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Adaptation of Chimeric Retroviruses In Vitro and In Vivo: Isolation of Avian Retroviral Vectors with Extended Host Range

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We have designed and characterized two new replication-competent avian sarcoma/leukosis virus-based retroviral vectors with amphotropic and ecotropic host ranges. The amphotropic vector RCASBP-M2C(797-8), was obtained by passaging the chimeric retroviral vector RCASBP-M2C(4070A) (6) in chicken embryos. The ecotropic vector, RCASBP(Eco), was created by replacing the *env*-coding region in the retroviral vector RCASBP(A) with the *env* region from an ecotropic murine leukemia virus. It replicates efficiently in avian DFJ8 cells that express murine ecotropic receptor. For both vectors, permanent cell lines that produce viral stocks with titers of about 5 × 10^6 CFU/ml on mammalian cells can be easily established by passaging transfected avian cells. Some chimeric viruses, for example, RCASBP(Eco), replicate efficiently without modifications. For those chimeric viruses that do require modification, adaptation by passage in vitro or in vivo is a general strategy. This strategy has been used to prepare vectors with altered host range and could potentially be used to develop vectors that would be useful for targeted gene delivery.

Retroviral vectors are widely used in studies of gene structure and function in cultured cells and in animal models. Retroviral vectors have also been used for clinical applications, including human somatic cell gene therapy. A number of retroviral vectors have been developed; most are based on avian and mammalian retroviruses. The majority of these vectors are replication-defective derivatives of the murine leukemia virus (MLV). In general, MLV vectors lack all genes for the viral structural proteins that are required for viral replication. The viral genes are usually expressed either by cotransfection or by a packaging cell line that supplies the viral proteins in *trans*. There are replication-competent MLV vectors; however, the insert size is limited (71, 72). Replication-competent vectors based on avian sarcoma/leukosis viruses (ASLV) can accept larger inserts. Naturally occurring ASLV can have several different envelopes (subgroups A to E). The various ASLV envelopes are distinguished based on host range; none of these envelopes allows the ASLV (or the vectors derived from them) to efficiently infect mammalian cells.

We developed the replication-competent chimeric retroviral vector RCASBP-M2C(4070A) by replacing the subgroup A *env* gene of the ASLV-based retroviral vector RCASBP(A) with the *env*-coding sequence of an amphotropic MLV (6). The original amphotropic RCASBP replicated poorly; passage of the virus selected for a variant that has a single change, P242I, in gp70. The adapted vector, RCASBP-M2C(4070A), replicates efficiently in chicken embryo fibroblasts (CEF) or in DF-1 cells (25, 64) and can efficiently transfer genes into cultured mammalian cells; however, the virus is replication defective in mammalian cells. The RCASBP-M2C(4070A) vector has advantages compared with replication-defective MLV-based vectors. Since the RCASBP-M2C(4070A) vector is replication competent in avian cells, it spreads rapidly through an avian cell culture following transfection and rapidly produces a high-titer viral stock. The vector has no sequence homology with endogenous mammalian retroviruses (except in the envelope region), which makes recombination with an endogenous mammalian retrovirus unlikely. This makes the vector safe as well as convenient. Although the RCASBP-M2C(4070A) vector has advantages, there is one problem: the virus is quite toxic to chicken cells. In practical terms, this means that the viral titer increases rapidly as the vector spreads through the culture and then falls as the infected cells die from the cytopathic effect of the virus. The parental vector RCASBP(A) causes no detectable cytopathic effect in cultured chicken cells, which suggests that the murine envelope causes the cytopathic effect.

We took two approaches to developing versions of the RCAS vector that would efficiently infect mammalian cells but not cause such profound cytopathology in avian cells. First, we asked whether an RCAS vector that used another MLV envelope (the ecotropic envelope) would still be cytopathic. Second, we attempted to select a less cytotoxic derivative of RCASBP-M2C(4070A) by passage in chicken embryos. Both of these approaches were successful; we have developed two new vectors.

One vector, RCASBP(Eco), contains the *env* gene from an ecotropic MLV. The vector replicates in avian (DF-1) cells that express the murine ecotropic receptor. This vector can efficiently infect murine cells and has only modest cytotoxicity in DF-1 cells that express the ecotropic receptor. The second vector, RCASBP-M2C(797-8), was derived by passaging the cytopathic amphotropic vector RCASBP-M2C(4070A) in chicken

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embryos. The resulting vector is considerably less cytotoxic for DF-1 cells than the 4070A parent. We were able to use both of the new vectors to establish permanent avian producer cell lines that produce high-titer viral stocks.

MATERIALS AND METHODS

Cell culture. The avian cell line DF-1 (25, 64) was kindly provided by Douglas Foster (University of Minnesota, Minneapolis). Construction of the avian cell line DFJ8, which expresses the murine ecotropic receptor, is described below. DF-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 5% newborn calf serum, 10% tryptose-phosphate broth (GIBCO BRL, Gaithersburg, Md.), 100 U of penicillin per ml, and 100 μg streptomycin (Quality Biological, Inc., Gaithersburg, Md.) per ml. DFJ8 cells were grown in the same medium supplemented with G418 (200 μg/ml; GIBCO BRL). The murine packaging cell line PA317 and the BAG2 retroviral vector-producing cell line CRE-BAG2 (57) were obtained from the American Type Culture Collection (Manassas, Va.). These cells were maintained in Dulbecco's modified Eagle's medium with 10% calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Recombinant DNAs. Plasmids were constructed using standard techniques (63). The recombinant retroviral vector RCASBP(Eco) was prepared as follows. The *env* coding region of an ecotropic Moloney MLV was PCR amplified from plasmid pRR88 (kind gift from Alan Rein, HIV Drug Resistance Program, National Cancer Institute), using primers RSV-ECOF2E (GCTTCGCCCGGC TCCAGT) and RSV-ECOR2 (ACACACGCGGCCGCCTATGGCTCGTACT CTATAGGC). The fragment spanning the unique *Kpn*I site, the *env* splice acceptor site, and the coding region of the signal peptide was PCR amplified from the retroviral vector RCASBP(A) (55), using primers RSV-ECOF1 (GA GTGGGAAAAAGGATGGAACG) and RSV-ECORIE (AGTGGACCCGGG CGAAGCAGCTCTTACCCCCGTAACCTCA). The resulting PCR fragments were fused and amplified by overlap extension PCR (32) with primers RSV-ECOF1 and RSV-ECOR2. The PCR product contained a gene for a chimeric Env consisting of the signal peptide from RCASBP(A) and the surface and transmembrane proteins from ecotropic MLV. To construct the retroviral vector RCASBP (Eco), an aliquot of the PCR product was cleaved with *Kpn*I and *Nco*I. A second aliquot of the PCR product was cleaved with *Nco*I and *Not*I in a separate reaction. The *env* region was removed from the retroviral vector RCASBP-M2C(4070A) (6) by cleavage with *Kpn*I and *Not*I, and the chimeric ecotropic *env* gene was inserted by three way-ligation of this fragment with the *Kpn*I-*Nco*I and *Nco*I-*Not*I PCR fragments, generating the plasmid RCASBP(Eco).

