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Ross River Virus Glycoprotein-Pseudotyped Retroviruses and Stable Cell Lines for Their Production

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Pseudotyped retroviruses have important applications as vectors for gene transfer and gene therapy and as tools for the study of viral glycoprotein function. Recombinant Moloney murine leukemia virus (Mo-MuLV)-based retrovirus particles efficiently incorporate the glycoproteins of the alphavirus Ross River virus (RRV) and utilize them for entry into cells. Stable cell lines that produce the RRV glycoprotein-pseudotyped retroviruses for prolonged periods of time have been constructed. The pseudotyped viruses have a broadened host range, can be concentrated to high titer, and mediate stable transduction of genes into cells. The RRV glycoprotein-pseudotyped retroviruses and the cells that produce them have been employed to demonstrate that RRV glycoprotein-mediated viral entry occurs through endocytosis and that membrane fusion requires acidic pH. Alphavirus glycoprotein-pseudotyped retroviruses have significant advantages as reagents for the study of the biochemistry and prevention of alphavirus entry and as preferred vectors for stable gene transfer and gene therapy protocols.

Alphaviruses are a group of enveloped arthropod-borne viruses that have a very wide geographic distribution and pose a serious threat to human health in many regions (28). Symptoms exhibited by infected individuals can include fever, rashes, arthralgia, severe headaches, myalgia, and persistent polyarthritis (28). The equine encephalitis viruses that are found in both North and South America can cause fatal encephalitis in humans. The alphaviruses, which include the Semliki Forest, Sindbis, and Ross River (RRV) viruses, have extremely broad host ranges, in terms both of the animals that can be infected (invertebrates and vertebrates, including birds, mammals, and reptiles) and of the cell types of the hosts within which the virus can replicate (28).

The alphavirus virion is composed of a single strand of RNA surrounded by 240 copies of a nucleocapsid protein that together form an icosahedral nucleocapsid that is encapsulated by a lipid bilayer (3, 7, 22). The viral transmembrane glycoprotein complex is responsible for the binding of the alphavirus to the surface of a susceptible cell and for the fusion of the viral and cellular membranes that occurs during the process of viral entry. It consists of a trimer of heterodimers, with the heterodimer composed of two transmembrane proteins, E1 and E2. There are 80 such complexes (spikes) in the alphavirus envelope (6).

The structural proteins of the alphaviruses (the capsid [C] and glycoproteins [E3, E2, and E1]) are synthesized as a polyprotein (C-E3-E2-6K-E1) that is processed proteolytically into the individual subunits (28). The capsid, present at the amino terminus of the polyprotein, is a protease, which cleaves itself from the nascent chain shortly after its synthesis. The amino-terminal section of the remainder of the polyprotein functions as a signal sequence that directs the translocation of the subsequent polypeptide region into the endoplasmic reticulum. A hydrophobic sequence approximately 400 residues after the signal sequence acts as a stop-transfer signal and as the membrane anchor for E2. The following 30 residues transiently function as a signal sequence for the carboxy-terminal half of the polypeptide. A proteolytic cleavage following this signal sequence results in the release of E3-E2 (referred to as pro-E2 or PE2) that is anchored in the membrane. A heavily palmitoylated and hydrophobic 6-kDa segment (8) at the amino terminus of the remaining portion of the polyprotein (referred to as 6K) is organized so that its carboxy-terminal 25 residues act as a signal sequence for E1. The signal sequence is cleaved, resulting in the release of the E1 glycoprotein, which is anchored in the membrane by a stop-transfer sequence that directly precedes the carboxy terminus of E1.

PE2-E1 heterodimers form in the endoplasmic reticulum and are transported to the Golgi apparatus, where PE2 is cleaved into E3 and E2 at a sequence recognized by the furin class of protein convertases (28). The E2/E1 spikes associate with the nucleocapsids during budding, and a T=4 symmetry enveloped virion is produced (3, 22). Whereas E2 appears to be involved in binding to host cell receptors and possesses most of the epitopes for neutralizing antibodies, E1 is believed to be responsible for the process of membrane fusion. In the case of Semliki Forest virus, it has been demonstrated that membrane fusion is a low-pH-dependent process and that viral entry requires the endocytosis of bound viral particles (12, 17, 28). Exposure of the E2-E1 complex to low pH induces irreversible conformational changes that result in the dissociation of E2 and the reorganization of E1 into a homotrimer that is believed to be the active membrane fusion-promoting entity (7, 32, 33). It has, however, been suggested that the effect of lysosomotropic weak bases, which prevent the acidification of endosomes, on Sindbis virus infection is mediated through the inhibition of viral RNA replication rather than through an abrogation of entry (2).

An experimental system that would allow the effects of mu-

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tations and chemical treatments on virus assembly and virus-genome replication to be disentangled from those on virus entry would possess major advantages in resolving a number of issues. One that would permit, in addition, the rapid and quantitative analysis of the effects of a large number of amino acid residue substitutions in the alphavirus glycoproteins would produce important insights into the biochemical and structural basis of their function. The entry of a virus into a cell is most readily examined in the context of a cell and, more pertinently, a virus particle. This is true mainly because entry involves the interaction of the viral proteins with membrane-bound proteins and/or cellular membranes, and our capacity to reconstitute such interactions in cell-free systems has lagged behind our success with soluble macromolecules. Two virtues of utilizing virus particles for these analyses are that the introduction of an expressed viral genome into a cell offers a quantitative and sensitive measure of the entry step and that virus particles are an enriched source of the viral proteins that facilitate entry.

