (10), with the exception that D17 cells, as well as CEF, were used. There was no more than a twofold variation in the amount of helper virus present in different stocks prepared with the same helper virus and same cell (data not shown).

prA8, a nonpermuted clone of 4070A (Am-MLV) (35) kindly provided by J. Sorge and p60BSal, a permuted clone of Rev (29, 41), were labeled by nick translation and used as probes to quantify Am-MLV and Rev production, respectively, by using the method referred to above (10).

CAT assay. The CAT assay was done by the method of Gorman et al. (14). A quantity of 10 to 20 μg of plasmid DNA

was transfected per 100-mm dish. These amounts of DNA were in a linear range for uptake and expression in chicken and dog cells (data not shown). Protein was quantified by the Bradford assay (4), and equal amounts of protein were assayed for each cell extract from a specific host cell. Transiently transfected dog and chicken cells gave increasing amounts of acetylation with time for all vectors tested with 2 to 50 μg of protein (data not shown).

The plasmids used were pREV133 (Fig. 1 [designated Rev in Fig. 2]) and pME138 (Fig. 1 and 2), which contain a Rev LTR 5′ to the CAT gene; pMLV120 (Fig. 1 [designated MLV in Fig. 2]) and pME141 (Fig. 1 and 2), which contain the Ha-MSV LTR 5′ to the CAT gene; pRSVCAT (13), which contains the RSV U3 region 5′ to the CAT gene (Fig. 1 [designated RSV in Fig. 2]); and pSV2CAT (14), which has the SV40 early gene transcriptional elements 5′ to the CAT gene (Fig. 1 [designated SV40 in Fig. 2]). Cells from each species were transfected with each plasmid at least twice. There was no more than a threefold variation in acetylation relative to pRSVCAT between different transfections.

An assumption used in interpreting this assay is that transfected DNA is used with the same efficiency as integrated proviral DNA. Also, the stability of the RNAs generated by the different vectors in the same cells is assumed to be the same.

To determine promoter strength in rat cells by the CAT assay, pools of TK^+ cell clones derived from infection of BRL TK^- cells with ME138(Rev) and ME141(Am-MLV) were grown to confluency, and extracts were made. Stocks of ME138(Rev) were made by cotransfection of CEF with pME138 and a molecular clone of Rev. Stocks of ME141(Am-MLV) were made by superinfection with Am-MLV of Neo^r D17 cells which had been cotransfected with pME141 and pSV2*neo* (37). Culture dishes containing 20 to 50 TK^+ cell clones were pooled, and 3 to 10 μg of protein was assayed.

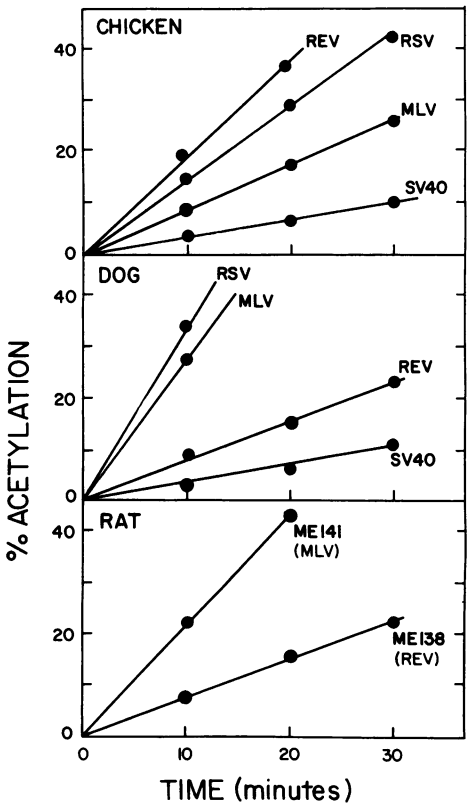


FIG. 2. Assay of CAT activity in chicken, dog, and rat cell extracts. Graphs show the percentage of conversion of chloramphenicol to its acetylated forms with time of incubation. REV, MLV, RSV, and SV40 refer to pREV133, pMLV120, pRSVCAT, and pSV2CAT, respectively. ME141 contains MLV LTRs, while ME138 contains Rev LTRs.

