Profound Amplification of Pathogenic Murine Polytopic Retrovirus Release from Coinfected Cells

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Previous studies indicate that mice infected with mixtures of mouse retroviruses (murine leukemia viruses [MuLVs]) exhibit dramatically altered pathology compared to mice infected with individual viruses of the mixture. Coinoculation of the ecotropic virus Friend MuLV (F-MuLV) with Fr98, a polytopic MuLV, induced a rapidly fatal neurological disease that was not observed in infections with either virus alone. The polytopic virus load in coinoculated mice was markedly enhanced, while the ecotropic F-MuLV load was unchanged. Furthermore, pseudotyping of the polytopic MuLV genome within ecotropic virions was nearly complete in coinoculated mice. In an effort to better understand these phenomena, we examined mixed retrovirus infections by utilizing *in vitro* cell lines. Similar to *in vivo* mixed infections, the polytopic MuLV genome was extensively pseudotyped within ecotropic virions; polytopic virus release was profoundly elevated in coinfecting cells, and the ecotropic virus release was unchanged. A reduced level of polytopic SU protein on the surfaces of coinfecting cells was observed and correlated with a reduced level of nonpseudotyped polytopic virion release. Marked amplification and pseudotyping of the polytopic MuLV were also observed in mixed Fr98–F-MuLV infections of cell lines derived from the central nervous system (CNS), the target for Fr98 pathogenesis. Additional experiments indicated that pseudotyping contributed to the elevated polytopic virus titer by increasing the efficiency of packaging and release of the polytopic genomes within ecotropic virions. Mixed infections are the rule rather than the exception in retroviral infection, and the ability to examine them *in vitro* should facilitate a more thorough understanding of retroviral interactions in general.

Infection of mice by exogenous ecotropic murine leukemia viruses (MuLVs) frequently results in the generation of recombinant viruses in which a portion of the envelope gene encoding the receptor-binding region has been replaced with the analogous envelope sequences derived from endogenous polytopic retroviruses (1–3, 6, 8, 11, 18, 20, 21, 27, 29, 39). The polytopic *env* gene encodes a protein (SU) that utilizes a cell surface receptor distinct from the ecotropic receptor, resulting in an altered infectious host range of the recombinants (4, 41, 46). Ecotropic MuLVs infect only cells of murine origin, whereas recombinant polytopic MuLVs infect murine cells as well as cells of numerous other species (16). Although some endogenous polytopic proviruses carry intact retroviral genes, replication-competent endogenous polytopic viruses have not been described (2, 39). The generation of recombinant polytopic MuLVs is thought to be instrumental in proliferative diseases resulting from ecotropic MuLV infection (7, 25, 35, 40, 42–45). A principal mechanism of oncogenesis ultimately involves the integration of MuLVs near proto-oncogenes and may involve either the exogenous ecotropic virus or the recombinant polytopic MuLVs (5, 17, 32, 44, 45). It is also apparent that *in vivo* interactions of the viruses in the mixed infections generated by recombination strongly influence the pathogenicity of the retroviruses. In this regard, we have described *in vivo* interactions between ecotropic and recombinant polytopic MuLVs that may facilitate a stepwise process of leukemogenesis in mice infected with Moloney murine leukemia virus (M-MuLV) (29). More recently, we examined the interactions of ecotropic and polytopic MuLVs that were inoculated as virus mixtures into mice (22). Coinoculation of the ecotropic virus Friend murine leukemia virus (F-MuLV) with one polytopic virus (Fr54) greatly extended the survival times of mice compared to those of mice inoculated with F-MuLV alone, while coinoculation of F-MuLV

with another polytopic virus (Fr98) induced a rapidly fatal neurological disease that was not observed in infections with either virus by itself (22). In both instances, we observed nearly complete pseudotyping of the polytopic virus RNA genome within ecotropic virions. Furthermore, the polytopic virus load was greatly elevated in coinoculated mice compared to that in mice inoculated with the polytopic virus alone. In contrast, the ecotropic virus load in coinoculated mice was unchanged. The processes leading to the extensive pseudotyping of polytopic MuLVs and the elevation of the polytopic virus load are not known, nor is it known if there is a relationship between pseudotyping and an elevated polytopic virus load.