To construct the recombinant murine retroviral vector LRNL-J8, the coding region for the ecotropic receptor MCAT-1 was isolated from plasmid pJET (3) by cleavage with *Bam*HI. The *Bam*HI ends were filled using T4 DNA polymerase. *Sal*I linkers (New England Biolabs, Beverly, Mass.) were ligated to the fragment. Subsequently, the fragment was cleaved with *Sal*I and inserted into the *Sal*I site of a murine retroviral vector, LRNL (79), generating LRNL-J8.

Transfection and preparation of viral particles. The DF-1 and DFJ8 cells were transfected by a modified $CaPO_4$ precipitation technique (22). Briefly, a $CaPO_4$ precipitate containing 10 μg of plasmid DNA was added to the culture medium, and the cultures were incubated at 37°C for 4 h. The cells were then incubated in the medium containing 15% glycerol for 5 min at 37°C, washed two times in phosphate-buffered saline (PBS), and grown in culture medium. The transfected cell cultures were passaged to allow the virus to spread through the culture.

To transfect PA317 cells, a $CaPO_4$ precipitate containing 10 μg of plasmid DNA was added to the culture medium, and the cultures were incubated at 37°C overnight. Twenty-four hours posttransfection, the culture medium was replaced.

To prepare viral proteins for Western blot analysis, culture medium from infected cells was clarified by centrifugation at 3,000 rpm for 10 min, and the viral particles were pelleted through a 15% sucrose cushion by centrifugation at 35,000 rpm for 1 h at 4°C in an SW41 rotor (Beckman, Fullerton, Calif.). The pellet was resuspended in protein gel sample buffer, heated at 100°C for 4 min, and loaded onto a gel as described below.

Western blot analysis. Viral proteins were fractionated by electrophoresis in a sodium dodecyl sulfate (SDS)–4 to 20% gradient polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, Mass.). ASLV capsid protein was detected by incubation of the membrane with rabbit antiserum that recognized p27 (generated by immunization of rabbits with virus particles). MLV envelope proteins were detected by incubation with goat antiserum against gp70 or with rabbit antiserum against p15E (kindly provided by Alan Rein). Protein bands were visualized by enhanced chemiluminescence detection with the alkaline phosphatase substrate CDP-Star (Boehringer Mannheim, Indianapolis, Ind.).

p27 antigen capture enzyme-linked immunosorbent assay (ELISA). Rabbit anti-p27 antibodies conjugated with horseradish peroxidase (anti-p27-HRP) were obtained from SPAFAS, Inc. (North Franklin, Conn.). Ninety-six-well plates were coated with anti-p27 antibodies (generated by immunization of rabbits with ASLV particles) in 100 mM sodium carbonate-sodium hydrocarbonate buffer (pH 9) overnight at 4°C. The wells were washed with PBS containing 0.1% Tween 20 and were incubated in a solution of 5% nonfat dried milk in PBS at 37°C for 1 h to block nonspecific binding. Samples were prepared by adding Tween 20 to the cell culture supernatants (to a final concentration of 0.5%), followed by three cycles of freezing at −70°C and thawing at 37°C. The anti-p27 antibody-coated wells were incubated with the supernatants at 37°C for 1 h. The wells were then washed with PBS containing 0.1% Tween 20 and incubated with anti-p27-HRP in 5% milk–PBS. The antigen-antibody complexes were detected using the trimethylbenzidine-hydrogen peroxide substrate reagent (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). The color reaction was measured using a Dynatech plate reader with a 405-nm filter.

Avian cell line DFJ8. The avian cell line DFJ8 was prepared by stably transferring the MCAT-1 cDNA, which encodes the receptor for ecotropic MLV, into DF-1 cells using a retroviral vector. PA317 cells were transfected with the retroviral vector LRNL-J8 (described above). Forty-eight hours posttransfection, culture medium containing the virus was harvested and used to infect DF-1 cells. Polybrene (final concentration, 8 μg per ml) was added to the culture medium obtained from the transfected PA317 cells. The mixture was added to 5×10^5 DF-1 cells in a 60-mm-diameter tissue culture dish. The cells were incubated for 4 h at 37°C, and 3.5 ml of culture medium was added. Forty-eight hours postinfection, the cells were trypsinized and plated in culture medium that contained G418 (400 μg/ml; GIBCO BRL). Two days later, the culture medium was replaced with the fresh medium containing G418 (200 μg/ml). Colonies of G418-resistant cells appeared 15 days postinfection. Ten clones were isolated by using cloning cylinders, and cell lines were developed. To test these cell lines for expression of the receptor, 5×10^5 cells were infected with the ecotropic retroviral vector BAG2 produced by CRE-BAG2 murine cells (57); 48 h postinfection, the cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (57).

Adaptation of RCASBP-M2C(4070A) in chicken embryos. Embryonated eggs (line EV-0) received 0.1 to 0.2 ml of the RCASBP-M2C(4070A) virus stock at day 6 of incubation. A 21-gauge, 1-in. needle was used to inject the virus into embryos through the air cell of the egg. At day 12 of incubation, the live embryos were processed. Under sterile conditions, the large end of the egg was opened, and the shell membrane was removed. To prepare the embryo extract, the embryos were placed into a 10-ml syringe fitted with an 18-gauge needle and then pushed through the needle into 10 ml of complete LM medium (8.75 g of Leibowitz L-15 medium, 5.0 g of McCoy 5A medium, 1.5 g of $NaHCO_3$ per liter) supplemented with 10% fetal bovine serum, amphotericin B (Fungizone; 3 μg/ml), and gentamicin (50 μg/ml). The suspension was vortexed vigorously. Embryo extract samples were centrifuged at $400 \times g$ to remove cells and debris; 0.5 ml of each sample was used to infect DF-1 cell cultures that were approximately 60% confluent. The infected DF-1 cells were passaged five times to prepare virus stocks.

Confocal microscopy. Cells infected with RCASBP(Eco) or with RCASBP-M2C(4070A) were grown in plastic petri dishes. Confocal microscopy was performed with an inverted laser confocal microscope (Zeiss, Jena, Germany).

Titration of retroviral vectors expressing the puromycin resistance gene on mammalian cells. Virus titers were determined on mammalian cells as described previously (6). Briefly, DF-1 cells were transfected with retroviral vectors that the expressed puromycin resistance gene. At each cell passage, serial dilutions of the culture medium from the transfected cells were prepared and used to infect D17 (dog) cells. Forty-eight hours postinfection, the cells were trypsinized and plated in culture medium that contained puromycin. Fifteen days postinfection, colonies of puromycin-resistant cells were Giemsa stained and counted.