TABLE 1. Progeny virus titers from Rev and Am-MLV-infected cells[a]

Cell	Virus	Virus titer (IU/ml)
Chicken	Rev	10^7
	Am-MLV	10^4
Dog	Rev	10^6
	Am-MLV	10^4
Rat	Rev	10^1
	Am-MLV	10^3

[a] CEF, dog (D17), and rat (Rat-1) cells were infected in parallel with Rev, and the titer of progeny virus was determined on CEF by cytopathic effect 5 days after infection. Am-MLV recovered from infected CEF, D17, and rat (BRL TK^-) cells was quantified by focus formation on S^+L^- cells 7 days after infection (2,3). The titer of Am-MLV recovered from NIH 3T3 cells infected in parallel was 10^6 IU/ml.

Thymidine kinase assay. The thymidine kinase assay was done by the method of Emerman and Temin (11). Pools of Neo^r cell clones derived from infection of BRL TK^- cells with ME123(Rev) and ME140(Am-MLV) were grown to confluency, and extracts were made. Stocks of ME123(Rev) were made by cotransfection of CEF with pME123 (10) and a molecular clone of Rev. Stocks of ME140(Am-MLV) were made by superinfection of Neo^r D17 cells which had been cotransfected with pME140 and SV2*neo* with Am-MLV. Culture dishes with 20 to 50 Neo^r BRL TK^- cell clones were pooled, and 40 μg of protein was assayed.

Viruses. Viruses were recovered from cells by four different methods.

(i) Superinfection. Stocks of replication-competent reticuloendotheliosis helper virus were derived by transfection of CEF with a DNA clone of Rev (pSW253) (42). Stocks of replication-competent Am-MLV were derived by transfection of NIH 3T3 cells by prA8 (35) or from a stock of mink cells chronically infected with Am-MLV (generously provided by R. Risser). D17-derived cell clones (D17-MVL110.A, D17-cistorneo.A3, and D17-REV111. HF3) harboring a provirus with a selectable gene or G418-resistant D17 cells which had been cotransfected with SV2*neo* (37) and retroviral vector DNAs (pME140 or pME141) in a 1:10 ratio were superinfected with stocks of either replication-competent Rev or Am-MLV. Superinfection and cotransfection of dog or chicken cells (see below) produced virus stocks with both defective and helper virus. Virus was harvested 5 to 7 days after infection and was subjected to three freeze-thaw cycles before infection of fresh cells.

(ii) Cotransfection of chicken cells. CEF were cotransfected with Rev DNA to supply helper virus and one of the recombinant plasmids in a ratio of 0.5 μg of helper virus DNA to 5 μg of recombinant viral DNA per 60-mm dish. Virus was harvested 4 to 6 days after transfection.

(iii) Transfection of a Rev helper cell line. D17-C3 cells in 60-mm dishes were transfected with 5μg of a given recombinant plasmid per dish. Virus was harvested 4 to 6 days after transfection and was subjected to three freeze-thaw cycles before infection of fresh cells.

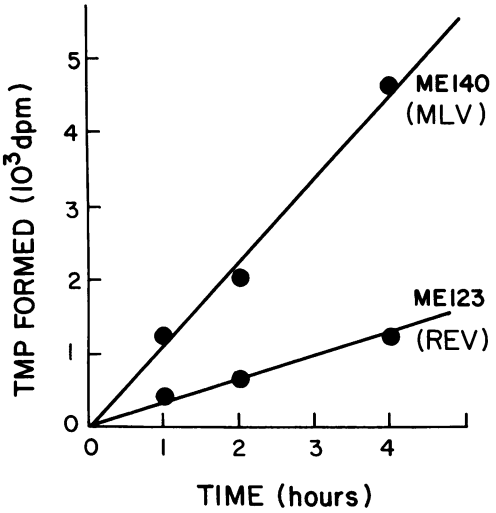


FIG. 3. Assay of thymidine kinase activity in ME123 and ME140 infected rat (BRL TK$^-$) cells. ME123 is a Rev-based vector. ME140 is a MLV-based vector. Extracts from pooled Neo^r TK$^-$ cell clones were analyzed for their thymidine kinase activity as previously described (11).

(iv) Cotransfection of dog (D17) cells with retroviral vector and helper viral DNAs. D17 cells were cotransfected with an *Eco*RI-digested and ligated permuted clone of 4070A (kindly provided by J. Horowitz) and recombinant plasmids in a ratio of 0.5 μg of helper virus to 5 μg of recombinant viral DNA per 60-mm dish. The virus was harvested 5 to 7 days after transfection.