Functional analysis of a variety of retroviral and filoviral (34, 35) glycoproteins has been accomplished through the use of recombinant retroviruses that have incorporated the glycoproteins into their envelopes. These chimeric retroviruses (referred to as pseudotypes) can be constructed so that they are competent for entry into a cell and transduction of a recombinant genome but are incapable of replicating in the transduced cells. The product of the recombinant genome can be one whose expression can be readily monitored, so that assays of its presence in a cell can be used as measures of the capacity of the pseudotyped retrovirus to enter into the cell. We present here our analysis of the Moloney murine leukemia virus (Mo-MuLV) pseudotyped with RRV glycoproteins. Our establishment of stable cell lines that produce such alphavirus glycoprotein-pseudotyped retrovirus may have major applications in the fields of gene transfer and gene therapy. The broad host specificity of the alphaviruses might permit the recombinant retroviruses to transduce genes into cells that are not normally susceptible to retrovirus infection.

MATERIALS AND METHODS

Cell lines and cell culture. Mouse NIH 3T3 fibroblasts and E86nlslacZ (30) cells (GP+E-86 cells [16] transfected with MFG.S-nlslacZ [21], a retroviral vector that encodes a nucleus-localized β-galactosidase) were grown in Dulbecco's modified eagle's medium (DMEM; Sigma) with 10% calf serum (CS; Gibco-BRL), streptomycin (0.1 mg/ml), and penicillin (10 U/ml) (PS; Sigma) (DMEM-CS/PS). BHK21 (hamster kidney), RK13 (rabbit kidney epithelial), SW13 (human adrenocortical carcinoma), VeroE6 (African green monkey kidney epithelial), HeLa (human cervical carcinoma), ϕNX (second-generation 293T-based retroviral packaging cells) (11, 23, 29), gpGFP (which produce envelope protein-deficient replication-incompetent Mo-MuLV particles carrying MFG.S-GFP-S65T, a retroviral vector encoding the *Aequorea victoria* green fluorescent protein S65T mutant [30]), and gpnlslacZ cells were grown in DMEM with 10% fetal bovine serum (FBS; Sigma) and PS (DMEM-FBS/PS). The gpnlslacZ cells were developed in our lab by cotransfecting MFG.S-nlsLacZ and pJ6Ωpuro (19) into ϕNX cells. Transfected cells were grown in DMEM-FBS/PS supplemented with puromycin (2 μg/ml) (Sigma), and antibiotic-resistant colonies were isolated and screened for the production of high-titer replication-incompetent virus resulting from transient transfection with penv1min, a vector that encodes the wild-type Mo-MuLV envelope protein (30). Vesicular stomatitis virus (VSV) G protein-pseudotyped retrovirus-producing 293GPGnlslacZ cells (21) were grown in DMEM-FBS/PS supplemented with puromycin (2 μg/ml) and tetracycline (1 μg/ml) (Sigma). Whereas expression of the VSV G protein in these cells is repressed by the presence of tetracycline in the medium, 48 h before collection of pseudotyped virus, the medium in which the 293GPGnlslacZ cells were grown was replaced with DMEM-FBS/PS.

RRV glycoprotein expression plasmid construction. The region encoding the RRV envelope glycoproteins was amplified from pRR64, which contains the full-length cDNA of the RRV genome (13), using *Taq* DNA polymerase (Promega Corporation) and two primers complementary to the viral cDNA at nucleotides 8376 (5′-CGGGATCCACCATGTCTGCCGCGCT-3′) and 11312 (5′-CGCTCTAGATTACCGACGCATTGTTATG-3′). The amplified fragment, which contained the RRV E3-E2-6K-E1 coding region, was digested with the restriction endonucleases *Bam*HI and *Xba*I and ligated into the *Bam*HI and *Xba*I sites of pBacPac, a baculovirus expression vector (Clontech). The resulting plasmid was digested with *Bam*HI and *Xba*I, and the fragment containing the RRV E3-E2-6K-E1 coding region was ligated into the *Bam*HI and *Xba*I sites in the pcDNA3 and pcDNA3.1/Zeo(+) mammalian expression vectors (Invitrogen). The resulting plasmids were designated pRRV-E2E1 and pRRV-E2E1A, respectively.

Transient expression of viral glycoproteins in retroviral packaging cells. In preparation for transfection, 500,000 ϕNX cells were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 27 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM K_2HPO_4 [pH 7.4]) prior to incubation with 2 ml of Opti-MEM (Gibco-BRL) for 30 min at 37°C in a 5% CO_2 atmosphere. Then 2 μg of pRRV-E2E1 and 2 μg of MFG.S-GFP-S65T were incubated with 300 μl of Opti-MEM and 24 μl of Lipofectamine (Gibco-BRL) for 30 min at room temperature prior to dilution with 2.4 ml of Opti-MEM. The resulting mixture was incubated with the cells for 7 h at 37°C. Medium was replaced with DMEM-FBS/PS for a further 48-h incubation at 37°C before collection of the supernatant medium for analysis of the transduction capacity of and level of glycoprotein incorporation into viral particles. When the gpnlslacZ cells were transfected, a similar protocol was followed except that the transfected DNA consisted solely of 4 μg of pRRV-E2E1 or pMD.G (20).