Cloning of adapted RCASBP(Eco) and RCASBP-M2C(4070A) genomes. The cloning of genomes of adapted viruses and the reconstruction of corresponding replication-competent retroviral vectors were performed as described (6). Briefly, Hirt DNA was extracted from infected DFJ8 or DF-1 cells and used to construct a library in the λZAPExpress cloning vector (Stratagene, La Jolla, Calif.). The library was probed with radioactively labeled fragments of the ecotropic or the amphotropic *env* gene. Positive phage clones were purified and converted into plasmid clones. The resulting plasmid DNAs were cut with *Sac*I and *Cla*I. The *Sac*I-*Cla*I fragments that contained the *gag, pol*, and *env* genes of the adapted retroviruses were used to replace the *Sac*I-*Cla*I fragments of non-

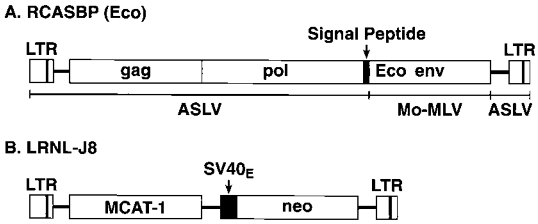

FIG. 1. Schematic of structures of chimeric retroviral vector RCASBP(Eco) (A) and murine retroviral vector LRNL-J8 (B) LTR, long terminal repeat; Mo-MLV, Moloney MLV; $SV40_E$, simian virus 40 early promoter.

adapted retroviral vectors, generating adapted replication-competent retroviral vectors.

Sequencing of the *env* gene. The cloned *env* genes were sequenced by the dideoxy-chain termination method with primers specific for the ecotropic and amphotropic *env* genes (6).

RESULTS

The avian cell line DFJ8 expresses a functional ecotropic receptor. Ecotropic Moloney MLV use the murine cationic amino acid transporter MCAT-1 as a cellular receptor. Most nonrodent cells are not permissive for ecotropic MLV infection; however, these cells become permissive if they express MCAT-1 (5, 8, 28, 51, 58, 67, 68). Since avian cells lack a functional receptor for ecotropic murine retroviruses, they are not efficiently infected by ecotropic murine retroviruses. We constructed an avian cell line, DFJ8, which stably expresses MCAT-1; this line is efficiently infected by ecotropic retroviruses. A replication-defective murine retroviral vector, LRNL (79), was used to transfer the MCAT-1 cDNA into the avian cell line DF-1. The vector LRNL-J8 expresses MCAT-1 from the long terminal repeat (Fig. 1B); this vector also expresses the G418 resistance gene (*neo*).

Ten G418-resistant DF-1 clones were isolated and tested for expression of a functional MCAT-1 by infection with the ecotropic retroviral vector BAG2 (57). All of the cell lines that we isolated could be infected with the murine vector; the parental DF-1 cell line could not (data not shown). The cell line that was most susceptible to infection by the BAG2 vector was designated DFJ8 and was used for experiments with the vector RCASBP-M(Eco) and its derivatives.

The chimeric retroviral vector RCASBP(Eco) replicates in DFJ8 cells but not in DF-1 cells. A chimeric ecotropic *env* gene was prepared in which the region encoding the ecotropic gp70 surface glycoprotein was fused to a sequence encoding the Env signal peptide of an ASLV (see Materials and Methods). The amphotropic *env* gene was removed from the vector RCASBP-M2C(4070A) (6) and replaced with chimeric ecotropic *env* gene (Fig. 1A), creating the vector RCASBP(Eco).

One of the RCASBP(Eco) clones turned out to be aberrant. It carried the fragment of the pBR322 plasmid DNA between the 3′ terminus of the ecotropic *env* gene and the 3′ long terminal repeat. In this clone, the sequence encoding the two C-terminal amino acid residues of p15E and the stop codon was replaced with a segment of the pBR322 sequence. This aberrant construct was designated RCASBP(Eco1) and was used in the experiments in parallel with the normal RCASBP(Eco) vector.

To generate virus stock, DFJ8 cells were transfected separately with RCASBP(Eco) and RCASBP(Eco1) plasmid DNAs, and the cells were passaged to allow the virus to replicate. Two plates of DFJ8 cells were independently transfected with each plasmid. To test the ability of the viruses to replicate specifically in DFJ8 cells, two plates of unmodified DF-1 cells were transfected in parallel with RCASBP(Eco) and RCASBP(Eco1), and the cells were passaged. Samples of culture media were collected at each passage, and then the samples were analyzed for the presence of viral particles using a p27 antigen capture ELISA (see Materials and Methods). The RCASBP(Eco) virus replicated rapidly in transfected DFJ8 cells. Virus production reached peak level by passage 2 (data not shown). However, the aberrant RCASBP(Eco1) virus initially replicated slowly. As shown in Fig. 2A, 24 h posttransfection, the cells transfected with RCASBP(Eco1) released virus particles that could be detected by ELISA. This initial burst of virus production was followed by a lag period, during which production of the virus dropped to a level that was practically undetectable by ELISA. Virus production rose again at passage 4 and reached a peak at passage 6, suggesting that the virus had spread through the culture (Fig. 2A). No virus production was observed in transfected DF-1 cells, showing that both RCASBP(Eco) and RCASBP(Eco1) replicated only in cells expressing the ecotropic receptor.

To show that the virus stocks were infectious for DFJ8 cells, aliquots of the cell culture medium obtained at passage 6 were used to infect fresh DFJ8 cells. DF-1 cells were also treated with the same culture medium. As in the previous experiment, we collected samples of cell culture medium at each passage and measured the production of virus by p27 ELISA. Both viruses were detected in the culture medium from DFJ8 cells at passage 1 and reached the peak level by passage 2, suggesting that both viruses replicated efficiently and rapidly infected all cells in the culture. No lag period was seen in the second infection by RCASBP(Eco1) (compare graphs in Fig. 2A and B). DF-1 cells did not produce detectable viral particles (Fig. 2B). This result suggests that the chimeric retroviral vectors RCASBP(Eco) and RCASBP(Eco1) have an ecotropic host range and that the RCASBP(Eco1) virus generated by transfection replicated slowly at first and adapted in the course of passaging on DFJ8 cells.

Sequence changes in the ecotropic *env* gene of adapted RCASBP(Eco1). To detect possible sequence changes in the rapidly replicating RCASBP(Eco) virus, the entire *env* region was PCR amplified from the total genomic DNA of infected DFJ8 cells and sequenced. As expected, no sequence changes were found, showing that RCASBP(Eco) did not undergo adaptation upon passaging in DFJ8 cells.

Given the delay in the appearance of rapidly replicating viruses after transfection of RCASBP(Eco1) in DFJ8 cells, we considered the possibility that the rapidly replicating virus was a genetic variant of the original vector RCASBP(Eco1). Molecular clones were prepared from the adapted viral stock, and the sequence of the *env* gene in each clone was determined. In each of the five clones sequenced, the aberrant amino acid sequence at the C terminus of the transmembrane protein p15E was changed to IEYGAGR, followed by a stop codon