Stocks of wild-type Rev were quantified by cytopathic effects on chicken cells by endpoint dilution (39). Stocks of Am-MLV were quantified on S$^+$L$^-$ cells by focus formation (2, 3).

Infections were carried out in the presence of 10 μg of Polybrene per ml for CEF and 100 μg of Polybrene per ml for all other cells. All transfections were done by the calcium phosphate method (15).

RESULTS

Chicken and dog cells are permissive and rat cells are semipermissive for Rev replication. Infection of chicken (CEF), dog (D17, an osteosarcoma cell line), and rat (Rat-1) cells with Rev produced progeny virus titers of 10^7, 10^6, and 10 IU/ml, respectively (Table 1). Previous work in our laboratory had shown that Rev proviruses are formed in chicken, dog, and rat (BRL TK$^-$) cells (1, 24). Chicken, dog, and rat cells are semipermissive for Am-MLV replication, producing 100- to 300-fold less virus than mouse cells (NIH 3T3) infected with the same amount of virus (Table 1).

Rat cells have less Rev RNA than do chicken cells early after infection. To determine whether Rev RNA was present in rat cells, rat and chicken cells were infected in parallel, and the amount of Rev-specific RNA was determined by RNA dot blots 2 days after infection. There was 250 times less Rev RNA in rat cells than in chicken cells (data not shown). This difference could be due to different synthesis rates or different RNA stabilities. To determine whether the block is at RNA synthesis (transcription), we used the CAT assay.

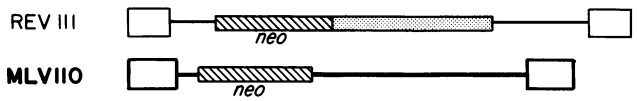
Rev LTR is transcribed in rat cells. To determine whether transcription from the Rev LTR is limiting for virus production in rat cells, we used the CAT assay as a measure of the relative transcriptional strength of the Rev LTR.

The results of the transient CAT assays for chicken and dog cells are shown in Fig. 2. In chicken and dog cells, the RSV, Rev, and MLV LTRs (pRSVCAT, pREV133, and pMLV120, respectively [Fig. 1]) are strong transcriptional elements, while the SV40 early gene transcriptional elements are weaker (pSV2CAT [Fig. 1]). Data from transient transfection of rat cells (Rat-1 and BRL TK$^-$) were too low to be interpreted (data not shown). Therefore, we used pooled TK$^+$ cell clones derived by infection of BRL TK$^-$ cells with ME138(Rev) and ME141(Am-MLV) (Fig. 1) for the CAT assay, and pooled Neo^r cell clones derived by infection of BRL TK$^-$ cells with ME123(Rev) and ME140(Am-MLV) (Fig. 1) for the thymidine kinase assay (see above) to determine the relative strength of the Rev and MLV LTRs in rat cells. The results (Fig. 2 and 3) show that the Rev LTR was about one-third the strength of the MLV LTR in rat cells. The difference between the strengths of the Rev and MLV LTRs may be less, since the MLV LTR is less suppressed than the Rev LTR when an internal *tk* promoter is selected, resulting in decreased expression from the Rev, but not the MLV LTR (M. Emerman, personal communication). These results show that the Rev LTR is actively transcribed in rat cells.

Efficiency of pseudotype formation of Rev- and MLV-derived genomes with Rev and MLV proteins is not reciprocal. To determine whether there is a block to Rev replication in rat cells before transcription, we produced Rev-Am-MLV pseudotypes. These pseudotypes contain *gag*, *pol*, and *env* proteins from either Rev or Am-MLV. Rev- and MLV-derived defective vectors (pREV111 and pMLV110, respectively [Fig. 4]) carrying a G418 resistance marker (*neo*) from the bacterial transposon Tn*5* were used so that the number of cells with at least one defective provirus could be determined by counting the number of Neo^r colonies after infection with each virus stock.

Data from only one of three methods used to form pseudotypes in dog cells are presented (see above for a description of the methods), since all three gave similar results for the efficiency of pseudotype formation.