Generation of stable cells producing RRV E2E1-pseudotyped Mo-MuLV. ϕNX or gpnlslacZ cells were transfected as above with 8 μg of pRRV-E2E1 and 0.4 μg of the pJ6Ωpuro plasmid except that the DNA mixture contained 48 μl of Lipofectamine and 600 μl of Opti-MEM. Selection with medium containing puromycin (2 μg/ml) began after 48 h. Clonal colonies of cells were isolated after 2 weeks of selection, and these were grown in DMEM-FBS/PS. The cell lines were then screened for production of high-titer replication-incompetent virus. The resulting ϕNX-derived and gpnlslacZ-derived cell lines were designated SafeRR and SafeRR-nlslacZ, respectively. Similar transfections of the gpnlslacZ cells with 8 μg of pRRV-E2E1A alone and selection with medium containing Zeocin (200 mg/ml) rather than puromycin resulted in the cell line SafeRR-nlslacZA.

Transduction by recombinant retroviruses. Supernatant medium from recombinant virus-producing cells was passed through a 0.45-μm filter, mixed with hexadimethrine bromide (Sigma) (final concentration, 8 μg/ml), and incubated with cells for 5 h at 37°C in a 5% CO_2 atmosphere. The recombinant virus-containing medium was then replaced with DMEM-CS/PS medium. Cells transduced with MFG.S-GFP-S65T were, 48 h after infection, washed with PBS and then lifted from the plate with PBS containing 1 mM EDTA. The cells were then analyzed with a Coulter XL-MCL flow cytometer using a 525-nm band-pass and a 488-nm air-cooled argon laser. Forty-eight hours after the infection, the cells transduced using virus bearing MFG.S-nlslacZ were fixed and stained as described (26, 30). Concentrated virus for transductions was obtained by passing supernatant medium from 10-cm tissue culture dishes of confluent cells through a 0.45-μm filter and centrifugation through a 30% sucrose cushion at 75,000 × *g* for 2 h in a Beckman 50.2 titanium rotor. The virus in the pellet was suspended in DMEM-CS/PS medium and incubated with cells under the conditions described above.

Immunological detection of RRV E2 and E1. Supernatant medium from 10-cm tissue culture dishes of confluent SafeRR-nlslacZA or gpnlslacZ cells was passed through a 0.45-μm filter and spun through a 25% sucrose cushion at 75,000 × *g* for 2 h in a Beckman 50.2 titanium rotor. The virus present in the pellet was suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer with β-mercaptoethanol. The sample was then analyzed by SDS-PAGE (10% acrylamide). The separated proteins were then transferred to a nitrocellulose membrane at 44 mA for 2 h in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.05% SDS). The membranes were blocked with blocking buffer (5% powdered milk in TNT [20 mM Tris-Cl (pH 7.6), 137 mM NaCl, 0.1% Tween 20]) for 1 h at 22°C. The membranes were then incubated in blocking buffer with a 1:5,000 dilution of either rabbit polyclonal anti-RRV E2 antibody (PAbE2) or rabbit polyclonal anti-RRV E1 antibody (PAbE1) for 2 h at 22°C. The membranes were washed twice with TNT and then incubated with 0.2 μg of horseradish peroxidase-linked goat anti-rabbit immunoglobulin secondary antibody (Chemicon) per ml in blocking buffer for 30 min at 22°C. Then the membranes were washed twice with TNT and incubated with enhanced

chemiluminescence detection reagents (Amersham Pharmacia Biotech). Immunoreactive proteins were visualized by exposure of the membrane to film. Lysates of the SafeRR-nlslacZA or gpnlslacZ cells were prepared by washing the cells with 10 ml of PBS and then incubating the cells with 2 ml of cell lysis buffer (20 mM Tris-Cl [pH 7.4], 0.5 M NaCl, 0.5% NP-40, 0.02% NaN_3) for 5 min at 22°C. Cell debris was removed by centrifugation at 16,000 × *g* in a microcentrifuge. Then 15 μl of the cell lysate was mixed with 15 μl of 2× SDS-PAGE buffer and analyzed by electrophoresis and immunoblotting as above. Wild-type RRV was propagated in BHK15 cells and isolated as previously described (3).

Antibody neutralization assays. Virus concentrated from supernatant medium from cultures of E86nlslacZ, 293GPGnlslacZ, or SafeRR-nlslacZ cells (as described above) was mixed in PBS with dilutions of anti-RRV E2 monoclonal antibody MAb10C9 (31) and 10-fold-diluted guinea pig complement for 1 h prior to infection of cells. This mixture was added to DMEM-CS/PS with hexadimethrine bromide (8 μg/ml) and overlaid on NIH 3T3 cells for 5 h prior to replacement with fresh DMEM-CS/PS. Cells were stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) after 48 h. A similar protocol for the determination of neutralization by polyclonal PAbE2 (rabbit anti-RRV E2 antiserum) was carried out, except that the source of the RRV E2E1-pseudotyped retrovirus was SafeRR cells that had been transiently transfected with 4 μg of MFG.S-nlslacZ and the source of the VSV G-pseudotyped retrovirus was gpnlslacZ cells that had been transiently transfected with 4 μg of pMD.G (20).