In the present study, we examined coinfection of *in vitro* cell lines by ecotropic and polytopic MuLVs. Our *in vitro* observations are remarkably similar to what was observed *in vivo* and have enabled us to examine the interactions of retroviruses in mixed infections in a less complex setting. Our results indicate that the release of polytopic MuLV virion RNA from the cell is greatly facilitated by pseudotyping within ecotropic virions and strongly suggest that the recombinant polytopic MuLV Env proteins are

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less efficient participants in virion assembly processes than ecotropic MuLV Env proteins.

MATERIALS AND METHODS

Cells, viruses, and vectors. NIH 3T3 cells were used for the propagation of viruses and for assays of MuLVs. All cells were maintained in tissue culture media supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (25 µg/ml), gentamicin (50 µg/ml), and amphotericin B (2 µg/ml). NIH 3T3 cells, the mouse microglial cell line 1094MG, and the neural stem cell line C17.2 (38) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated bovine calf serum. The 1094MG cell line was obtained from William Lynch, Iowa State University. *Mus dunni* cells (28) were maintained in RPMI medium containing 10% heat-inactivated fetal bovine serum. The ecotropic MuLV F-MuLV 57 (31), the amphotropic MuLV 4070A (24), and the polytropic MuLVs Fr98 (34) and Fr54 were obtained as virus stocks after transfection of NIH 3T3 cells with plasmids encoding each provirus and subsequent infection of *Mus dunni* cells. The ecotropic MuLVs M-MuLV 1387 and AKR 2A (13) were harvested from infected NIH 3T3 cells, and the polytropic MuLVs FrNx (1), 383-2T (21), M-RV 2A, and M-RV 7A (2) were harvested from infected *Mus dunni* cells. The AKV recombinant viruses AKR 13, MCF 247, and Akv-2-C34 (14) were originally obtained as virus stocks from Miles Cloyd and Janet Hartley (14). M73P-S is a polytropic virus isolated from AKR/J mice in our laboratory (23). Virus stocks of the AKV recombinants were harvested from chronically infected *Mus dunni* cells. The retroviral vector G1nβgSVNa (Gene Therapy Inc., Gaithersburg, MD) carries the *Escherichia coli* β-galactosidase (β-Gal) gene with the simian virus 40 (SV40) large T-antigen nuclear localization signal driven by the M-MuLV long terminal repeat. LAPSIN (Clontech Laboratories, Inc., Palo Alto, CA) contains the human placental alkaline phosphatase (AP) gene driven by the M-MuLV long terminal repeat.

Infectivity, transduction assays, and MABs. Infectious MuLVs were quantified using monoclonal antibodies (MAB) specific for the SU proteins of the viruses in a focal immunofluorescence assay (FIA) (37). Briefly, NIH 3T3 cells were seeded and infected with serial dilutions of samples containing 8 µg/ml of Polybrene (Sigma-Aldrich). The cells were allowed to grow to confluence (5 days). The monolayers were incubated with MABs (~0.1 ml per dish of medium harvested from hybridoma cell lines producing the MAB), rinsed, and then incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Ig) to bind to the MABs on infected cells. Foci of infected cells were detected and quantified by fluorescence microscopy. The MABs employed in the assays included MAB 538, which is specifically reactive with M-MuLV (29); MAB 48, which is specifically reactive with F-MuLV (10); and MAB 514 and MAB 516 (9), which react specifically with polytropic MuLVs. For transduction assays, NIH 3T3 cells were initially transfected with plasmids carrying the retroviral vector LAPSIN or G1nβgSVNa and then cultured in the presence of G418 to select for transduced cells. The transduced cultures were then infected with the polytropic MuLV Fr98 to establish cell lines releasing progeny polytropic MuLVs as well as retroviral vectors pseudotyped within polytropic virions. The polytropic MuLVs released from the cultures were assessed using the FIA described above. Transduction by released vectors (LAPSIN or G1nβgSVNa) encapsulated in MuLV virions was quantified as previously described, using assays for AP or β-Gal activity (26) in NIH 3T3 cells.