Figure 4 shows the G418 resistance titers (NEO TU per



VIRUS		VIRUS TITER (NEO TU/ml)	
GENOME	PROTEINS	DOG	RAT
REV 111	REV	2×10^5	5×10^4
MLV110	REV	1×10^2	50
MLV110	Am-MLV	1×10^5	5×10^4
REV 111	Am-MLV	1×10^4	2×10^3

FIG. 4. NEO TU titers of REV111 and MLV110 pseudotypes on different species. Large open boxes in pREV111 and pMLV110 represent the Rev and MLV LTRs, respectively. Thin straight lines represent Rev-derived sequences, while the heavy straight lines represent Ha-MSV-derived sequences. Hatched boxes represent the bacterial aminoglycoside 3′ phosphotransferase gene (*neo*). The dotted box represents the herpes simplex virus thymidine kinase gene (*tk*). Virus stocks were derived by superinfection of dog cell clones. Stocks were titered for their Neo^r transforming ability on dog (D17) and rat (BRL TK$^-$) cells.

milliliter) on dog cells of virus stocks produced by superinfection of dog cell clones. The virus titers where the homologous proteins packaged and subsequently reverse transcribed and integrated the viral genomes forming a provirus [i.e., REV111 (Rev) and MLV110(Am-MLV)] are relatively high and very similar, indicating that Rev and Am-MLV replicate with about the same efficiency in dog cells. The efficiency of murine amphotropic proteins interacting with a heterologous versus a homologous genome [i.e., REV111 (Am-MLV) versus MLV110(Am-MLV)] is 10%, while the efficiency of Rev proteins interacting with a heterologous versus a homologous genome [i.e., MLV110(Rev) versus REV111(Rev)] is 0.05%.

pMLV110 is derived from Ha-MSV and has VL30 E. To determine the efficiency of pseudotype formation of a genome containing MLV E with Rev proteins, an Am-MLV *neo*-containing pseudotype (cistorneo [35]) was prepared. The efficiency of Rev proteins interacting with the cistorneo genome is comparable to that of MLV110 relative to the REV111 genome (0.1%, Table 2).

Block(s) to Rev replication in rat cells is posttranscriptional. Once the efficiency of pseudotype formation was determined, we infected rat cells with these stocks to determine whether there is a block to replication before transcription.

REV111(Rev) had similar NEO TU titers on dog and rat cells (Fig. 4). Therefore, all REV111(Rev) pretranscriptional events in dog and rat cells are equally efficient. Since all replication events through integration and expression for REV111(Rev) are equally efficient in dog and rat cells, while very little progeny virus is produced from Rev-infected rat cells, there is a posttranscriptional block to Rev replication in rat cells. MLV110(Am-MLV), MLV110(Rev), and REV111(Am-MLV) each have approximately the same NEO TU titers on both types of cells, indicating that each pseudotype forms a provirus and expresses the *neo* gene with approximately the same efficiency in dog and rat cells.

DISCUSSION

We made retroviral vectors and produced pseudotypes of these vectors to determine (i) the specificity of the interactions of retroviral and host proteins with retroviral nucleic acids and (ii) the nature of the block to Rev replication in rat cells.

Pseudotype formation. We determined the frequency at which Rev and Am-MLV proteins can form an infectious virus containing a Rev E and other Rev-derived control sequences, a MLV E and MLV-derived control sequences, or a VL30 E and MLV-derived control sequences.

We found that the Rev-derived vector, pREV111, is pseudotyped, and proviruses are formed efficiently by Am-MLV proteins. The relatedness of Rev to MLV may account for the ability of the MLV proteins to complement the Rev genome in the formation of pseudotypes (43, 44). However,

TABLE 2. NEO TU titers of Rev and Am-MLV pseudotypes on dog cells

Virus[a]		Virus titer (NEO TU/ml)
Genome	Protein	
REV111	Rev	5×10^4
Cistorneo	Rev	50
Cistorneo	Am-MLV	3×10^4
REV111	Am-MLV	8×10^3

[a] Virus stocks were derived by superinfection of dog cell clones and were assayed for their Neo^r transforming ability on dog cells.