Treatment of cells with lysosomotropic bases. Filtered supernatant medium from E86nlslacZ, Safe-RRnlslacZ, or 293GPGnlslacZ cells containing hexadimethrine bromide (8 μg/ml) and various concentrations of the lysosomotropic bases chloroquine and NH_4Cl were incubated with NIH 3T3 cells that had been treated for 1 h with corresponding concentrations of the bases. After 5 h the supernatant medium was removed and replaced with fresh DMEM-CS/PS. Transduction of the cells was quantified after 48 h by staining the cells with X-gal. In each case, the titer of virus on untreated cells was approximately 2.5×10^4 transducing units (TU) per ml.

Cell fusion assays. gpnlslacZ and SafeRR-nlslacZA cells were grown to near confluence, washed with PBS, and incubated in cell fusion buffer (10 mM MES [morpholineethanesulfonic acid], 10 mM HEPES) at either pH 7.0 or 5.5 for 1 min, followed by further growth for 4 h in DMEM-FBS/PS. Cells were fixed with methanol at 4°C and treated with Giemsa stain (1 mg/ml) prior to microscopic photography.

RESULTS

Generation and characterization of RRV glycoprotein-pseudotyped retroviruses. In order to determine whether RRV glycoproteins could be incorporated into recombinant Mo-MuLV virions and convey an expanded tropism upon the recombinant retroviruses, ϕNX cells, which produce envelope-deficient replication-incompetent Mo-MuLV particles (11, 23), were transfected with plasmids encoding the RRV glycoproteins (pRRV-E1E2) and MFG.S-GFP-S65T (a retroviral vector encoding the *Aequorea victoria* green fluorescent protein S65T mutant). Recombinant retroviral particles present in the supernatant medium of these transfected cells transduced murine NIH 3T3 cells with a titer of 2×10^4 TU/ml. The RRV glycoprotein-pseudotyped retroviruses also exhibited the expected expanded tropism. RRV glycoprotein-pseudotyped viruses produced through transient transfection of gpnlslacZ cells transduced a variety of mammalian cell lines, including ones not originating from rodents, in a similar fashion to VSV G-pseudotyped retroviruses (Table 1); the parent Mo-MuLV virus infects only rodent cells.

We also determined whether expression of β-galactosidase activity by NIH 3T3 cells incubated with the supernatant medium of the transfected gpnlslacZ cells was maintained as would be expected if the NIH 3T3 cells were successfully transduced by pseudotyped recombinant retroviruses. Over the course of 2 weeks, no reduction in the percentage of cells that expressed β-galactosidase activity was observed (data not shown).

TABLE 1. Transduction of cells by RRV and VSV glycoprotein-pseudotyped Mo-MuLV

Cells	Mean transduction (TU/ml) ± SD	
	RRV	VSV
NIH 3T3	$1.4 (\pm 0.5) \times 10^4$	$1.6 (\pm 0.2) \times 10^4$
BHK21	$5.3 (\pm 0.7) \times 10^3$	$4.2 (\pm 0.7) \times 10^3$
HeLa	$4.8 (\pm 0.6) \times 10^3$	$1.2 (\pm 0.5) \times 10^4$
Vero E6	$2.5 (\pm 0.1) \times 10^3$	$1.6 (\pm 0.1) \times 10^3$
SW13	$1.0 (\pm 0.1) \times 10^2$	$3.0 (\pm 0.8) \times 10^3$
RK13	$1.2 (\pm 0.1) \times 10^3$	$2.6 (\pm 0.4) \times 10^4$

Generation and characterization of stable cell lines that produce RRV glycoprotein-pseudotyped retroviruses. Many applications of the RRV glycoprotein-pseudotyped retroviruses for gene transfer and gene therapy would be facilitated by the construction of stable cell lines that are capable of producing the recombinant retroviruses indefinitely. ϕNX cells were transfected as above with pRRV-E2E1 and the antibiotic resistance-conveying plasmid pJ6Ωpuro and selected for puromycin resistance. A clonal colony of cells, designated SafeRR cells, was isolated, which, upon transient transfection with the retroviral vector MFG.S-nlslacZ (21), produced RRV glycoprotein-pseudotyped Mo-MuLV with a titer of 2×10^4 TU/ml with NIH 3T3 cells as the targets. A stable cell line, designated SafeRR-nlslacZA, has been identified that produces RRV glycoprotein-pseudotyped Mo-MuLV bearing MFG.S-nlslacZ with a titer of 10^5 TU/ml with NIH 3T3 cells as the targets. The stable cell lines can be maintained in culture for at least 2 months without diminution of the titer of recombinant virus. The RRV glycoprotein-pseudotyped virus can be concentrated >500-fold by ultracentrifugation, with 66% recovery of infectious particles.