Pseudotyping of polytropic genomes within ecotropic or amphotropic virions was assessed by infectivity assays using uninfected target cells and cells which were chronically infected with ecotropic or amphotropic MuLVs. The chronically infected cells were completely refractory to infection by virions utilizing the same receptor, including virions containing pseudotyped polytropic genomes. The degree of pseudotyping was assessed by comparisons of infectivity for uninfected target cells susceptible to both pseudotyped and nonpseudotyped polytropic virions and infectivity for chronically infected cells susceptible only to nonpseudotyped polytropic virions. Similarly, pseudotyping of the LAPSIN and

G1nβgSVNa vectors was assessed by the AP or β-Gal assay on NIH 3T3 cells as well as on NIH 3T3 cells infected by the ecotropic MuLV, to assess the degree of pseudotyping of the vectors within ecotropic virions.

Cell surface staining and flow cytometry. Cells infected with the polytropic virus Fr98 or infected with Fr98 and superinfected with F-MuLV were treated with phosphate-buffered saline (PBS) containing 0.002 M EDTA to detach the cells from the tissue culture plate. The cells were incubated with MAB 514, reactive with the SU protein of Fr98, followed by incubation with FITC-conjugated goat anti-mouse Ig. Similarly, cells infected with the ecotropic virus F-MuLV or infected with F-MuLV and superinfected with Fr98 were detached and incubated with MAB 48, reactive with the SU protein of F-MuLV, followed by incubation with FITC-conjugated goat anti-mouse Ig. Data were collected by an LSRII (BD Biosciences) flow cytometer and were analyzed using FlowJo (Tree Star).

Quantification of virion RNA. Comparisons of the amounts of Fr98 virion RNA released from cells were accomplished using an RNase protection assay (RPA). Total RNA from a virus pellet equivalent to 1 ml of tissue culture supernatant was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was then analyzed for specific viral RNA by use of a Riboquant system (BD Biosciences). A probe specific to Fr98 envelope RNA was generated using the plasmid pc1579, which contained nucleotides 310 to 503 of the Fr98 envelope gene. The plasmid was linearized using EcoRI and transcribed with T7 polymerase, using Riboquant reaction mix and ³²P-labeled UTP (NEN Life Sciences). The probe was quantified and then mixed with RNA samples. Samples containing the probe were heated to 90°C and then incubated at 56°C overnight. Following hybridization, samples were treated with RNase A, phenol-chloroform extracted, and ethanol precipitated following the Riboquant protocol (BD Biosciences). Samples were analyzed on a precast Tris-borate-EDTA (TBE)-urea gel (Bio-Rad). The ³²P-labeled protected probe was quantified using a Storm phosphorimager (Amersham Biosciences, Piscataway, NJ) and ImageQuant software. Samples without viral RNA were used as a negative control to ensure RNase degradation of unbound labeled probe.

RESULTS

Amplification of polytropic MuLVs released from cell lines coinfecting with ecotropic MuLVs. In previous studies, we observed that inoculation of mice with mixtures of the ecotropic virus F-MuLV and the polytropic isolate Fr98 or Fr54 resulted in a profound elevation of the polytropic virus load (22, 33). To determine if this occurred *in vitro*, we compared infectious polytropic viruses released from cell lines infected with only the polytropic virus to polytropic viruses released from cells infected with both the polytropic virus and F-MuLV. Chronically infected NIH 3T3 cell lines were established that released stable levels of polytropic MuLVs. The cells were then superinfected with an ecotropic MuLV at a high multiplicity of infection to achieve complete ecotropic MuLV infection of the culture. This approach was taken to readily establish ecotropic viral interference, thereby minimizing reinfection of the cells by the polytropic MuLV because of ecotropic viral pseudotyping.

F-MuLV superinfection of NIH 3T3 cells chronically infected with either Fr54 or Fr98 resulted in a profound increase in the polytropic MuLV titer compared to that in chronically infected cells that were mock infected (Fig. 1A). This observation is consistent with our *in vivo* results indicating highly elevated polytropic MuLV replication in coinoculated mice (22, 33). Similar results were observed when *M. dunni* cells chronically infected with Fr98 were superinfected with F-MuLV.

