A Phenotypic Host Range Alteration Determines RD114 Virus Restriction in Feline Embryonic Cells

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We have characterized the restriction mechanism for RD114 virus replication in embryonic feline cells (FeF). By comparing growth properties of the virus in FeF cells with its behavior in a fetal feline glial cell line (G355) permissive for RD114, we showed that both cell lines were readily infectible by virus grown in permissive cells and that no significant differences in viral integration or viral RNA expression could be detected. However, analysis of viral protein expression revealed differences in viral *env* gene processing in the two cell types. Envelope precursor pR85 was produced, but the expected processed gp70 product was detectable only in permissive (G355) cells. An envelope product of 85 kDa was packaged into virions produced by FeF cells, while virions produced by G355 cells contained the expected RD114 gp70. While the gp85 *env*-containing virions were infectious for permissive G355 cells, they were unable to infect FeF cells. The block to infection by the gp85-containing particles in FeF cells could be abrogated by treatment with the glycosylation inhibitor tunicamycin. Our results indicate that restriction of RD114 virus involves a novel mechanism dependent on two factors: altered glycosylation of the envelope to a gp85 form and an altered RD114 receptor in FeF cells.

Naturally occurring, nonimmunologically based mechanisms which limit retrovirus replication are known to exist in a variety of organisms, with the *Fv-1* system in mice the best-characterized example (15, 21, 34). Developing an understanding of how such resistance occurs may lead to new opportunities to develop or augment such resistance in natural retroviral hosts and thus lead to new approaches to antiviral therapy for animals or humans. RD114 is a type C oncornavirus endogenous to domestic cats (1, 16, 25, 32). Although the genome of cats contains approximately 20 copies of RD114, only one encodes a replication-competent viral genome (22). RD114 virus is generally noninfectious in cat cells (8, 16) and has been previously classified as a xenotropic virus (8, 33), although it had been shown previously that functional receptors for RD114 virus existed on nonpermissive feline cells (6). Furthermore, an embryonic feline glial cell line, G355, which permits efficient, high-titer replication of RD114, has been described previously (12).

The observation that RD114 pseudotypes of transforming type C viruses could infect and transform nonpermissive feline cells while the replication-competent RD114 virus failed to replicate in these same cells (6) suggested that an intracellular, postpenetration step in the viral life cycle was blocked. Since little is known about how such events occur in any restricted cell-virus system, we undertook to analyze the mechanism of RD114 restriction in feline embryo fibroblasts. We compared various stages of the viral life cycle in restricted cells with the behavior of RD114 in the permissive G355 cell line and contrasted both to the behavior of feline leukemia virus (FeLV), a feline type C virus which replicates efficiently in both cell types. These studies indicated that differences in infection, proviral DNA synthesis and integration, viral RNA transcription, or translation or virus release was not responsible for the observed restricted growth. We

did observe, however, that the nonpermissive cells, in contrast to the permissive cells, produce a glycosylated gp85 *env* gene product which is incorporated into virions and that these virions, while fully infectious for the permissive G355 cell line, are essentially unable to infect nonpermissive FeF cells, leading to a block to RD114 spreading in nonpermissive cells. This block, however, can be overcome by treatment of the nonpermissive cells with tunicamycin, a glycosylation inhibitor, suggesting that a glycosylation-induced difference in the RD114 receptor in the nonpermissive cells or alternatively, the production of an unidentified glycosylated ligand which blocks this receptor is responsible for the observed restriction. This two-step phenotypic modification of a type C retrovirus represents a novel mechanism of virus restriction in mammalian cells.

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MATERIALS AND METHODS

Cells. Feline cells include G355, a fetal feline glial cell line obtained from the Naval Bioscience Laboratory; G355-5, a single cell clone of G355 cells; FeF, a feline embryonic cell (7) obtained from Peter Fischinger, Medical University of South Carolina, Charleston; and PG-4, isolated from a Moloney sarcoma virus-induced focus of transformed G355 cells (12). Both G355 and FeF cells show a normal feline diploid karyotype. All cells were subcultured weekly at a split ratio of 1:5 and maintained in McCoy's 5A medium containing 15% fetal bovine serum.