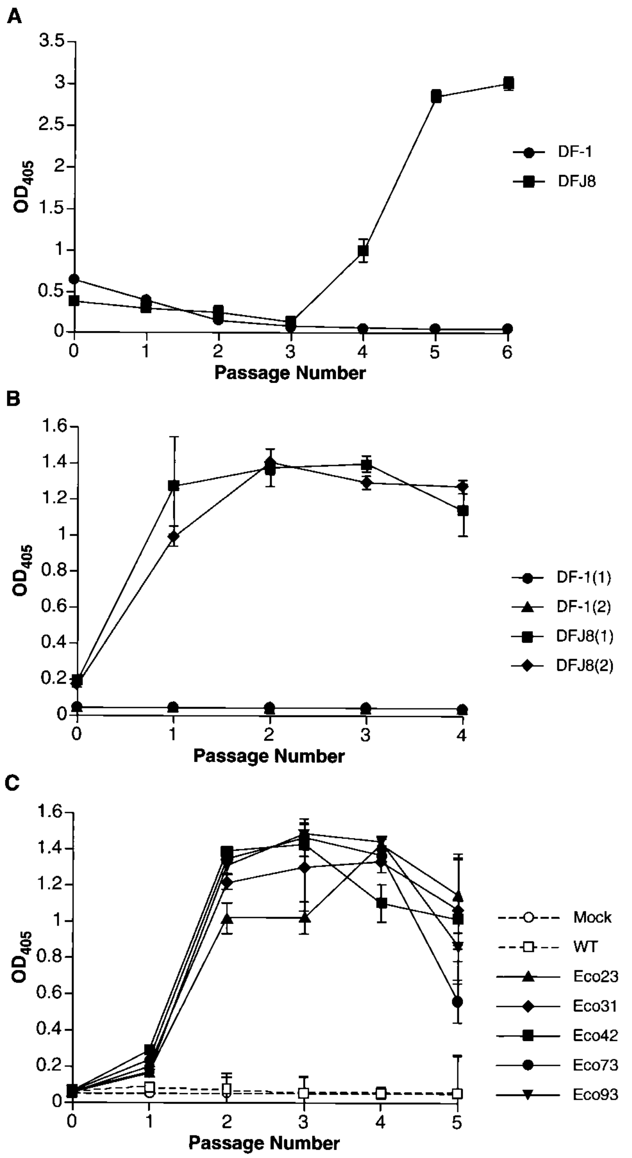

FIG. 2. Replication and adaptation of the ecotropic vector RCASBP(Eco1) in avian cells. DF-1 and DFJ8 cells were transfected with RCASBP(Eco1) plasmid DNA or infected with RCASBP(Eco1) virus and passaged every other day. At each passage of the transfected cells, samples of the culture medium were harvested and the level of virus production was quantified by p27 capture ELISA (see Materials and Methods). (A) Replication of unadapted virus. Cells were transfected with plasmid DNA. (B) Replication of the adapted virus. DF-1 and DFJ8 cells (two plates of each) were infected with cell culture medium harvested at passage 6 of the unadapted virus (A). (C) Replication of molecular clones of adapted RCASBP(Eco1). DFJ8 cells were transfected with plasmid DNAs of adapted RCASBP(Eco1) and, separately, with plasmid DNA of unadapted RCASBP(Eco1). Mock, uninfected cells; WT, wild-type (unadapted) vector. Values are averages of two independent determinations. OD_{405}, optical density at 405 nm.

(Fig. 3). Comparison of the sequences at the 3′ end of the *env* gene and the downstream pBR322 fragment in the adapted and unadapted RCASBP(Eco1) showed that in the adapted virus, a portion of the pBR322 sequence was deleted. This deletion resulted in a fusion of the C-terminal p15E sequence with the downstream segment of pBR322 encoding the amino acid sequence GAGR and a stop codon. These changes were identical in all five ecotropic *env* genes. No other mutation was found in any of the five ecotropic *env* genes.

DFJ8 cells were transfected with each of the adapted RCASBP(Eco1) clones that we obtained. Replication was measured by testing culture medium for the presence of p27 by ELISA. As shown in Fig. 2C, all of the cloned viruses spread rapidly through DFJ8 cultures. In contrast, the cells transfected with nonadapted RCASBP(Eco1) began to produce virus only at passage 6 (data not shown).

Cytopathic effect of RCASBP(Eco) and the adapted RCASBP (Eco1) viruses in DFJ8 cells. The amphotropic vector RCASBP-M2C(4070A) (6) caused pronounced cytopathic effects (vacuolization of cytoplasm and a formation of syncytia) when the virus replicated in CEF. Similar morphological changes were seen in DF-1 cells infected by RCASBP-M2C(4070A). The peak of the cytopathic effect in DF-1 cells was seen three to four passages after transfection (or infection) with RCASBP-M2C(4070A) and was preceded by a peak of viral production measured by ELISA (data not shown). The infected DF-1 cultures went through a crisis, and most of the cells died by passage 5 or 6. A small number of cells survived, replicated, and formed a monolayer 2 to 3 weeks later. These cells released substantially less virus into the culture medium than the acutely infected cells. This suggested that only those cells that expressed relatively low amounts of viral proteins (and produced fewer viral particles) survived.

To determine whether the adapted ecotropic vector RCASBP(Eco1) would cause cytopathic changes in DFJ8 cells, the cells were transfected with a cloned adapted RCASBP (Eco1), RCASBP(Eco73) (clone 73; Fig. 2C and 3). Separate cultures of DFJ8 cells were transfected with RCASBP-M2C(4070A). At passage 4, RCASBP(Eco73) caused formation of only a few syncytia. A similar effect was seen in cells infected with the RCASBP(Eco) vector (data not shown). In contrast, there were profound cytotoxic effects in the cells infected with RCASBP-M2C(4070A); vacuoles were seen in the cytoplasm, and there were numerous syncytia (data not shown).

We investigated whether it was possible to establish a permanent cell line that would produce high-titer stocks of either the ecotropic or the amphotropic RCASBP vector. A gene that confers resistance for hygromycin B (24) was inserted into the *Cla*I site of the adapted retroviral vectors RCASBP(Eco73) and RCASBP-M2C(4070A), generating vectors RCASBP (Eco73)Hyg and RCASBP-M2C(4070A)Hyg. These vectors were introduced into DFJ8 or DF-1 cells by transfection; the cells were passaged and, at different passages, treated with hygromycin B. DFJ8 cells transfected with RCASBP(Eco73) Hyg and passaged one to two times formed a monolayer in the presence of hygromycin B. These cells produced a viral stock with a titer of up to 5×10^6 CFU per ml (data not shown). In contrast, the DF-1 cells transfected with RCASBP-M2C (4070A)Hyg showed signs of cytotoxicity. The cells died rapidly during selection with hygromycin B, and we were not able to establish a permanently infected vector-producing cell line (data not shown). We believe that the difference in the behavior of RCASBP(Eco73)Hyg- and RCASBP-M2C(4070A)Hyg-

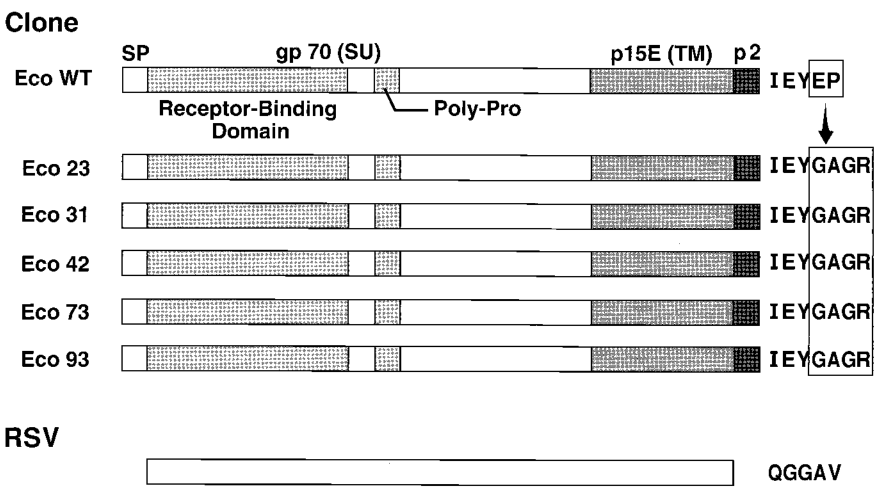

FIG. 3. Changes in the amino acid sequence of the Env protein in molecular clones of adapted RCASBP(Eco1) viruses. Eco WT, wild-type ecotropic Env protein; Eco 23 to Eco 93, Env proteins in molecular clones of the adapted virus. RSV (Rous sarcoma virus), the envelope of the avian virus used to construct RCASBP(A), showing the sequence at the end of the transmembrane protein (TM). SP, signal peptide; SU, surface protein.

infected cells is due to a difference in the cytopathogenicity of the retroviral vectors.