It was of interest to determine if the amplification of the polytropic MuLVs by coinfection with ecotropic viruses extended to virus combinations other than the ecotropic virus F-MuLV and its

FIG 1 Amplification of polytypic viruses but not ecotropic viruses released from coinfecting cells. (A) NIH 3T3 cells chronically infected with the polytypic MuLV Fr54 or Fr98 were mock infected or superinfected with the ecotropic virus F-MuLV. The cells were grown to confluence, the medium was changed, and the virus was harvested after 6 h. Polytypic MuLV titers were assessed on uninfected NIH 3T3 cells by use of an FIA using MAb 514. Fr54 and Fr98, polytypic viruses released after mock infections of the respective cultures; Fr54 + F-MuLV and Fr98 + F-MuLV, polytypic viruses released after superinfection of the cultures with F-MuLV. Values represent the means and standard errors of the means (SEM) for duplicate or triplicate determinations of experiments on three separate cultures. (B) NIH 3T3 cells chronically infected with the ecotropic virus F-MuLV were mock infected or superinfected with the polytypic MuLV Fr98. After the cells reached confluence, the medium was changed, and the virus was harvested after 6 h. F-MuLV titers were assessed on uninfected NIH 3T3 cells by use of an FIA using MAb 48. F-MuLV, ecotropic virus released after mock infections of the F-MuLV-infected cultures; F-MuLV + Fr98, ecotropic virus released after superinfection of the cultures with Fr98. Values represent means and SEM for duplicate or triplicate determinations of experiments on three separate cultures. FFU, focus-forming units.

derivative polytypic recombinant viruses. We examined the effects of coinfections of various combinations of MuLVs. These included other ecotropic MuLVs coinfecting with their derivative polytypic MuLVs, ecotropic MuLVs coinfecting with polytypic MuLVs from a different ecotropic parent MuLV, and an amphotropic MuLV coinfecting with polytypic MuLVs. The viruses used included both class I leukemogenic polytypic MuLVs and class II nonleukemic polytypic MuLVs derived from AKR mice (27, 32, 33).

The ecotropic MuLV component of *in vitro* mixed infections is not amplified. Our previous *in vivo* studies of mixed infection revealed a profound elevation of the polytypic MuLV titer in coinfecting mice compared to that in mice infected with only the polytypic MuLV. In contrast, the level of ecotropic MuLV in coinfecting mice was the same as that in mice inoculated with only the ecotropic MuLV. To examine this phenomenon *in vitro*, cell lines chronically infected with the ecotropic virus F-MuLV were superinfected with the polytypic MuLV Fr98, and the ecotropic infectivity was determined. The level of ecotropic MuLV released from the coinfecting cells was not significantly altered compared to that for mock-infected control cells (Fig. 1B).

Amplification of retroviral vectors released from coinfecting cell lines. To determine if the amplification of polytypic infectivity reflected a distinctive property of polytypic genomic RNA, we

examined the effect of coinfection on the release of retroviral vectors from *in vitro* cell lines. Cell lines harboring retroviral vectors encoding either β -Gal (G1n β gSVNa) or AP (LAPSN) were infected with the polytypic MuLV Fr98 and passaged in culture several times to establish a chronic infection. Such cells release a mixture of virions, i.e., those which have packaged the vector genome and those which have packaged the polytypic Fr98 genome. The cells were then superinfected with F-MuLV, and the titers of the packaged vectors as well as the packaged Fr98 genome released from the superinfected cell lines were compared to titers released from cells infected with the polytypic virus alone. All titers were assessed using uninfected NIH 3T3 cells and the AP assay, the β -Gal assay, or an FIA using MAb 514, which is reactive with the polytypic SU protein. With both vectors, a greatly elevated transduction titer was observed for viruses released from the coinfecting cells, and it closely paralleled the increase in polytypic MuLV titers measured by FIA (Fig. 2). These results indicate that amplification of infectivity of the polytypic MuLV in mixed infections is not due to an inherent property of the polytypic MuLV RNA.

Polytypic MuLV genomes released from coinfecting cells are extensively pseudotyped within ecotropic virions. Polytypic viral genomes released from coinfecting mice are extensively pseudotyped by ecotropic MuLVs (22). This is true with coinoculated mice as well as with mice which have generated polytypic viruses after infection with an ecotropic MuLV (29, 36). To determine if the polytypic MuLVs released from coinfecting *in vitro* cell lines are also pseudotyped within ecotropic virions, the infectious titers of polytypic MuLVs were assessed on uninfected NIH 3T3 cells as well as NIH 3T3 cells that were chronically infected with the ecotropic MuLV. Polytypic MuLV genomes packaged in polytypic virions or pseudotyped within ecotropic virions can infect uninfected target cells because the receptors recognized by the SU proteins of the polytypic or ecotropic virions are not blocked. However, target cells chronically infected with the ecotropic MuLV are refractory to infection by all ecotropic virions, including those containing pseudotyped polytypic RNA genomes. Thus, extensively pseudotyped polytypic viruses exhibit a greatly reduced titer on ecotropic MuLV-infected cells, in which the ecotropic MuLV receptor is blocked, in contrast to the case with uninfected target cells. The polytypic MuLVs released from all of the cultures coinfecting with ecotropic and polytypic MuLVs exhibited extensive pseudotyping of the polytypic MuLV (Table 1). Amplification of the retroviral vectors described earlier (Fig. 2) was also associated with extensive pseudotyping of the vectors within ecotropic virions (data not shown).