We wished to confirm that the RRV glycoproteins E2 and E1 were expressed in the packaging cells and incorporated into the recombinant Mo-MuLV particles. Proteins from the lysates of SafeRR-nlslacZA cells and virus particles collected from the supernatant medium of these cells were analyzed by immunoblotting for the presence of the RRV E1 and E2 proteins (Fig. 1). Two immunoreactive proteins were detected in the analysis of the SafeRR-nlslacZA cell lysate using anti-RRV E2 antibodies (Fig. 1A, lane 5). These proteins had mobilities consistent with identification as the unprocessed RRV E2 (PE2) and the mature E2 glycoproteins. The mobility of the immunoreactive proteins detected in the pseudotyped retrovirus particles (Fig. 1A and B, lane 4) corresponded to that of the mature E2 and E1 proteins found in RRV virions (Fig. 1A and B, lane 1).

Inhibition of entry of RRV glycoprotein-pseudotyped retrovirus by anti-RRV E2 antibodies. The recombinant virus produced by the SafeRR-nlslacZ cells was used to characterize further the properties of the pseudotyped viruses and their usefulness for gene transfer and for analysis of RRV inhibition reagents. Superntant medium containing recombinant virus pseudotyped with the RRV glycoproteins, VSV G protein, or the Mo-MuLV envelope (Env) protein (produced by SafeRR-nlslacZ, 293GPGnlslacZ, and E86nlslacZ cells, respectively) was treated with dilutions of anti-RRV E2 polyclonal antiserum or monoclonal antibody 10C9, which binds the cell receptor-binding region of RRV E2 (27), prior to incu-

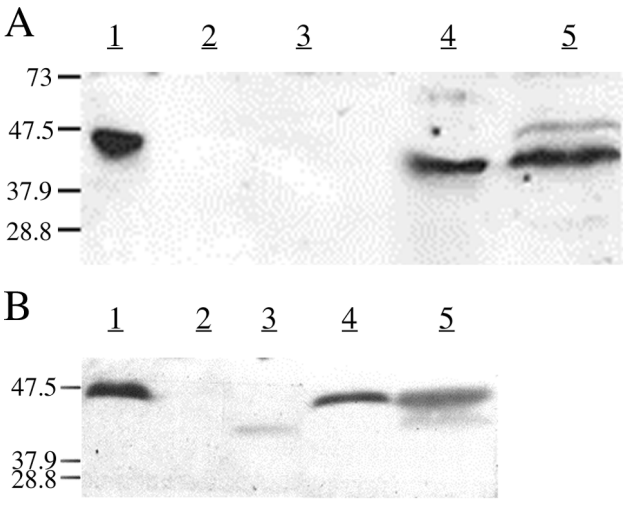

FIG. 1. Immunoblot analysis of RRV E2E1-pseudotyped virus-producing cells and virus. Purified RRV (lane 1) and supernatant medium and lysates of gpnlslacZ cells (lanes 2 and 3, respectively) and of SafeRR-nlslacZA cells (lanes 4 and 5, respectively) were separated by SDS-PAGE and immunoblotted with polyclonal rabbit antibodies to (A) RRV E2 (PAbE2) and (B) RRV E1 as described in Materials and Methods. A cross-reactive protein of higher mobility than E1 is found in the lysates of both the gpnlslacZ and SafeRR-nlslacZA cells. Sizes are shown in kilodaltons.

bation with NIH 3T3 cells. Whereas there was no significant inhibition of transduction by VSV G protein- or Mo-MuLV Env-bearing retroviruses, transduction by the RRV glycoprotein-pseudotyped viruses was specifically inhibited in a concentration-dependent manner (Fig. 2). These results demonstrate that entry of the pseudotyped viruses is mediated by the RRV glycoproteins and that the pseudotyped viruses may be useful for screening reagents that can block RRV entry into cells.

Inhibition of entry of RRV glycoprotein-pseudotyped retrovirus by lysosomotropic weak bases. It is generally accepted that alphaviruses enter cells through receptor-mediated endocytosis and that fusion of the viral and cellular membranes occurs in acidified endosomes (28). Part of the evidence supporting this conclusion is the fact that treatment of cells with lysosomotropic weak bases, such as chloroquine and ammonium chloride, which inhibit the acidification of endosomes, prevents the entry of viruses such as Semliki Forest virus. It has, however, been suggested that the effect of these agents on Sindbis virus infection is mediated through the inhibition of viral RNA replication rather than through the prevention of viral entry (2).

The mode of entry of RRV, which is closely related to Semliki Forest virus, has not yet been investigated. We decided to use the RRV glycoprotein-pseudotyped retrovirus to examine this issue. This approach has the advantage that only the entry step is mediated by proteins from RRV; the other steps in the virus life cycle that lead to transduction are performed by retroviral proteins. Therefore, the effects of treatment of cells with various concentrations of chloroquine and ammonium chloride upon transduction by the RRV glycoprotein-pseudotyped retrovirus were compared with those upon transduction by retroviruses bearing the Mo-MuLV Env or VSV G proteins (Fig. 3). Incubation of the cells with the lysososomotropic bases had little effect on transduction by viruses bearing the Mo-MuLV Env, consistent with the entry of Mo-MuLV through membrane fusion at the cell surface. This result also indicates that the reagents had at most very moderate effects on the other steps of transduction. In contrast, transduction by the VSV G protein-pseudotyped or RRV glycoprotein-pseudotyped retroviruses, which contain the same Mo-MuLV cores, was dramatically inhibited. This is consistent with the known utilization by VSV of the endocytic pathway for entry (18) and with the conclusion that RRV also depends upon acidified endosomes for entry.