Processing of the transmembrane protein p15E in the adapted RCASBP(Eco73) virions. Earlier work showed that in the particles of amphotropic vector RCASBP-M2C(4070A), the transmembrane protein p15E is efficiently processed (6). In the ecotropic vector RCASBP(Eco73), the aberrant C-terminal amino acid sequence of p15E was changed to IEYGAGR during passage in DFJ8 cells. We examined whether this change affected the cleavage of p15E. Virus particles from DFJ8 cells infected with RCASBP(Eco73) were analyzed for the presence of processed p15E by immunoblotting with anti-p15E antibodies. As shown in Fig. 4, in the virions of RCASBP (Eco73), p15E is processed with approximately the same efficiency as in the virions of amphotropic vector RCASBP-M2C(4070) or amphotropic MLV. This suggests that the processing of the modified p15E in the chimeric ecotropic retrovirus particles is relatively efficient.

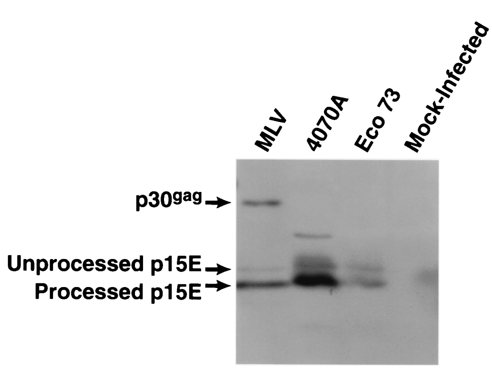

FIG. 4. Proteolytic processing of the pre-p15E in RCASBP(Eco73) virions. Virus particles produced by infected DFJ8 cells were recovered by ultracentrifugation. Viral proteins were fractionated on SDS–16% polyacrylamide gels and transferred to Immobilon P membranes (see Materials and Methods). Virion-associated transmembrane protein was detected with antibodies against p15E. MLV, wild-type amphotropic MLV (positive control); 4070A, RCASBP-M2C(4070A) (positive control); Eco 73, ecotropic vector RCASBP(Eco73).

Passaging of RCASBP-M2C(4070A) in chicken embryos selects viruses with reduced cytopathogenicity. The cytopathic effect of RCASBP-M2C(4070A) limits its usefulness. We first tried to select a less cytopathogenic virus in vitro by passaging of RCASBP-M2C(4070A) in DF-1 cells. However, even prolonged passaging of the virus in culture failed to produce a virus that had significantly reduced cytotoxicity (data not shown). We decided to try to select a less cytotoxic virus in embryonated eggs.

DF-1 cells were transfected with RCASBP-M2C(4070A), and the cells were passaged several times to generate a high-titer virus stock. Culture medium containing the RCASBP-M2C(4070A) virus was injected into EV-0 chicken embryos at day 6 of incubation. EV-0 embryos do not have endogenous retroviruses that are closely related to RCASBP. After 6 days of incubation in the embryos, the virus was recovered and used to infect DF-1 cells. Separately, control DF-1 cells were infected with the original cytopathic virus. At each passage, the morphology of infected cells was monitored. In parallel, the culture medium was collected and tested for the production of virus particles by p27 ELISA. To prove that the recovered virus was the amphotropic vector and not a recombinant retrovirus that could have been generated by recombination between the RCASBP-M2C(4070A) and a distantly related endogenous chicken retrovirus, we also tested the viral particles for the presence of both ASLV p27 capsid protein and amphotropic MLV gp70.

As shown in Fig. 5A, RCASBP-M2C(4070A) caused considerable vacuolization of cytoplasm in the infected DF-1 cells by passage 4. In contrast, viruses isolated from two separately infected chicken embryos caused only minor alterations of the morphology in the cells (compare the cells in Fig. 5B and C with the cells in Fig. 5A). These viruses were designated RCASBP-M2C(797) and RCASBP-M2C(808).

The data in Fig. 6A show that all three viruses grew rapidly

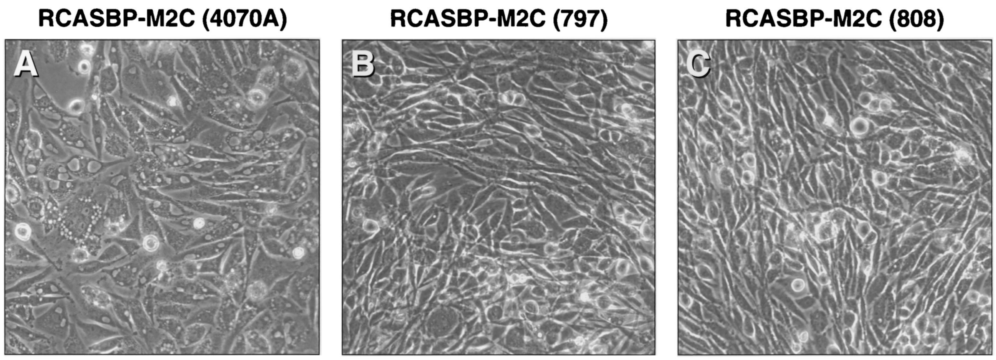

FIG. 5. Cytotoxic effect of RCASBP-M2C(4070A) and chicken embryo-adapted viruses RCASBP-M2C(797) and RCASBP-M2C(808) in DF-1 cells. The cells were infected either with the cytotoxic amphotropic vector RCASBP-M2C (4070A) or with viruses recovered from two of the infected chicken embryos. The morphology of the infected cells at passage 4 is shown.

in infected DF-1 cells. All virus-infected cell cultures reached the same level of virus production at passage 3. Particles of both RCASBP-M2C(797) and RCASBP-M2C(808) contained the amphotropic glycoprotein gp70, as shown by immunoblotting (Fig. 6B). This confirmed that the viruses recovered from chicken embryos were adapted versions of the amphotropic vector RCASBP-M2C(4070A) with substantially reduced cytotoxicity.