Amplification and pseudotyping of the polytypic virus Fr98 are observed in cells of CNS origin. All of the *in vitro* experiments described above employed fibroblastic cell lines. It was of interest to determine if the results could be extended to other cell types. In previous studies, we found that coinfection of Fr98 and F-MuLV resulted in a severe neurological disease that was not seen when either virus was inoculated alone (22). The disease, which culminated as early as 10 days after infection, was associated with a massive infection of the central nervous system (CNS) by the polytypic Fr98 virus that was not observed in mice infected with Fr98 alone. Thus, it seemed appropriate to determine if the amplification and pseudotyping observed upon coinfection of fibroblasts occurred in cells of CNS origin. A microglial cell line (1094MG) and a neural stem cell line (C17.2) were infected with Fr98, and the

FIG 2 Release of polytropic MuLV and retroviral vectors after superinfection with F-MuLV. NIH 3T3 cells harboring the retroviral vector LAPSN or GlnβgSVNa and chronically infected with the polytropic MuLV Fr98 were mock infected or superinfected with F-MuLV. After the cells reached confluence, the medium was changed, and the virus was harvested after 6 h. The release of infectious virions that had packaged the Fr98 genome, the LAPSN vector, or the GlnβgSVNa vector was quantified by an FIA or by AP or β-Gal assay of uninfected NIH 3T3 cells, respectively. (A) Release of infectious virions that had packaged the Fr98 genome as assessed by FIA. Fr98, Fr98 released from mock-infected cultures; Fr98 + F-MuLV, Fr98 released from F-MuLV-superinfected cultures. (B) Release of infectious virions as assessed by AP assay. AP(Fr98), transduction titers of LAPSN released from mock-infected cultures that harbored LAPSN and were chronically infected with Fr98; AP(Fr98) + F-MuLV, transduction titers from these cells after superinfection with F-MuLV. (C) Release of infectious virions as assessed by β-Gal assay. βgal(Fr98), transduction titers of GlnβgSVNa released from mock-infected cultures that harbored GlnβgSVNa and were chronically infected with Fr98; βgal(Fr98) + F-MuLV, transduction titers from these cells after superinfection with F-MuLV. Values for all measurements represent the means and SEM for duplicate or triplicate determinations of experiments on three separate cultures.

cultures were passed several times to establish chronic infections. The cells were then superinfected with F-MuLV or mock infected, and the progeny viruses released from the cultures were assessed for polytropic virus titers as well as for pseudotyping of Fr98 within ecotropic virions. With both CNS cell lines, we ob-

served amplification of the polytropic virus that was quite similar to our observations with fibroblasts (Fig. 3). The level of Fr98 pseudotyped with ecotropic virions was >95% for the C17.2 neural stem cell line and >97% for the 1094MG microglial cells. Similar results were obtained using Fr54, which does not induce a rapid neurological disease (22, 33).

Cell surface expression of the polytropic surface envelope protein (SU) is depressed on coinfecting cells. The large increase

TABLE 1 Amplification and pseudotyping of polytropic MuLVs in mixed infections

Polytropic MuLV	Coinfected MuLV	Amplification ^a (fold increase)	Pseudotyping ^b (%)
F-MuLV recombinants			
Fr98	F-MuLV	39	>99
	M-MuLV	69	>99
	4070A	8	>99
Fr54	F-MuLV	231	98
	F-MuLV	54	>99
FrNx	M-MuLV	69	>99
M-MuLV recombinants			
383-2T	M-MuLV	57	>99
	F-MuLV	23	97
	AKV	38	>99
M-RV 7A	M-MuLV	16	93
	M-MuLV	13	98
AKV recombinants			
AKR 13	AKV	9	ND
	M-MuLV	7	ND
MCF 247	AKV	10	ND
Akv-2-C34	F-MuLV	7	>90
M73P-S	F-MuLV	8	>90

^a Calculated from duplicate or triplicate determinations as the titer of infectious polytropic MuLV released from coinfecting cells divided by the titer of infectious polytropic MuLV released from cells infected with the polytropic MuLV alone, as determined by serial dilution assays on NIH 3T3 cells.
^b Calculated from titers assessed on NIH 3T3 cells and titers assessed on NIH3T3 cells chronically infected with the coinfecting MuLV. ND, not determined.