DNA. A pBR322 clone of Gardner-Arnstein feline leukemia proviral DNA designated pLHR2 (18) was a gift from James Casey, Cornell University, Ithaca, N.Y. Intact RD114 provirus cloned into pBR322, designated Sc3c, and a subclone of the RD114 long terminal repeat (LTR) in pBR322, designated Sc1e, were gifts from Roger Reeves, The Johns Hopkins University, Baltimore, Md. (22). The pSV2orineo plasmid in which the neomycin resistance gene from

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pSV2neo (28) was cloned into pUC8 was obtained from Tom Wood, John Sealy Center for Molecular Science, Recombinant DNA Laboratory, University of Texas Medical Branch, Galveston, Tex. The pSV0cat, pSV2cat, and pRSVcat constructs were described by Gorman et al. (10, 11) and obtained from George Khoury (National Institutes of Health). The pMov3 (13) plasmid contains Moloney leukemia virus proviral DNA cloned into pBR325. DNA restriction and other enzymes were obtained from New England Biolabs or Bethesda Research Laboratories and used under conditions described by the manufacturer and as described by Sambrook et al. (24).

DNA was nick translated as directed with the Nick Translation Kit 5000 purchased from Amersham. Random-primed probes were prepared with the Stratagene Prime-It Random Primer Kit.

Preparation and assay of viral stocks. G355 or PG-4 cells were infected with a multiplicity of infection (MOI) of 0.1 and subcultured once before being harvested. Viral supernatant was filtered through a 0.45- μ m-pore-size Nalgene filter, aliquoted, and stored at -70°C .

Stocks of Moloney murine sarcoma virus were prepared from PG-4 cells infected with RD114 or FeLV. Moloney sarcoma virus titer in focus-forming units/ml and leukemia focus-inducing units/ml were determined by assaying virus stocks on G355 and PG-4 cells, respectively. Neomycin-resistant pseudotyped viral stocks were obtained by infecting G355 cells containing a transfected recombinant viral construct expressing the *neo* gene (14) with RD114 or FeLV. Neomycin-resistant virions were assayed on G355 cells and selected in 200 μg of G418 per ml.

Viral stocks were also obtained from G355 cells transfected with cloned proviral DNA by the calcium phosphate method, as previously described (3).

For virus assays, G355, PG-4, or FeF cells were seeded at 2×10^5 cells per 60-mm-diameter culture plate 24 h prior to infection in McCoy's 5A supplemented with 15% fetal bovine serum and 4 μg of Polybrene per ml. Infections were performed by removing growth medium, adding 0.1 ml of virus, and incubating the plates for 1 h at 37°C . Cultures were then fed 4 ml of fresh medium, the medium was replaced on days 2 and 5 after infection, and plates were scored 6 or 7 days postinfection.

DNA transfection. Transfection was performed essentially as previously described (3), except that normal medium was placed with medium containing 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer 2 to 3 h before the addition of DNA precipitate. Sample DNA was cotransfected with pSV2neo DNA at a ratio of 10:1. Twenty-four hours later, cells were subcultured in 60-mm-diameter plates. Cells expressing the selectable marker were selected in growth medium containing 200 μg of G418 per ml.

Protein analysis. (i) **Western immunoblotting.** Cells were grown in T-75 flasks until 80 to 90% confluent and then washed with phosphate-buffered saline (PBS) and lysed in 4.0 ml of radioimmunoprecipitation assay buffer (0.15 M NaCl, 0.01 M Tris [pH 7.5], 0.001 M EDTA, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% aprotinin [Sigma]). The lysate was sonicated for 10 s and then cleared by centrifugation at $100,000 \times g$ for 60 min. The protein concentration was determined, and aliquots were stored at -70°C until they were used. In some cases, cell extracts were first immunoprecipitated as described below and the immune precipitates were analyzed. Prior to electrophoresis, Nonidet P-40 was added to 0.03% and the sample was incubated at 37°C for 20 min. An equal

volume of sample buffer (0.05 M Tris [pH 6.8], 1% SDS, 10% glycerol, 0.01% bromophenol blue, 0.015% β -mercaptoethanol) was added, and the sample was boiled for 5 min and then analyzed on either 7 or 4 to 20% polyacrylamide gels (Novex, Inc.). Proteins were electrophoretically transferred to nitrocellulose filters (31), and protein bands were visualized with specific antibodies and protein A-gold (Bio-Rad).

For the analysis of viral protein, virus-containing supernatant was filtered through a 0.45- μ m-pore-size filter and pelleted, and the virus pellets were resuspended, lysed in 0.03% Nonidet P-40, and then treated as described above.