pH dependence of cell-cell fusion. Another prediction of the hypothesis that RRV enters through the endocytic pathway and that membrane fusion is triggered by exposure to acid pH is that cells expressing the RRV glycoproteins should fuse membranes in a pH-dependent manner. SafeRR-nlslacZA cells, which express the RRV glycoproteins, and the parent gpnlslacZ cells were exposed to a buffer at pH 5.5 or 7.0 for 1 min, cultured in normal medium for 5 h, and then observed (Fig. 4). Large multinuclear cells, syncytia, formed from the fusion of adjacent cells were detected among the SafeRR-nlslacZA cells that had been incubated at pH 5.5, whereas none were detected among the gpnlslacZ cells incubated at either pH 5.5 or 7.0. Only a few small syncytia were detected among the SafeRR-nlslacZA cells that had been incubated at pH 7.0. These results confirm that RRV glycoprotein-mediated membrane fusion is dependent upon exposure of the glycoproteins to acidic pH.

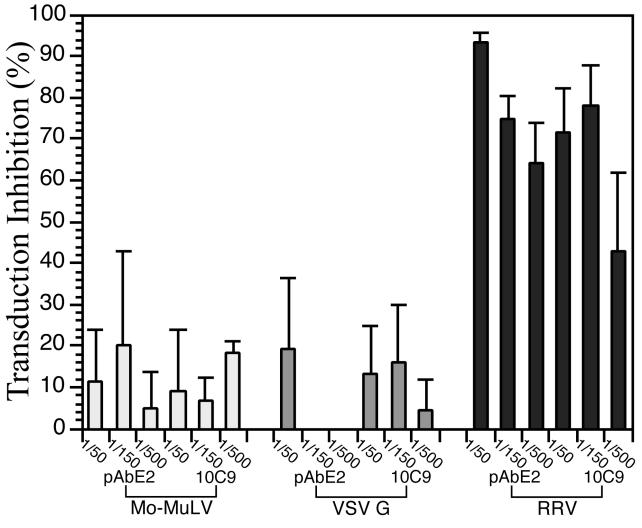

FIG. 2. Antibody-mediated neutralization of RRV E2E1-pseudotyped retroviral infection. RRV E2E1- (black), Mo-MuLV Env- (white), or VSV G- (gray) pseudotyped retroviruses (produced as described in Materials and Methods) were treated with the indicated dilution of anti-RRV E2 monoclonal antibody (MAb10C9) or polyclonal rabbit antiserum (PAbE2) as well as 10% guinea pig complement in PBS for 1 h at room temperature. The antibody-treated virus was diluted in DMEM-CS/PS and used to infect NIH 3T3 cells in the presence of hexadimethrine bromide (8 μg/ml). The data are presented as the percent inhibition of transduction by antibody-treated virus relative to the level of transduction by virus treated only with the 10% guinea pig complement in PBS. The data are representative of three independent experiments

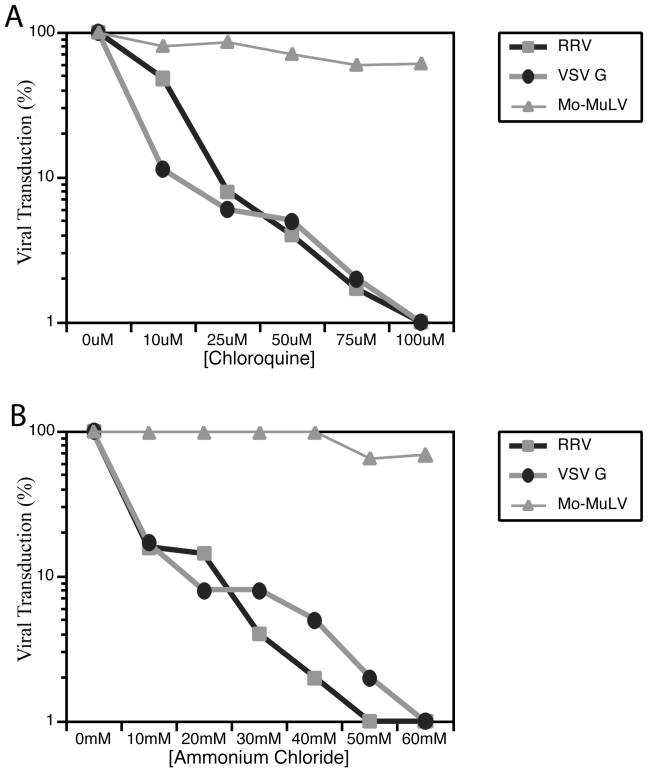

FIG. 3. Inhibition of transduction by RRV E2E1-pseudotyped retrovirus by lysosomotropic weak bases. NIH 3T3 cells were treated for 1 h with the indicated concentrations of (A) chloroquine or (B) ammonium chloride in PBS. Medium carrying pseudotyped nlslacZ-conveying retroviruses (RRV glycoproteins, Mo-MuLV virus envelope protein, or VSV G protein) containing the indicated concentration of base as well as hexadimethrine bromide (8 μg/ml) was incubated with the cells in a CO_2 incubator at 37°C. The cells were stained with X-gal at 48 h postinfection, and blue cells were counted. Viral transduction of cells treated with the indicated concentrations of reagent is represented as a percentage of the level of transduction of untreated cells. The data are representative of three independent experiments.