FIG 3 Polytropic viruses released from CNS cell lines chronically infected with the polytropic MuLV Fr98 after superinfection with the ecotropic virus F-MuLV. The microglial cell line 1094MG and the neural stem cell line C17.2 were chronically infected with the polytropic MuLV Fr98 and subsequently mock infected (Fr98) or superinfected with F-MuLV (Fr98 + F-MuLV). The cultures were grown to confluence, the medium was changed, and the virus was harvested after 6 h. Polytropic MuLV titers were assessed on NIH 3T3 cells by using an FIA with Mab 514. The values represent the means and SEM for duplicate determinations on 3 separate cultures.

FIG 4 Decrease in nonpseudotyped polytypic virions released after superinfection with F-MuLV. NIH 3T3 cells chronically infected with the polytypic MuLV Fr98 were mock infected or superinfected with the ecotropic virus F-MuLV. The cells were grown to confluence, the medium was changed, and the virus was harvested after 6 h. The nonpseudotyped polytypic virus titer was assessed on NIH 3T3 target cells that were chronically infected with F-MuLV. These target cells are permissive to infection by nonpseudotyped polytypic virions but not by polytypic viruses pseudotyped within ecotropic virions. Fr98, nonpseudotyped polytypic virions released from mock-infected cells; Fr98 + F-MuLV, nonpseudotyped polytypic virions released from cells superinfected with F-MuLV. The values represent the means and SEM for 2 to 4 replicate experiments using two separate chronically Fr98-infected cultures. Each of the cultures released distinct levels of infectious polytypic virions before superinfection that were suppressed by a similar percentage after superinfection with F-MuLV.

in the infectious titers of polytypic viruses appears to be the result of pseudotyping, but coinfecting cells continue to release nonpseudotyped polytypic virus at levels only moderately lower than those in cells infected with the polytypic MuLV alone. This was evident from assessments of the levels of polytypic MuLV infection on target ecotropic MuLV-infected cells (Fig. 4). These target cells are refractory to infection by ecotropic virions but permissive to infection by polytypic virions and thus provide a measure of the level of virions utilizing the polytypic SU protein. The decrease in polytypic virion release in coinfecting cells correlated with a decreased level of polytypic SU on the surfaces of cells, as assessed by flow cytometry analyses using a monoclonal antibody specifically reactive to the protein. These observations are consistent with the possibility that the level of cell surface SU influenced the level of nonpseudotyped polytypic virions released from the cells (Fig. 5A). The level of cell surface F-MuLV ecotropic SU protein was unchanged on coinfecting cells (Fig. 5B).

Increased efficiency of packaging of the Fr98 genome contributes to the amplification of Fr98 after coinfection with F-MuLV or amphotropic MuLV. The marked increase in polytypic MuLV titers attributed to pseudotyped polytypic viruses could proceed by at least two mechanisms. If the ecotropic virion were exceedingly more infectious than the polytypic virion, pseudotyping of the polytypic genomic RNA within the ecotropic virion would result in a greatly increased polytypic MuLV titer. A second mechanism, not contingent upon differences in infectivity, could be an increased efficiency of virion assembly and RNA

FIG 5 Cell surface expression of SU proteins after superinfection of cells chronically infected with F-MuLV or Fr98. (A) NIH 3T3 cells infected with the polytypic virus Fr98 were subsequently coinfecting with the ecotropic virus F-MuLV (solid line) or mock coinfecting (shaded area) and then stained with MAb 514, which recognizes the polytypic envelope. Uninfected control cells are represented by the dashed line. (B) NIH 3T3 cells infected with the ecotropic virus F-MuLV were subsequently coinfecting with the polytypic virus Fr98 (solid line) or mock coinfecting (shaded area) and then stained with MAb 48, which recognizes the ecotropic envelope. Uninfected control cells are represented by the dashed line.