(ii) **^{35}S labeling and immunoprecipitation.** Rapidly growing cells were labeled in 100-mm-diameter culture dishes in RPMI 1640 methionine-free medium containing 1% dialyzed fetal bovine serum and 250 μCi of [^{35}S]methionine. For pulse-chase experiments, chases with complete growth medium were done for 0, 1, 2, 3, and 4 h. Cells were washed in PBS and lysed in lysis buffer (10 mM sodium phosphate [pH 7.6], 100 mM sodium chloride, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS). Cell extracts were then aliquoted and stored at -70°C . For analysis, a volume of extract containing 5×10^6 cpm was precleared with normal goat serum twice and then treated with fixed *Staphylococcus aureus* protein A in order to remove nonspecific binding proteins. Samples were then treated with polyclonal antisera to RD114 or FeLV, and the complexes were precipitated with *Staphylococcus aureus* protein A. Pellets were resuspended in loading buffer and boiled for 3 min prior to gel loading. Polyacrylamide gels were either 7 or 4 to 20% gradient gels (Novex, Inc.). Polyclonal goat antibodies to the envelope gene (gp70) for RD114 and FeLV were obtained from National Institutes of Health Resources and are identified by catalog no. 79S741 and 77S310, respectively.

(iii) **Endoglycosidase H treatment.** Extracts were treated with endoglycosidase H (Boehringer Mannheim Biochemicals) according to the manufacturer's specifications. Extracts were then immunoprecipitated and analyzed as described above, except that the samples were not treated with normal goat serum.

RESULTS

Restriction prevents RD114 virus spread but not viral infection. We initially determined the rate of spread of RD114 virus in permissive (G355) and restricted (FeF) cells by infectious-center assays. Cells were infected with an MOI of 0.1, and the number of infected cells was measured 1 and 7 days after infection. Both wild-type RD114 and virus generated from cloned proviral RD114 DNA spread rapidly in permissive (G355) cells (Table 1); however, virus could clearly infect, but not spread, in restricted (FeF) cells. In contrast, FeLV could infect and rapidly spread in both cell types, consistent with the fact that both G355 and FeF are known to be permissive for this virus.

Similar results were obtained by comparing the ability of RD114 pseudotype stocks of a defective retroviral vector capable of inducing resistance to the neomycin analog G418 to generate drug-resistant colonies in permissive and non-permissive cells. Following infection and selection, the number of neomycin-resistant colonies observed was only slightly reduced (2- to 10-fold) in restricted cells relative to permissive cells. Our results were consistent with previous results with Moloney murine sarcoma virus-RD114 pseudotypes (6) and showed that both permissive (G355) and restricted (FeF) cells were essentially equally susceptible to

TABLE 1. Rate of virus spread for RD114 and FeLV in permissive G355 and restricted FeF cells

Virus (description) and day	% Positive cells ^a	
	Nonpermissive FeF	Permissive G355
RD114 (wild type)		
1	0.2	2.0
7	0.2	67.0
Sc3cV (RD114 clone)		
1	0.5	13.0
7	0.2	40.0
pLHR2V (FeLV clone)		
1	10.0	10.0
7	100.0	100.0

^a Cells were infected with an MOI of 0.1, predicting 10% virus-positive cells on day 1. Percents were determined by infectious-center assays.

infection with RD114 itself or defective viruses pseudotyped by RD114.

These experiments confirm that restriction is not due to the lack of the appropriate receptor in nonpermissive cells and demonstrate that RD114 virus can integrate efficiently in both permissive and restricted cells. The susceptibility of nonpermissive cells to RD114 infection could be demonstrated over a wide range of viral MOIs (Fig. 1). A linear response was observed for G355 cells at MOIs of 2 to 2×10^{-4} , and the restricted FeF cells showed a linear response at MOIs of 2×10^{-1} to 2×10^{-3} . Reproducibly, however, we observed that at higher MOIs (>0.2), the number of infected FeF cells reached a plateau and could not be increased, despite a 10-fold increase in virus. The mechanism of this resistance is unknown, but it could suggest that in FeF cells

FIG. 1. Kinetics of RD114 infection of G355 and FeF cells. Cells were infected with serial dilutions of RD114 pseudotypes of a virus encoding the *neo* gene (14) as described in Materials and Methods. Twenty-four hours later, the infected cells were selected in media containing 200 μ g of G418 per ml. For selection of FeF cells, the media also contained 50% conditioned media from rapidly growing uninfected FeF cells. The number of colonies was determined 10 to 12 days after infection. Each point represents the average of at least two plates.