DISCUSSION

It has been demonstrated that the glycoproteins of a number of enveloped viruses are capable of being incorporated onto the surface of retrovirus particles and of substituting for the retroviral envelope protein in the process of entry. We describe here the construction of recombinant retroviruses bearing the glycoproteins of the alphavirus RRV that are capable of transducing a variety of cell lines. We also describe stable cell lines that produce RRV glycoprotein-pseudotyped virus that may have applications for gene transduction experiments in the cells of a broad variety of animals and tissues. Alphaviruses are insect-borne viruses that are also capable of infecting many cell types in their mammalian hosts, so we anticipate that these pseudotyped viruses will be capable of introducing genes into dividing cells of virtually all higher animals. It is likely that, through the construction of alphavirus glycoprotein-pseudotyped lentiviruses, the range of cells that can be transduced will be further extended. It should be noted that phenotypic mixing of Sindbis glycoprotein antigens with Rous sarcoma virus has been previously observed (37)

The pseudotyped retrovirus with broad host specificity that is currently employed most often in gene transfer experiments is based upon the incorporation of the VSV G protein into the viral envelope (21, 36). Retroviruses pseudotyped with VSV G protein have a venerable history; they were the first pseudotypes between a retrovirus and a different enveloped virus that were identified. Applications of the currently available wide-specificity pseudotyped retroviruses, those based upon VSV G protein pseudotyping (21, 36), are limited by the toxicity of expression of the VSV G protein in the recombinant retrovirus-producing cell and the occurrence of pseudotransduction (9, 14). This latter phenomenon has been observed when, instead of stable gene transduction by VSV G-pseudotyped virus, the protein product of the gene that is carried by the recombinant retrovirus is transferred into the cell that is the target of infection. The presence of the transferred protein is detectable, of course, for only a limited period. The transfer vehicles may be vesicles containing the protein that is being assayed that arise from the propensity of the VSV G protein to form virus-like particles on its own when expressed on cellular membranes (24). In contrast, we have found that there appear to be no toxic effects of expression of the RRV glycoproteins on the packaging cells and that cells transduced by the RRV glycoprotein-pseudotyped retrovirus express the transduced genes over the course of weeks, which is an indication that pseudotransduction is not taking place.

The availability of RRV glycoprotein-pseudotyped recombinant viruses has allowed us to take a new approach to the investigation of a subject of controversy. In the case of Semliki Forest virus, it has been demonstrated that membrane fusion is a low-pH-dependent process and that viral entry requires the endocytosis of bound viral particles (28). In the case of Sindbis virus, however, it has been proposed that infection occurs through entry at the cell surface consequent upon conformational changes (5) induced by glycoprotein disulfide bond reduction, with no requirement for endocytosis (1). It was furthermore suggested that the effect of lysosomotropic weak bases is mediated through inhibition of alphaviral replication rather than through an abrogation of entry resulting from the inhibition of the acidification of endosomes (2). Recently, two alternative approaches to the question have been taken (4, 10). The results of both series of experiments support the hypothesis that alphavirus entry requires clathrin-dependent endocytosis (4) and acidification of the endosomes (10). Our findings that the entry of RRV glycoprotein-pseudotyped retrovirus is inhibited by lysosomotropic agents provides critical support for the hypothesis, because the possible effects of these agents are limited in our system specifically to the viral entry step. It remains possible, nevertheless, that both endocytosis and disulfide bond rearrangements participate in alphavirus glycoprotein-mediated membrane fusion and viral entry (25).

The pseudotype system offers a number of additional distinct advantages for the study of alphavirus entry. This experimental approach allows direct quantification of the capacity of a particular glycoprotein mutant to promote viral entry and permits the effects on membrane fusion to be distinguished from those on alphavirus budding, which is dependent upon glycoprotein-core interactions. Another benefit is that the emergence of revertant viruses, which can sometimes confuse mutational analyses (notwithstanding the great value of studies of such viruses), cannot occur under our assay con-