packaging by the ecotropic MuLV compared to the polytypic MuLV. In contrast to an increase in specific infectivity, an increase in the efficiency of polytypic RNA packaging would be reflected in the level of virion polytypic RNA released from the cell. To examine these possibilities, we quantified the polytypic RNA released from cells coinfecting with Fr98 and F-MuLV, cells coinfecting with Fr98 and the amphotropic virus 4070A, and cells infected with Fr98 alone. In addition, we assessed the polytypic virus titers and the degree of pseudotyping of the polytypic genome within ecotropic or amphotropic virions. A close correlation was observed between the level of polytypic infectivity and the level of virion polytypic RNA released from the cells (Fig. 6). Coinfection with either F-MuLV or 4070A resulted in >99% pseudotyping of the Fr98 genome.

FIG 6 Levels of polytropic Fr98 infectivity and virion RNA released from Fr98-infected cultures superinfected with the ecotropic virus F-MuLV or the amphotropic MuLV 4070A. NIH 3T3 cells chronically infected with the polytropic MuLV Fr98 were mock infected or superinfected with the ecotropic virus F-MuLV or the amphotropic MuLV 4070A. The cells were grown to confluence, the medium was changed, and the virus was harvested after 6 h. (A) Polytropic MuLV titers were assessed on uninfected NIH 3T3 cells. The values represent the means and SEM for triplicate determinations of the polytropic MuLV released from the cell lines. (B) Fr98 virion RNA released from cells chronically infected with Fr98 and superinfected with F-MuLV, superinfected with 4070A, or mock superinfected was assessed using an RNase protection assay. The lanes are labeled according to the superinfecting MuLV. The RNA control lane contained an irrelevant RNA sample taken through the assay procedure to control for complete digestion of the RNA probe in samples devoid of virion RNA.

DISCUSSION

Our previous studies of *in vivo* mixed retrovirus infections revealed remarkably altered pathology compared to that after infection with individual viruses of the mixture (19, 22). In those studies, the polytropic virus load in the coinoculated mice was markedly enhanced, while the ecotropic F-MuLV load was unchanged. Furthermore, the polytropic MuLV was nearly completely pseudotyped within ecotropic virions in coinoculated mice. It was possible that these observations reflected events restricted to *in vivo* infection, e.g., spread of the polytropic MuLV to different cell types facilitated by pseudotyping within ecotropic virions, ecotropic MuLV-induced expansion of target cells for the polytropic virus, or infection of cells that preferentially assemble ecotropic rather than polytropic envelope proteins into virions. Alternatively, some of these observations may reflect more basic features of coinfection by ecotropic and polytropic viruses, independent of the complex *in vivo* environment.

Our *in vitro* studies revealed a striking elevation of the polytropic MuLV infectivity released from coinfecting cells compared

to that released from cells infected with the polytropic MuLV alone. In all experiments, amplification was restricted to the polytropic MuLV component of the mixture, similar to our earlier *in vivo* results (22). It is apparent that this is a general phenomenon, as amplification of the polytropic MuLV was observed with many different polytropic-ecotropic MuLV pairs as well as with polytropic-amphotropic MuLV mixtures. In all instances, the amplification of the polytropic titer was accompanied by extensive pseudotyping of the polytropic genome within ecotropic or amphotropic virions. Thus, all or nearly all of the increase in polytropic MuLV infectivity observed was likely the result of pseudotyped polytropic MuLV genomes. Furthermore, the increased infectivity closely paralleled the level of polytropic virion RNA released from cells, indicating an increase in infectious particles rather than a large difference in specific infectivity between polytropic and ecotropic virions.

The explanation for the predominant packaging of the polytropic genome into ecotropic particles is unclear. Although polytropic and ecotropic SU proteins are present on coinfecting cells, most released virions exhibit an ecotropic host range. It is possible that virions are mosaic with respect to the SU proteins and that the infectivity of virions depends on the density of the receptor-binding SU proteins on the virion surface. The ecotropic SU protein, if present in a large excess over the polytropic SU protein, might simply dilute out the polytropic SU protein below a threshold density required for infection. If this were the case, one would predict that the release of nonpseudotyped polytropic viruses would be severely depressed. Our results do not support this hypothesis. We found that ecotropic pseudotyping of the polytropic genome did not severely supplant the release of polytropic virions, as evidenced by the continued release of virions utilizing the polytropic SU protein in coinfecting cells (Fig. 4). Rather, pseudotyping of the polytropic genome within ecotropic virions exerted an additive effect, augmenting the total polytropic infectious titer (pseudotyped and nonpseudotyped) released from the coinfecting cells.