FIG. 2. Northern blot analysis of total cellular RNA from uninfected and RD114-transfected G355 (permissive) and FeF (nonpermissive) cells. RNA was isolated, separated on formaldehyde-agarose gels, blotted onto nitrocellulose, and hybridized to a probe prepared from a cloned RD114 LTR fragment as described in Materials and Methods. (A) Lanes: 1, uninfected FeF cells; 2, uninfected G355 cells; 3 and 4, RD114-transfected G355 cells; 5 and 6, RD114-transfected FeF cells; 7 and 8, G355 cells infected by virus harvested from cells shown in lanes 5 and 6. Sedimentation coefficients appear on the left. (B) Both lanes: actin probe hybridization to lanes 1 and 2 after the hybridized probe shown in panel A was stripped.

receptors are more easily saturated by competing noninfectious particles, perhaps because there is a lower number of functional receptors on FeF cells.

Analysis of viral and virus-related RNA expression in permissive and nonpermissive cells. To directly analyze viral RNA expression and processing in permissive and nonpermissive cells, cellular RNA was extracted from uninfected, infected, and proviral DNA transfectants and subjected to Northern (RNA) analysis. An RD114 LTR-specific probe (*Xba*I-*S*tyI fragment) showed that uninfected FeF cells (Fig. 2A, lane 1) expressed relatively high levels of endogenous RD114 sequences. We observe four endogenously expressed bands of 7.0, 3.3, 3.0, and 1.8 kb. In contrast, the endogenous RD114 sequence expression is not detected (Fig. 2A, lane 2) in uninfected G355 cells, although long exposures sometimes show low levels of expression of ~3-kb species. Southern analysis detects multiple copies of endogenous RD114-related sequences in G355 cells (data not shown). Figure 2B shows the results following washing and reprobing of this blot with an actin probe to confirm the presence and level of RNA in both lanes. No viral reverse transcriptase activity was detected in the supernatant, nor were RD114 viral proteins detected in FeF cell extracts (data not shown), suggesting that the RNA expression represents defective copies of endogenous RD114 sequences. The lack of protein expression is particularly surprising in view of the high levels of RNA detected, but repeated analysis with sera directed against p28, gp70, and whole virus failed to detect any virus-related proteins. We cannot rule out, however, that low levels of some viral proteins are expressed in FeF cells.

Following RD114 proviral DNA transfection, both permissive cells (Fig. 2A, lanes 3 and 4) and restricted cells (Fig. 2A, lanes 5 and 6) show expression of the expected 8.5-kb genomic and 3.2-kb subgenomic envelope mRNA. The genomic RD114 RNA is clearly distinguishable from the endogenous 7.0-kb band expressed in uninfected FeF cells (Fig. 2A; compare lane 1 with lanes 5 and 6). Thus, both

FIG. 3. RD114 RNA in virions produced by G355 and FeF cells. RNA from purified virions was analyzed by Northern blot analysis with an RD114-specific probe. Lanes: 1 and 2, virions from RD114-transfected G355 cells; 3 and 4, virions from RD114-transfected FeF cells. Sizes appear on the left.

permissive and nonpermissive cells express RD114 virus in a similar fashion. The observation that the nonpermissive cell expressed high levels of RD114-related endogenous sequences, however, raised the possibility that this might play a role in the restriction process, so we analyzed the ability of these sequences to be packaged into virions and transmitted to uninfected cells.

Endogenous RD114-related sequences produced in FeF cells are packaged into virions. RNA from virions produced by permissive (G355) and restricted (FeF) cells transfected by RD114 proviral DNA was isolated and analyzed by Northern analysis. Virions produced by permissive (G355) cells contained only the expected 8.5-kb genomic RD114 mRNA (Fig. 3, lanes 1 and 2). However, virions produced by restricted (FeF) cells contained both an 8.5- and a 7.0-kb RD114 RNA species (Fig. 3, lanes 3 and 4). No viral RNA was detected in extracts of pelleted supernatants from uninfected FeF or G355 cells (data not shown). This 7.0-kb species was also packaged when FeLV was grown on FeF cells (data not shown), indicating that it contains fully functional type C viral packaging signals. We have been unable, however, to detect transmission of this 7.0-kb endogenous RD114 mRNA species to G355 cells. When RD114 virus produced from restricted (FeF) cells was used to infect permissive (G355) cells and RNA prepared from these infected cells was subjected to Northern blot analysis, only the 8.5-kb genomic RNA was detected (Fig. 2A, lanes 7 and 8).