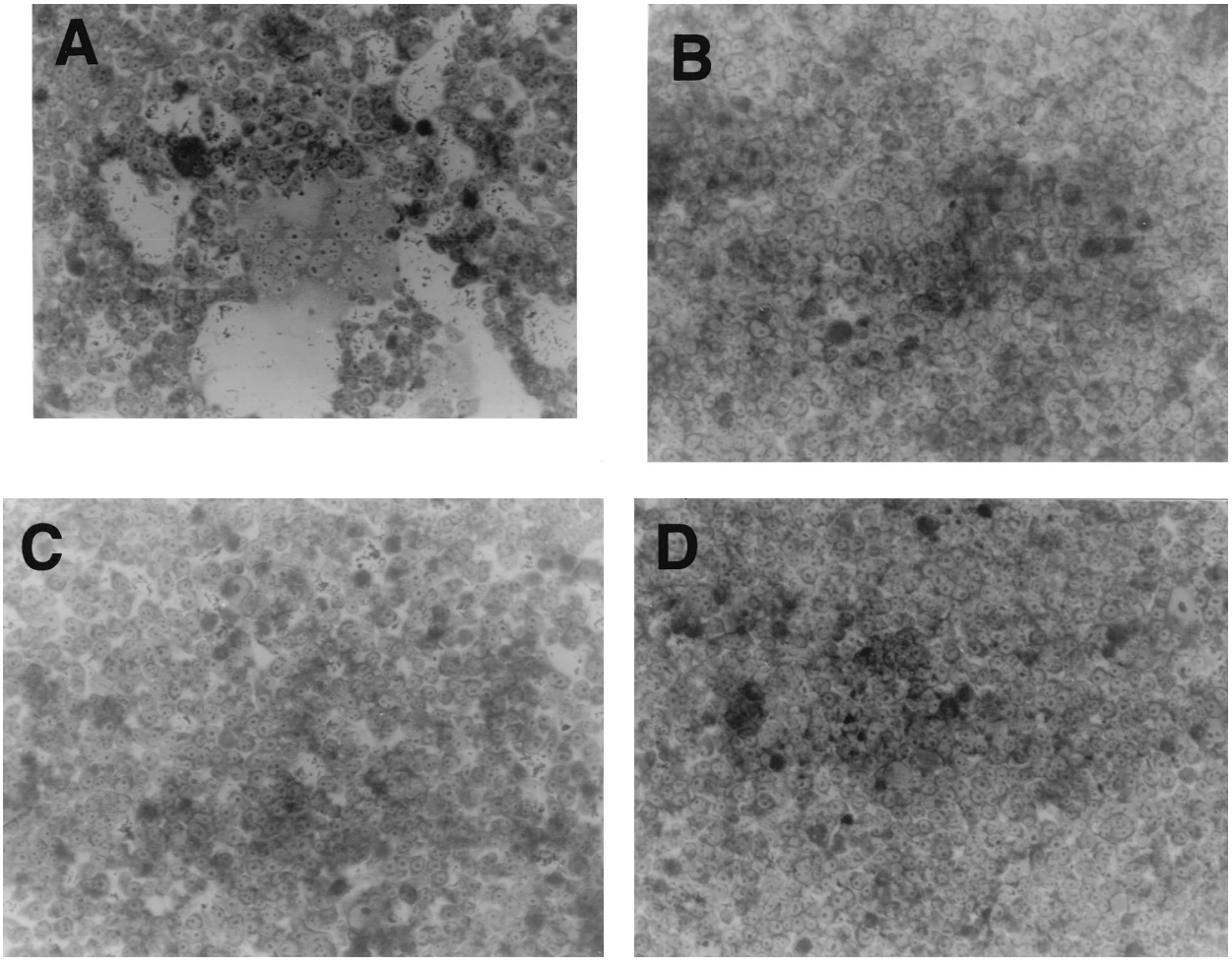

FIG. 4. Cell fusion in low-pH buffer. SafeRR-nlslacZA (A and C) and gpnlslacZ (B and D) cells were grown to near confluence, washed once with PBS, and overlaid with cell fusion buffer (10 mM MES 10 mM HEPES [pH 5.5]) (A and B) or neutral buffer (10 mM MES, 10 mM HEPES [pH 7.0]) (C and D) for 1 min. They were then grown for 4 h with DMEM-FBS/PS in a CO_2 incubator at 37°C to allow syncytium formation.

ditions. An additional consideration is that when the effects of mutations in the glycoprotein genes on alphavirus replication are being examined, there is always the potential for unforeseen consequences of the sequence changes for alphaviral RNA negative- or positive-strand synthesis. In the pseudotype system no such effects are possible. Finally, nucleotide alterations can be introduced into DNA constructs that can be directly transfected into cells, which facilitates investigations into the phenotypic consequences of large numbers of individual mutations.

It has been previously demonstrated that the association of foreign glycoproteins with budding retroviral particles is not a purely random event; the site of glycoprotein expression dictates the site of budding of the retrovirus in polarized epithelial cells even when the foreign glycoprotein has no discernible amino acid identity in the membrane-spanning or cytoplasmic domains with the native retroviral glycoprotein (15). It is noteworthy in this context that the formation of the RRV glycoprotein pseudotypes indicates that functional viral glycoproteins consisting of a trimer of heterodimers each possessing a membrane-spanning domain can be incorporated into a retroviral particle. Previously it had been demonstrated that foreign viral glycoprotein trimers with only three membrane-spanning domains (as opposed to the six present in the alphavirus multimers) could be incorporated into a retrovirus particle. These data suggest both that other alphaviral glycoproteins will be capable of forming functional retroviral pseudotypes and that the repertoire of viral glycoproteins that can be incorporated into functional retroviral particles is greater than previously thought. We anticipate that the alphaviral glycoprotein pseudotypes will have applications in the investigation of viral receptor identity and distribution and in clarifying the role of the various steps in alphavirus glycoprotein posttranslational processing (28).

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