Cytotoxicity and sequence analysis of the *env* gene of the chicken embryo-adapted viruses RCASBP-M2C(797) and RCASBP-M2C(808). Based on our previous work with adaptation of RCASBP-M2C(4070A) in CEF (6), we expected that the adapted variants of the virus would contain mutations in their *env* genes. The original amphotropic RCASBP replicated poorly in chicken cells; RCASBP-M2C(4070A) has a single mutation in gp70, P242I, that substantially enhances the replication of the chimeric virus. Molecular clones of the viruses RCASBP-M2C(797) and RCASBP-M2C(808) were prepared from Hirt-fractioned DNA isolated from infected DF-1 cells as described previously (6). DF-1 cells were transfected with two clones of RCASBP-M2C(797), 797-1 and 797-8, and three clones of RCASBP-M2C(808), 808-1, 808-4, and 808-8, and then passaged. Separately, the DF-1 cells were transfected with the control plasmid, RCASBP-M2C(4070A). Cells transfected with clones 808-1 and 808-4 produced very limited amounts of viral particles at passage 4 as determined by p27 ELISA (Fig. 7A), suggesting that these clones were replication defective. In contrast, the cells transfected with clones 797-1, 797-8, and 808-8 produced about the same amount of p27 as those transfected with the parental virus, RCASBP-M2C(4070A) (Fig. 7A). As can be seen in Fig. 7B, the cells transfected with clones 797-1, 797-8, and 808-8 grew normally, and there was only limited vacuolization of the cytoplasm. In contrast, cells transfected with RCASBP-M2C(4070A) stopped growing and displayed substantial vacuolization. We easily established continuously growing cultures from the cells transfected with adapted viral clones. In contrast, the majority of cells transfected with RCASBP-M2C(4070) died at passage 5 after transfection. This

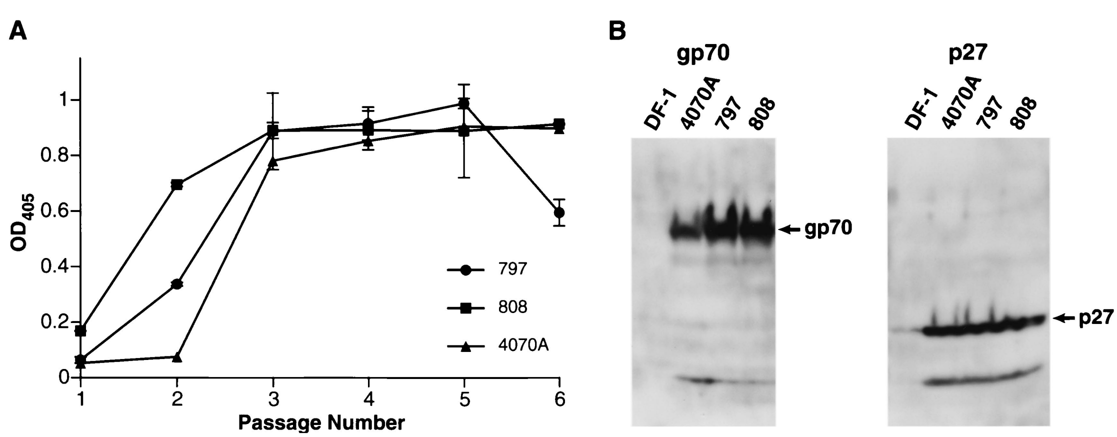

FIG. 6. Replication of chicken embryo-adapted amphotropic vectors in DF-1 cells. (A) p27 capture ELISA. The cells were infected with viruses obtained from infected chicken embryos or, separately, with the cytotoxic amphotropic virus RCASBP-M2C(4070A) and passaged every other day. At each passage, virus production was measured by quantitation of the p27 level in samples of the culture medium by ELISA. Values are averages of two independent determinations. (B) Virus was recovered from culture medium by ultracentrifugation, fractionated on SDS-gradient (5 to 20%) polyacrylamide gels, and detected by immunoblotting. Virion-associated Env and capsid proteins were analyzed by immunoblotting with antibodies against gp70 and p27. DF-1, supernatant from uninfected DF-1 cells; 4070A, supernatant from cells infected with RCASBP-M2C(4070A); 797 and 808, supernatant from cells infected with viruses recovered from chicken embryos. OD_{405}, optical density at 405 nm.

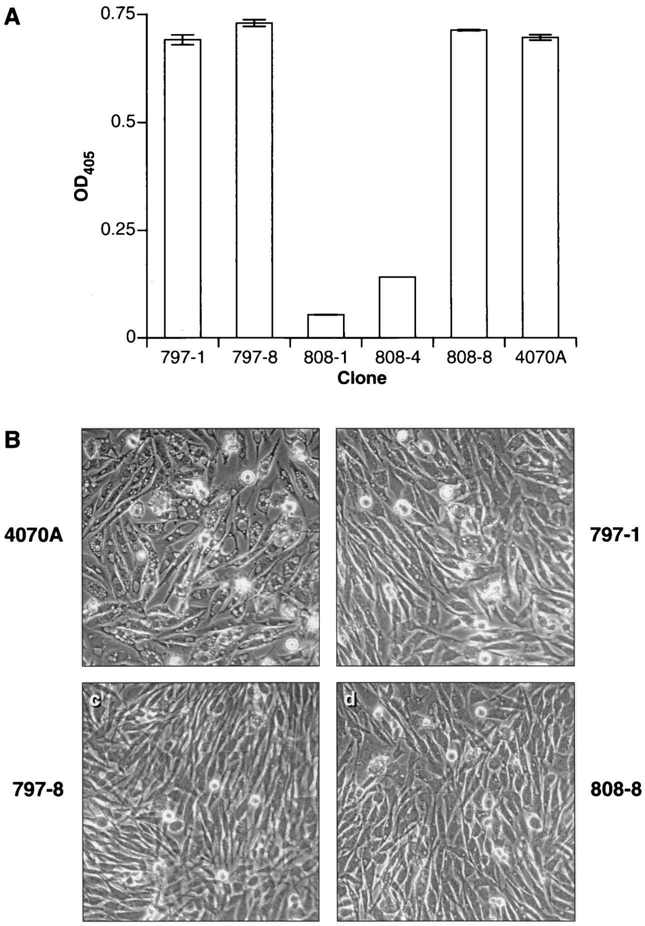

FIG. 7. Replication and cytotoxic effects of molecularly cloned chicken embryo-adapted amphotropic vectors in DF-1 cells. (A) p27 capture ELISA. DF-1 cells were transfected with plasmid DNA clones of chicken embryo-adapted vectors and passaged. Separately, the cells were transfected with cytotoxic amphotropic vector RCASBP-M2C(4070A). At passage 4, the amount of p27 capsid protein in the culture medium of the transfected cells was quantified by ELISA. Values are averages of two independent determinations. OD_{405}, optical density at 405 nm. (B) Cytotoxic effect in DF-1 cells at passage 4. 4070A, cells transfected with RCASBP-M2C (4070A); 797-1, 797-8, 808-1, 808-4, and 808-8, cells transfected with molecular clones of chicken embryo-adapted vectors.

shows that the clones 797-1, 797-8, and 808-8 are variants of the vector RCASBP-M2C(4070A) that have a reduced cytopathic effect.