Permissive and nonpermissive cells show differences in processing of viral *env* gene products. Analysis of intracellular viral proteins and those present in released virions revealed no apparent differences in size or relative level of reverse transcriptase or major *gag* proteins (data not shown). However, analysis with RD114 gp70 antiserum showed significant differences between permissive and nonpermissive cells in both intracellular and virion envelope proteins. While this antiserum recognized two bands of ~70 and 85 kDa in producer G355 extracts (Fig. 4A, lane 3), nonpermissive cells (Fig. 4A, lanes 4 and 5) showed a single diffuse band of ~85 kDa, approximately the size of the larger of the two bands seen in permissive cells. Purified virions released by permissive cells contained only the smaller 70-kDa band (Fig. 4B, lane 3), while nonpermissive cells produce virus containing the larger 85-kDa protein (Fig. 4B, lane 4). Analysis of FeLV grown on FeF and G355 cells revealed a similar size difference in the released virus protein recognized by an anti-FeLV-specific gp70 antiserum (data not shown), suggesting that FeF cells process both restricted and nonrestricted viral envelopes in a similar fashion.

The size differences seen with intracellular and viral *env* proteins indicated that FeF and G355 cells processed viral

FIG. 4. Analysis of proteins from infected, transfected, and uninfected cell extracts and cell-free virus with an RD114-specific gp70 antiserum. Unlabeled total cell lysates (A) or ^{35} S-labeled extracts of purified virions (B and C) were immunoprecipitated with a polyclonal RD114 anti-gp70 antiserum (A and B), or the labeled virus was run without immunoprecipitation (C) (see Materials and Methods). Solubilized precipitates were separated on 4 to 20% (A and B) or 7% (C) SDS-polyacrylamide gels and analyzed by Western blotting with protein A-gold (Bio-Rad) (A) or by autoradiography (B and C). (A) Lanes: 1, molecular weight markers (in thousands); 2, uninfected G355 cell extract; 3, RD114-transfected G355 cell clone G21; 4, RD114-transfected FeF cell clone F2J; 5, RD114-infected FeF cell clone 1916-5; 6, uninfected FeF cells. (B) Lanes: 1, molecular weight markers (in thousands); 2 to 5, purified ^{35} S-labeled virions or the pelleted media from uninfected control cells; 2, uninfected G355 control cells; 3, RD114-transfected G355 cells; 4, RD114-transfected FeF cells; 5, uninfected FeF cells. (C) Purified, labeled virions as described for panel B, run without immunoprecipitation.

envelope components differently. To investigate this further, we performed pulse-chase experiments on cells producing RD114. Following a 1-h pulse with [^{35} S]methionine, cells were incubated in the presence of excess cold methionine and harvested at 1-h intervals and cell extracts were analyzed by immunoprecipitation and polyacrylamide gel electrophoresis. Figure 5 (lanes 4 to 7) shows that in permissive cells RD114 *env* proteins are chased from an approximately 85-kDa form into a 70-kDa form, the size of which coincides with the size of the protein seen in mature virions (Fig. 4B, lane 3). In contrast, in nonpermissive cells (Fig. 5, lanes 8 to 12), the 85-kDa form is retained and moves into even higher-molecular-mass forms at later times. Note that the reduced levels of all forms seen in Fig. 5, lanes 11 and 12, reflect lower levels of total protein in these lanes. The

FIG. 5. Pulse-chase analysis of RD114 envelope protein processing in permissive and nonpermissive cells. RD114-transfected G355 (G21) or FeF (F2J) cells were labeled for 1 h with [^{35} S]methionine and chased for 1, 2, 3, or 4 h in cold media as indicated above the lanes, and extracts were prepared, precleared with normal goat serum, and immunoprecipitated with anti-RD114 antisera and subjected to SDS-polyacrylamide gel electrophoresis on 7% gels. Lanes: 1, molecular weight markers (in thousands); 2 and 3, uninfected G355 cells after the pulse (lane 2) and a 1-h chase (lane 3); 4 to 7, RD114-transfected G355 cells after the pulse (lane 4) and chases of 1, 2, and 3 h (lanes 5, 6, and 7, respectively); 8 to 12, RD114-transfected FeF cells after the pulse (lane 8) and chases of 1, 2, 3, and 4 h (lanes 9, 10, 11, and 12, respectively); 13 and 14, uninfected FeF cells after a pulse (lane 14) and a 1-h chase (lane 13).