A more likely explanation for the predominance of ecotropic virions in mixed infections is that the polytropic SU protein participates less efficiently in the virion assembly process than the ecotropic SU protein. Polytropic viruses are chimeric recombinant viruses comprised of genes from different retroviral genomes which have diverged along different evolutionary pathways. Indeed, many recombinant polytropic MuLVs encode a chimeric SU protein consisting of sequences derived from both the endogenous polytropic and the exogenous ecotropic parent. A chimeric protein may function less efficiently than either of the parental proteins. In this regard, functional impairment has been observed in chimeric viruses constructed between very closely related ecotropic MuLVs (30).

The decrease in cell surface polytropic SU protein in coinfecting cells may be pertinent to the moderately reduced levels of nonpseudotyped polytropic viruses released from the cell. The decreased cell surface polytropic SU protein on coinfecting cells could reflect competition for processing through the Golgi apparatus or competition for deposition on available plasma membrane sites. It is also possible that the ecotropic virus interferes at other stages in the polytropic MuLV replication cycle, such as RNA splicing to generate *env* mRNA or transport of RNA to the cytoplasm. Investigations of these aspects of interactions between

viruses in mixed infections should be facilitated greatly in an *in vitro* system that mirrors *in vivo* mixed infections.

We found that the levels of polytopic virion RNA released from cells infected with Fr98 or superinfected with either F-MuLV or an amphotropic MuLV correlated closely with the infectious polytopic titers released from the cells (Fig. 6). Furthermore, the polytopic genome was extensively pseudotyped in coinfections with either ecotropic or amphotropic MuLVs. It is possible that superinfection by either virus results in an elevated level of polytopic transcripts available for packaging. However, we observed nearly identical amplification of the polytopic MuLV titers and the titers of two different retroviral vectors after superinfection with F-MuLV (Fig. 2). It seems unlikely that transcriptional activation of different integrants would account for precisely the same elevation of transduction or infection. Rather, these results further support the premise that the increased efficiency in the assembly process of the ecotropic virus compared to the polytopic virus is responsible for the elevated polytopic virion RNA level.

Although differences in assembly efficiency contribute to the increase in infectious virus observed in these studies, it is likely that the specific infectivity of different virions also contributes. In this regard, recombinant polytopic MuLVs differ greatly in their ability to infect mouse cells (12, 20, 23). Ecotropic pseudotyping of a poorly infectious polytopic MuLV would be expected to increase its relative infectivity to a greater extent than pseudotyping of a polytopic virus that readily infects mouse cells.

In this study, we examined coinfection of *in vitro* cell lines by ecotropic and polytopic MuLVs. Our *in vitro* observations are remarkably similar to what we observed previously *in vivo*. Furthermore, these observations extend to different *in vitro* cell lines, including cells of CNS origin which are targets for the polytopic MuLV Fr98. Our studies strongly suggest that the effects of mixed infections on replication and pathology in the host reflect fundamental features of the interactions among retroviruses in mixed infections. Retroviral mixed infections are common in nature, and it is clear that interactions among retroviruses can have profound pathological consequences. In this regard, our results may be pertinent to early studies of polytopic viruses from AKR mice as well as from certain other mouse strains (14, 15). In those studies, which identified both pathogenic and nonpathogenic viruses, pathogenic recombinant viruses were identified by examining the ability to accelerate disease in mice which harbor and express high levels of endogenous ecotropic MuLVs. The pathogenicity of the viruses was not apparent in mouse strains that did not express ecotropic MuLVs, suggesting that mixed infection was a prerequisite for pathogenicity. Considering that pseudotyping and amplification of polytopic MuLVs are observed as general occurrences with a variety of virus combinations and with different cell types, it seems quite likely that these interactions underpin many pathological consequences of mixed retrovirus infections. Further studies using *in vitro* cell lines that mimic *in vivo* mixed infections should facilitate a more thorough understanding of these interactions.

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