The *env* genes of the adapted viral clones were sequenced to determine the genetic changes responsible for the reduced cytopathogenicity of the RCASBP-M2C(4070A) variant. In all five clones, the isoleucine at position 242 of gp70 was replaced with threonine (Fig. 8). Clones 797-1 and 797-8 were identical and carried only the I242T mutation. Clone 808-8 carried two additional mutations, L59F in the N-terminal portion of gp70 and E574L in the transmembrane protein p15E. These mutations, which were present in only the one clone, apparently have no effect on the ability of the virus to replicate in DF-1 cells (Fig. 7A). One of the replication-defective clones, 808-1, carried a large deletion in the N-terminal segment of gp70-coding region and had several additional mutations (L175S, L176S, and L450F) in the gp70 and one mutation, A545V, in the p15E-coding region. The other replication-defective clone, 808-4, carried a small duplication in the N-terminal portion of gp70, as well as mutations in gp70 (V342M) and in p15E (A545V). Both of the replication-defective clones also carried the I242T mutation, which was present in all clones derived from viruses that had been passaged in chicken embryos. The mutation I242T was the only difference between the *env* gene of adapted viruses 797-1 and 797-8 and the *env* gene of the cytopathic RCASBP-M2C(4070A).

Amphotropic MLV passaged in the DF-1 cells contains P242 in the *env* gene. The vector RCASBP-M2C(4070A) was derived by replacing avian subgroup A *env* gene in RCASBP(A) by the *env* gene from the amphotropic MLV 4070A. To better understand the nature of the mutation in the *env* gene of the adapted retroviral vector RCASBP-M2C(4070A), we transfected the DF-1 cells with the infectious molecular clone of the amphotropic MLV that contained the 4070A *env* gene and passaged the cells to allow the virus to spread. Unlike the unadapted RCASBP-M(4070A), the amphotropic MLV replicated rapidly in DF-1 cells (data not shown). After five passages, the genomic DNA was isolated from the infected cells, and the region of the amphotropic *env* gene encoding amino acid 242 was amplified by PCR and sequenced. After passage in DF-1 cells, the *env* gene of the passaged MLV contained a proline codon in the position 242. These data suggest that the amphotropic MLV grows well on DF-1 cells and that the change seen at position 242 of the RCASBP-M2C viruses is not related to binding to the avian version of the amphotropic receptor.

Gene transfer into mammalian cells by the chicken embryo-adapted RCASBP-M2C(797-8) vector. The vector RCASBP-M2C(797-8) is substantially less cytotoxic for DF-1 cells than RCASBP-M2C(4070A), making it easier to derive a high-titer stock. However, the mutation I242T in RCASBP-M2C(797-8) could potentially affect the ability of mutant envelope protein to recognize the mammalian amphotropic receptor, which could reduce the titer of the virus on mammalian cells. To address this issue, we constructed RCASBP-M2C(797-8)Puro, a version of the vector that expresses the puromycin resistance gene. DF-1 cells were transfected with the vector and passaged. At each passage, a sample of culture medium was harvested. Tenfold serial dilutions of all samples were prepared and used to infect D17 cells, which were then cultured in the presence of puromycin to select for puromycin-resistant colonies. In parallel, D17 cells were infected with serial dilutions of tissue culture medium obtained from DF-1 cells that were infected with the vector RCASBP-M2C(4070A)Puro (6). Figure 9 shows that the titer of RCASBP-M2C(4070A)Puro rose initially but at passage 5 dropped significantly. At passage 5, the infected culture contained large numbers of dead or dying cells. In contrast, DF-1 cells infected with RCASBP-M2C(797-8)Puro showed no significant cytotoxic changes at passage 5. The titer of the RCASBP-M2C(797-8)Puro virus was 1.1×10^6 CFU per ml. We passaged the infected DF-1 cells several more times and established permanent DF-1 cell culture that produced RCASBP-M2C(797-8)Puro stock with titer of 3×10^6 to 6×10^6 CFU/ml (data not shown). In addition, we passaged DF-1 cells infected with RCASBP-M2C(797-8)Puro in the

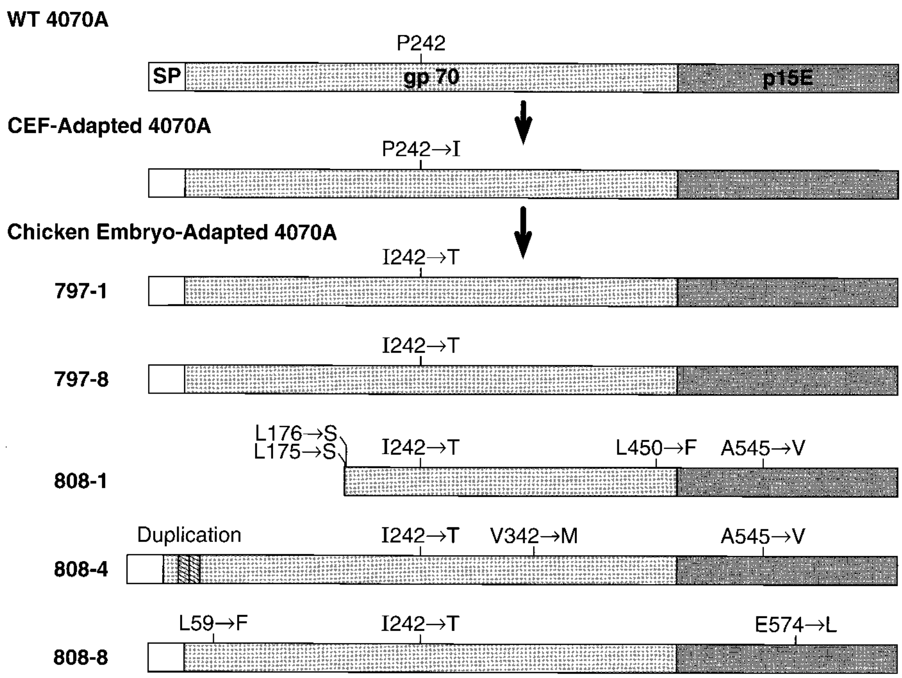

FIG. 8. Structures of Env proteins encoded by wild-type amphotropic MLV (WT 4070A) and by molecular clones of the chicken embryo-adapted amphotropic vectors.

presence of puromycin. The cells grew rapidly and produced virus with the titer of 5×10^6 CFU/ml (data not shown).

DISCUSSION

Retroviruses readily incorporate envelope glycoproteins from other retroviruses and from viruses that belong to other taxonomic groups. Pseudotypes between the groups such as avian and murine leukemia/sarcoma viruses, murine and feline retroviruses, and murine retroviruses and lentiviruses, between different groups of lentiviruses, and between retroviruses and nonretroviruses have been described (1, 2, 4, 7, 9, 10, 12, 16, 19–21, 23, 26, 27, 29, 30, 35, 36, 39, 41, 44, 45, 52–54, 61, 65, 70, 73, 77, 78, 81–84). These pseudotyped retroviruses acquire the host range of the incorporated envelope glycoprotein.