FIG. 6. Endoglycosidase H analysis of RD114 envelope proteins in infected G355 and FeF cells. Extracts of transfected cells labeled for 1 h with [^{35} S]methionine and chased for 4 h with cold media were treated with endoglycosidase H and then immunoprecipitated with anti-RD114 antisera and analyzed on 7% polyacrylamide gels. Molecular weight markers (lane 1), extracts of uninfected G355 cells without (lane 2) or with (lane 3) endoglycosidase H treatment, untreated (lane 4) or endoglycosidase H-treated (lane 5) extracts of RD114-transfected G355 cells, untreated (lane 6) or endoglycosidase H-treated (lane 7) extracts of RD114-transfected FeF cells, and extracts of untreated (lane 8) or endoglycosidase H-treated (lane 9) infected FeF cells are shown.

85-kDa form is the size of the *env* protein found in the virions produced by FeF cells (Fig. 4B, lane 4). This serum also detects a variable amount of a smaller protein in both infected and uninfected cells, but this is not packaged into virions (see Fig. 4B). Thus, nonpermissive cells process the viral *env* protein aberrantly, and both cells and the viruses they produce contain a larger form similar in size to the precursor form seen in permissive cells.

To determine whether this size difference represents a failure to cleave the p15E fragment from the unglycosylated 85-kDa *env* gene product, we analyzed the total viral protein in mature RD114 virions. Figure 4C, lanes 3 and 4, shows that RD114 produced by G355 or FeF cells expresses similar amounts of a 15-kDa protein. The presence of this band is consistent with the cleavage of pR85 into gp70 and p15E. We have also detected an ~15-kDa protein in FeF cells infected with FeLV (data not shown) with a p15E-specific monoclonal antiserum (kindly provided by J. Elder, Scripps Clinic), further indicating that FeF cells are able to cleave envelope precursors properly. These observations are also consistent

with the results of experiments in which intracellular RD114 *env* proteins are treated with endoglycosidase H to remove sugar moieties. Proteins precipitated with gp70 antisera from either permissive (Fig. 6, lanes 4 and 5) or nonpermissive (Fig. 6, lanes 6 and 7) cell extracts generate a 56-kDa band upon treatment with endoglycosidase H. The larger forms observed in Fig. 6, lane 5, are most probably due to incomplete digestion.

RD114 virus produced by nonpermissive cells shows an altered ability to infect nonpermissive cells. The differences in gp70 present in virus produced by permissive and nonpermissive cells raised the question of whether these viruses might exhibit altered patterns of infectivity. Since previous evidence had indicated (see above) that RD114 virus was infectious for the permissive G355 cells, we studied in more detail whether these virions were also infectious for the nonpermissive FeF cells. We prepared RD114 pseudotypes of a *neo*-containing defective virus and measured the abilities of these stocks to generate drug-resistant colonies on permissive and nonpermissive cells. As had been shown previously, virus grown on G355 cells generated essentially equal numbers of colonies on G355 and FeF cells (Fig. 7, row 1, lanes A and B). In contrast, RD114 pseudotypes grown on FeF cells failed to induce G418^r colonies on FeF cells (Fig. 7, row 2, lane B), although they generated large numbers of colonies on G355 cells (Fig. 7, row 2, lane A). Thus, virions containing the high-molecular-weight form of the RD114 gp70 protein were unable to infect FeF cells, consistent with the observation that RD114 failed to spread in the nonpermissive cell line. In contrast, FeLV grown on FeF cells was fully capable of infecting and spreading in FeF cells despite the fact that these virions also contained a higher-molecular-weight form of gp70 (data not shown).

The inability of RD114 viral pseudotypes containing an altered gp70 to induce G418^r FeF colonies was suggestive of a surface block. We had evidence from previous experiments that some nonfeline cells resistant to RD114 infection could be rendered infectible by treatment with glycosylation inhibitors (5), so we tested the effect of tunicamycin, a well-known inhibitor of N-linked glycosylation, on the susceptibility of FeF cells to RD114 infection. Treatment of FeF cells with tunicamycin did not affect the ability of G355-grown virus to induce colonies (Fig. 7, row 1, lane C), but

FIG. 7. Infectivity of RD114 virus for FeF and G355 cells. RD114 pseudotypes of a *neo*-containing virus were used to infect either G355 cells, FeF cells, or FeF cells treated with tunicamycin (0.1 μ g/ml for 18 h). After 24 h, G418 (200 μ g/ml) was added and cells were grown for 9 or 10 days until selection was complete. Plates were then fixed with 70% methanol and stained with Giemsa. Virus grown on G355 (permissive cells) (row 1) or FeF (nonpermissive cells) (row 2) was used to infect G355 (A), FeF (B), or tunicamycin-treated FeF (C) cells. Designations for the *env* glycoproteins in the virions (see text) are given in parentheses.

their sensitivity to FeF-grown virus was increased several hundredfold following treatment (Fig. 7, row 2, lane C). Similar experiments utilizing FeLV grown on G355 and FeF show no differences in infectivity and no effect of tunicamycin treatment (data not shown). These results were consistent with the presence of an RD114-specific, glycosylation-sensitive receptor modification or a glycosylation-sensitive interfering factor in FeF cells which induces resistance to infection by RD114 viruses produced by FeF cells.