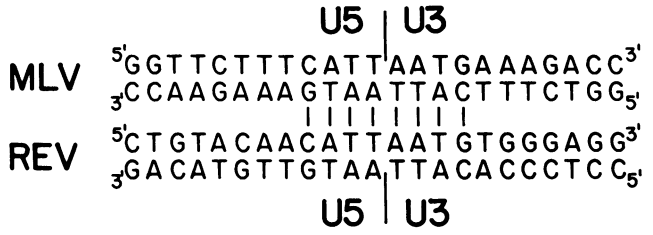


FIG. 5. Comparison of the nucleotide sequences surrounding the murine leukemia virus (34, 35) and Rev (29, 30) *att* sites. Homologies are marked by vertical lines.

the Ha-MSV-derived vector, pMLV110, and the Am-MLV-derived vector, cistorneo, showed a much lower efficiency of pseudotype and provirus formation with Rev proteins. This result indicates that heterologous pseudotype formation is not reciprocal for Rev-MLV pseudotypes. The low production of MLV110(Rev) and cistorneo(Rev) probably is the result of competition with wild-type Rev at packaging, since similar results were seen from virus produced by cotransfection of CEF with pMLV110 or cistorneo and Rev helper DNA (data not shown).

The ability to form pseudotypes may be due to the recognition of E by viral proteins. The retroviral nucleic acid sequences that are necessary for encapsidation have been defined by deletion analysis for Rev (41), Moloney-MLV (28), and Am-MLV (35). To determine if the E sequences of MLV and Rev have any homologies, a "best fit analysis" was done with the University of Wisconsin Genetics Computer Users Group programs (6). For Rev, Moloney-MLV, and Am-MLV, no significant homologies between reticuloendotheliosis and murine virus sequences were found in the regions determined by deletion analysis to be E sequences (data not shown). Therefore, the interaction of viral RNA (E) with viral proteins in virus formation is not sequence specific or sequences other than the wild-type E (as defined previously) are used for packaging.

Other viral protein-viral nucleic acid interactions occur during reverse transcription and integration; for example, the *pol* gene encodes an endonuclease which acts on the *att* site. The *att* sites for MLV and Rev show an 8-bp region of perfect homology surrounding the LTR-LTR junction (Fig. 5). This identity may be responsible for the ability of the *pol* gene product(s) from Rev and MLV to act on heterologous *att* sites.

Blocks to Rev replication in rat cells. (i) There is less Rev RNA and viral DNA in Rev-infected rat versus chicken cells. The lack of Rev progeny virus from Rev-infected rat cells indicated a significant block to Rev replication in these cells (Table 1). Analysis of unintegrated viral DNA in Rev-infected rat and chicken cells showed little or no unintegrated viral DNA present in Rev-infected rat cells (unpublished data). We found there was 250 times less Rev RNA in infected rat than chicken cells. These data indicate that the block(s) to Rev replication probably occurs during the initial round of infection and results in less viral RNA available for translation or packaging. To determine whether the block is at the level of transcription, we determined the relative strength of the Rev LTR by using the CAT assay.

(ii) Rev LTR is active in rat cells, but the strength of an LTR does not always correlate with virus production. We compared the relative efficiency of the Rev and MLV LTRs in both transient (CAT) and stable (*tk*) transcriptional assays in rat cells. CAT and *tk* data (Fig. 2 and 3) showed that the Rev

and MLV LTRs are transcriptionally active in rat cells and are approximately the same strength. However, transcriptional strength, determined by the CAT assay, is not a good indicator of virus replication. In chicken and dog cells the Rev and MLV LTRs are similar strengths, yet Rev replicates well in both species, while Am-MLV is semipermissive in these cells. Rat cells are semipermissive for Am-MLV and Rev replication, yet Am-MLV progeny titers are 3 logs higher than Rev progeny titers from Am-MLV- and Rev-infected rat cells, respectively.

Surprisingly, when the CAT gene is placed at a position analogous to that of the *gag* gene in the Rev genome, and the CAT mRNA terminates in the 3′ LTR rather than in SV40 sequences, the Rev LTR is not a strong transcriptional element in chicken and dog cells (pREV118 [data not shown]).

(iii) Rev replication is blocked posttranscriptionally in rat cells. To determine the efficiency of Rev replication through the steps of integration and expression, we compared the titers of Rev-Am-MLV pseudotypes on dog and rat cells. For each virus, the titers were similar on both dog and rat cells, with the titer on the dog cells always being higher (Fig. 4). This difference probably reflects the relative strengths of the Rev and MLV LTRs in dog versus rat cells (i.e., the Rev LTR is four- to fivefold stronger and the MLV LTR twofold stronger in dog than rat cells). Alternatively, different RNA stabilities could be involved. Since the titers of each of the viruses on dog and rat cells are similar, the efficiency of viral replication through integration and expression is the same in both species.

Since Rev is not produced from Rev-infected rat cells, these results indicate that there is a posttranscriptional block which limits Rev production in rat cells.

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