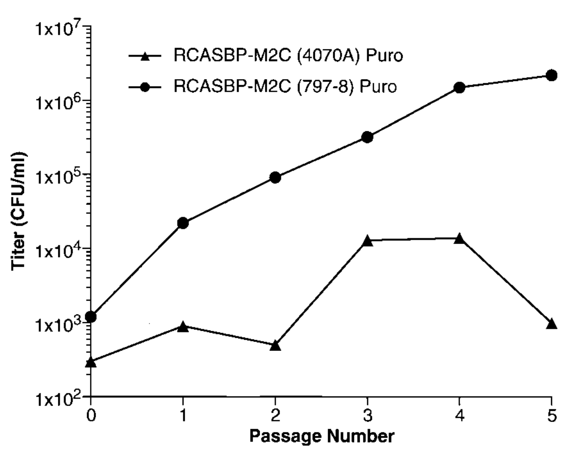

FIG. 9. Passage of cells infected with RCASBP-M2C(4070A)Puro and RCASBP-M2C(797-8)Puro. Infection was initiated by transfection. Cells were passaged, and the virus was titered at each passage (see Materials and Methods). The lower titer of the RCASBP-M2C(4070A)Puro virus was a result of death of the cells infected with this virus.

It is possible to permanently change the host range of a retrovirus by replacing its *env* gene with the *env* gene from another retrovirus. Replacing the Env-coding sequence of RCASBP(A) with the envelope gene from an amphotropic MLV and adapting the virus by passage in avian cells allowed us to generate the stable chimeric retroviral vector RCASBP-M2C(4070A) (6). Relative to the parental amphotropic envelope, the envelope gene in this adapted vector contains a point mutation, P242I, in the surface glycoprotein gp70. The adapted virus replicates efficiently in CEF and DF-1 cells and infects, but does not replicate in, mammalian cells. However, this version of the amphotropic RCASBP is cytopathic in avian cells. Two RCASBP derivatives that can infect mammalian cells, but are much less cytopathic, have been developed. One virus uses the ecotropic envelope glycoprotein. The other virus uses a version of the amphotropic envelope that has undergone a second round of selection. This amphotropic virus has a second amino acid change at position 242; the isoleucine found in the cytotoxic version of the amphotropic virus was converted to threonine. The chimeric ecotropic retrovirus maintained the original proline residue at position 242; there were no genetic changes in the ecotropic envelope gene. Passage of an ecotropic virus with an aberrant C-terminal amino acid sequence of p15E selected for a virus in which the C terminus of p15E was modified.

How do the mutations in the amphotropic envelope help the RCASBP viruses to replicate? The chicken amphotropic receptor appears to be different from its mammalian counterpart (47, 74). In the case of the amphotropic envelope, one possibility was that the envelope was selected so that it could better use the avian amphotropic receptor. In contrast, the ecotropic vectors RCASBP(Eco) and RCASBP(Eco1) use the normal mammalian ecotropic receptor that is expressed in DFJ8 cells. However, amphotropic MLV replicated rapidly in the DF-1 cells with no delay. In addition, there was no change at position 242 upon passage of MLV in DF-1 cells. This suggests that the wild-type 4070A Env glycoprotein interacts efficiently with the chicken amphotropic receptor, permitting infection by both amphotropic MLV and the amphotropic chimeric retroviral vector RCASBP-M2C(4070A). Thus, it appears unlikely that the mutation P242I is involved in adapting the chimeric retrovirus to the avian amphotropic receptor.

The fact that the ecotropic envelope does not need to adapt suggests that there is nothing fundamentally wrong with the interaction of a murine envelope glycoprotein and an ASLV virion. The data also shows that both the naturally occurring (YEYEP) and mutant (YEYGAGR) variants of the C-terminal sequence of p15E are suitable for the formation of infectious ASLV particles. Moreover, we previously showed that the unadapted amphotropic Env glycoprotein is efficiently incorporated into the RCASBP particles (6). Therefore, it appears to be unlikely that the P242I mutation significantly increases the level of incorporation of the amphotropic Env glycoprotein into ASLV particles. What is the effect of the P242I mutation on the amphotropic envelope? The P242I mutation is in the surface glycoprotein but is at the C-terminal amino acid position in the available crystal structure (17). It is unclear from the structure how the mutation would enhance the ability of the amphotropic envelope to function in an ASLV particle. The fact that a similar mutation is not required for the ecotropic envelope to function in an ASLV particle only complicates the problem.

Env proteins are among the determinants responsible for the cytopathic effect of retroviruses in infected cells (11, 13, 14, 32–34, 37, 38, 42, 43, 48, 50, 56, 59, 60, 62, 69, 80). Among the ASLV, the subgroup B viruses are substantially more cytopathic than subgroup A viruses. Replacing the subgroup A *env* gene of the noncytopathic retrovirus RCASBP(A) with the *env* gene of an amphotropic MLV produced, after one round of adaptation in cell culture, a highly cytopathic retrovirus, RCASBP-M2C(4070A). The virus was passaged in chicken embryos to select a variant that was less cytopathic. In doing this experiment, we hoped not only to obtain a more useful vector but also that the mutations in the embryo-adapted virus might help us understand the role of the P242I mutation. The genetic variant of the vector RCASBP-M2C(4070A), isolated after passage in chicken embryos, had the isoleucine residue in position 242 of the gp70 converted to threonine, which markedly reduced the cytotoxicity of the virus for avian cells. These data have two implications. First, they demonstrate a strong selective pressure in vivo for a formation of less cytopathic retroviruses, suggesting that this strategy could be used to improve other retroviral vectors. Second, they underscore the potential structural and functional importance of position 242 in gp70, since the single amino acid change in this position appears not only to adapt the amphotropic murine retrovirus Env glycoprotein to allow it to function in the chimeric ASLV particles but also to affect the cytotoxicity of chimeric retroviruses in avian cells. Unfortunately, it does not make it easier to understand why this residue is so important.

Numerous retroviral vector systems that exploit the ability of retroviruses to form pseudotypes to alter the host range of the vector have been described (15, 18, 29, 31, 40, 45, 46, 49, 66, 75, 76). The majority of retroviral vectors of this type are replication defective. Replication-defective vectors are able to infect target cells; however, our data suggest that at least in some cases, an unmodified Env may not be optimal. The amphotropic RCASBP vector initially replicated very slowly in avian cells. Passaging the chimeric retrovirus selected variants that grew rapidly in avian cells. In contrast, replication-defective retroviruses cannot be passaged to select variants with enhanced infectivity. Since our current understanding of the structure-function relationship of retroviral Env proteins is limited, it is not possible to rationally construct an optimized Env protein. This suggests a general strategy that can be employed to optimize *env* genes for use with particular retroviruses, whether the ultimate goal is the development of a replication-competent or a replication-defective vector. The strategy takes advantage of the ability of replication-competent chimeric retroviruses to spread, even if the initial replication efficiency is low. Mutations arise, and those that increase the replication of the chimeric virus are selected. Our results suggest that at least in some cases, selection may be more efficient in a developing embryo or in an intact animal. Once the appropriate adapted virus is selected, its genome can be molecularly cloned and used in the construction of more efficient vectors. In some cases, identification of sequence changes in the adapted chimeric retroviruses could potentially provide information that could be used to engineer optimized Env proteins. We have identified one type of sequence change that can adapt the ASLV-MLV chimeric amphotropic retroviruses and alter their cytopathogenesis. We hope that further studies of chimeric retroviruses would allow us to develop other chimeric retroviral vectors with various viral surface glycoproteins providing expanded host range. Such retroviral vectors could potentially be used for targeted gene delivery.

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