DISCUSSION

RD114 virus had been previously shown to replicate poorly on feline cells and on that basis had been classified as a xenotropic virus (33). However, RD114 virus had also been shown to undergo one round of infection in restricted feline cells (6), indicating that lack of an RD114 receptor could not be responsible for the inability of RD114 to replicate. We undertook the studies described in this report in order to determine the mechanism of RD114 virus restriction and to evaluate its potential as a model for cellular control of viral replication and pathogenesis.

We confirmed that RD114 virus could infect but not spread on nonpermissive FeF cells utilizing both biologically and molecularly cloned RD114 virus. RD114 virus stocks showed linear kinetics in G355 cells at MOIs from 2 to 2×10^{-4} . However, RD114 virus kinetics in restricted FeF cells was linear only between MOIs of 2×10^{-1} and 10^{-4} and reproducibly plateaued at MOIs above 2×10^{-1} . This plateau did not appear to be due to the presence of a resistant subpopulation of FeF cells, since 10 randomly selected cell clones were equally susceptible to infection (5). It is possible that the number of functional receptors is low on FeF cells and that competition with noninfectious particles at high MOIs leads to a high degree of interference. Fewer RD114 receptors could contribute to the RD114 restriction mechanism (see below).

The efficient generation of G418-resistant FeF colonies by RD114 viral pseudotypes indicates that viral uptake and integration are efficiently achieved by the RD114-encoded proteins in nonpermissive cells. Southern analysis confirms that both the number and structure of integrated proviruses are similar in both cell lines. The 2- to 10-fold reduction in colonies seen in FeF relative to G355 could be due to the lower plating efficiency of the nonestablished FeF cells at low cell densities, and it is not sufficient to account for the nonpermissive phenotype of these cells.

RD114 virus restriction in FeF cells does not appear to occur at the level of transcription. Transient transfection chloramphenicol acetyltransferase assays (5) showed that the RD114 LTR in restricted FeF cells was equal to or 1.5 times better than that in permissive cells and that the level of expression was similar to that of the nonrestricted FeLV LTR. This observation is consistent with the results of Spodick et al. (29, 30), who showed that LTRs of both defective and replication-competent endogenous viruses were able to function in several different cell types. Northern blot analysis of RNA expression in restricted and permissive cells showed that while uninfected permissive cells expressed no readily detectable RD114-specific RNA, the nonpermissive FeF cells express multiple species detectable with an LTR-specific probe. This is consistent with other studies which indicate that endogenous RD114-related species are frequently expressed in feline tissues (4, 19). However, we were unable to detect endogenous expression in FeF cells with polyclonal anti-RD114 antisera directed

against whole virus, p28, or gp70, and no viral particles or reverse transcriptase activity was detected in the culture supernatant. This lack of expression is surprising in view of the high level of viral RNA expressed in these cells, but it could reflect evolutionary selection against protein expression and the generation of functional viruses in cats.

Our data did show that the largest species of endogenous RNA was efficiently packaged by RD114, and we also detected this species in FeLV grown on FeF cells. No transmission to and subsequent expression by G355 cells was ever observed. Murine VL30 sequences, which represent an endogenous retrovirus-like sequence which encodes no viral proteins, are both packaged and transmitted by replication-competent mammalian viruses (2, 26, 27). It is possible that RD114 endogenous sequences are transmitted at a frequency too low to be detected in our analysis. Alternatively, since we were unable to detect expression of endogenous RD114 sequences, G355 may restrict integration or expression of endogenous RD114 LTRs. We have observed similar FeF-like levels of RD114-related RNA expression in other primary and established feline cell lines, and the presence of these sequences in virus particles could play a role in feline retroviral disease or the generation of retrovirus recombinants in vivo. Further analysis will be required to determine whether the endogenous sequences are specifically blocked in G355 cells and if so, to elucidate the mechanism of that block. The presence of endogenous RD114-related sequences represents a clear difference between virus grown on the permissive cell lines and that grown on the nonpermissive cell lines, but the fact that FeLV, which is not restricted, also packages these sequences effectively makes it unlikely that the defective viruses play a role in RD114 restriction.

Of greater significance to viral restriction, however, are the differences in *env* proteins observed between the two cell lines. Pulse-chase experiments showed that in G355 cells the RD114 glycosylated precursor, pR85^{env}, was efficiently processed to gp70. However, pR85^{env} was aberrantly processed in FeF cells to an 85-kDa form (gp85). Consistent with these results, steady-state levels of *env* proteins measured in cell extracts showed the expected precursor and gp70 proteins in RD114-infected or -transfected G355 cell extracts. pR85 was found in transfected or infected FeF cell extracts, but virions produced by FeF cells principally contain an 85-kDa *env* product, while virions produced by G355 cells contain an apparently normal 70-kDa product. The pattern of gp70 seen in pulse-chase experiments resembles that seen in Moloney murine leukemia virus mutants blocked in *env* gene processing (9). When infectivity of the gp70- or gp85-containing virions was measured directly, our data showed that the gp70-containing RD114 produced by G355 cells could efficiently infect either G355 or FeF cells. However, while RD114 gp85 virions could infect G355 cells efficiently, they were essentially unable to infect FeF cells. Thus, RD114 virus growth in FeF cells is restricted to one round of infection, and the virus progeny from the initially infected FeF cells is unable to spread to other FeF cells in the culture. This resistance to a second cycle of infection is most probably due to a surface block, since it could be overcome by tunicamycin treatment of the FeF cells.

It is unlikely that the modified behavior of the FeF-grown RD114 is due to the acquisition of a new viral envelope protein, since the gp70 or gp85 envelope-containing RD114 virions for RD114 virus exhibited the same interference properties on G355 cells (data not shown). RD114 virions containing either gp70 or gp85 envelope proteins are infec-

tious for permissive G355 cells, so it is likely that the RD114 viral receptor in G355 cells interacts with both forms of the envelope protein. However, our results suggest that the RD114 receptor on the restricted FeF cells can interact functionally with only RD114 gp70-containing and not gp85-containing virions. The fact that this exclusion of gp85-containing virions can be abrogated by tunicamycin treatment suggests two possible explanations. (i) The RD114 receptor could be glycosylated in FeF cells in a manner which blocks the gp85 envelope-carrying virion from functionally interacting with the receptor. Upon tunicamycin treatment, this modification is removed, and infection can occur. (ii) Alternatively, FeF cells produce a glycosylated protein which competes for the RD114 receptor. Tunicamycin treatment could then inhibit the competitor from binding to the receptor, allowing virus infection of FeF cells by both gp70- or gp85-containing RD114 virions, analogous to the manner that retrovirus interference is abrogated by glycosylation inhibitors such as tunicamycin (23). The fact that FeF cell receptors may be saturated more easily than those of G355, as suggested by the plateau observed at high MOIs, could also make it easier for any other potential competitor to saturate these receptors and to block infection.

It is interesting that changes in infectivity patterns induced by glycosylation inhibitors have recently been described for CHO cells (18). We have observed that treatment of murine cells with glycosylation inhibitors renders them susceptible to infection by G355-grown RD114 virus, similar to what is observed for FeF cells. This may suggest that the RD114 receptor protein may be a glycoprotein whose glycosylation pattern and ability to function as a receptor vary in a species- and tissue-specific manner. In the studies described by Miller and Miller (17), there was evidence that in some cases the resistant cells released a soluble interfering factor into the media whose function was glycosylation sensitive. However, we have been unable to detect a factor in media conditioned by FeF cells which can interfere with RD114 infection of either FeF or G355 cells with or without tunicamycin treatment.

It is unclear at this point whether the mechanism which we have described is the only one responsible for RD114 resistance in feline cells. However, the majority of feline cell lines which we have tested will not support the spread of RD114. Of four additional fetal or embryonic cell lines and one adult-derived cell line tested, all but one are sensitive to infection by RD114 virus bearing gp70 but are resistant to the gp85 form and thus resemble FeF cells. The sole exception, AH927, is susceptible to both gp70- and gp85-bearing RD114. Significantly, this line was shown to generate FeLV *env* protein of about 70 kDa (20), which would be consistent with its permissiveness, like that of G355, for RD114 bearing either size class of SU glycoprotein.

This report describes the first case in which restriction of retrovirus replication is due to a phenotypic alteration of glycosylation of the envelope gp70 product. Unlike other restriction systems, this restriction is only phenotypic and not genotypic. It would be of interest to determine whether other viruses employ such a mechanism and whether this is involved in either pathogenesis or targeting of virus to specific cells or tissues in vivo.

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