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Gene Transfer into Mammalian Cells by a Rous Sarcoma Virus-Based Retroviral Vector with the Host Range of the Amphotropic Murine Leukemia Virus

EUGENE V. BARSOV AND STEPHEN H. HUGHES[1]

ABL-Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201

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We have constructed and characterized a Rous sarcoma virus-based retroviral vector with the host range of the amphotropic murine leukemia virus (MLV). The chimeric retroviral genome was created by replacing the *env* coding region in the replication-competent retroviral vector RCASBP(A) with the *env* region from an amphotropic MLV. The recombinant vector RCASBP-M(4070A) forms particles containing MLV Env glycoproteins. The vector replicates efficiently in chicken embryo fibroblasts and is able to transfer genes into mammalian cells. Vector stocks with titers exceeding 10^6 CFU/ml on mammalian cells can be easily prepared by passaging transfected chicken embryo fibroblasts. Since the vector is inherently defective in mammalian cells, it appears to have the safety features required for gene therapy.

Retroviral vectors are powerful tools that can be used to transfer genes into mammalian cells and animals, including humans. As an obligate part of the retrovirus life cycle, reverse transcriptase (RT) generates, from the single-stranded viral RNA genome, a double-stranded DNA copy that is subsequently integrated into the host genome (55). This ability to introduce new genetic information into the chromosomes of target cells provided the inspiration for the development of retroviral vectors as vehicles for the stable transfer of genes. Retroviral vectors have been used for a variety of experimental applications, including the expression of foreign genes, insertional mutagenesis, cell lineage studies, and the creation of transgenic animals.

A number of retroviral vectors have been described, including those based on murine leukemia virus (MLV) (7, 8), mouse mammary tumor virus (41), gibbon ape leukemia virus (30), human immunodeficiency virus (5, 36, 45), and avian retroviruses (4, 9, 20, 24, 38, 47). Of the available retroviral vectors, replication-defective derivatives of Moloney MLV are the most widely used. These vectors encode all of the *cis*-acting elements necessary for viral replication but lack the genes for the viral structural proteins, which can be provided in *trans* by a packaging cell line. However, vectors of this type have major disadvantages. Recombination events between the packaging genome and the vector that can result in the generation of wild-type virus can occur (35). As a consequence it is often difficult to obtain high-titer stocks that are entirely free of recombinant replication-competent virus. Contamination of the recombinant retroviral vector stock with replication-competent MLV can interfere with gene transfer experiments and can present potentially serious problems for gene therapy, such as the induction of lymphomas in primates infected by wild-type MLV present in retroviral vector stocks (12, 49).

We have developed a series of a replication-competent vectors based on Rous sarcoma virus (RSV) (20, 38). RSV is the only known naturally occurring replication-competent retrovirus that has acquired a cellular gene, the v-*src* oncogene. In the replication-competent avian splice (RCAS) vectors (38), the v-*src* sequences have been replaced with a unique restriction site (*Cla*I), which can be used to insert a gene of interest. In the parental RSV, *src* is flanked by direct repeats; in RCAS, the upstream direct repeat has been removed, increasing the stability of the vector. In contrast to replication-defective vectors, the RCAS vectors do not require a packaging cell line. Viral stocks are prepared by transfecting a plasmid encoding the vector into cultured chicken embryo fibroblasts (CEF). High-titer virus stocks can be prepared simply by passaging transfected cells and allowing the virus to spread. To prepare viral stocks, we use CEF that lack any endogenous viral sequences that are closely related to the RCAS vectors (1), which prevents recombination with endogenous elements. Because of the simplicity of preparing high-titer stocks of the replication-competent virus, RCAS vectors have been used to express a number of genes and to make transgenic chickens (25, 38, 42, 43).

The main limitation of RSV-based vectors is their host range, which is defined by the viral envelope (Env) glycoprotein and is restricted to avian cells. In order to infect a host cell, the Env glycoprotein must specifically bind to a cognate receptor on the surface of the cell. One method that can be used to overcome this limitation is to make cell lines or transgenic mice that express the cellular receptor for subgroup A avian leukosis viruses. The RCAS vectors can transfer and stably express genes in mammalian cells and transgenic mice that express this receptor (2, 15). Although the transfer of genes is efficient, avian leukosis viruses do not replicate in mammalian cells. Thus, the RCAS vectors are replication defective in mammalian cells (15).

Because high-titer viral stocks can be prepared quickly and easily in avian cells and because the resulting vectors are inherently defective in mammalian cells, we sought to develop an RCAS vector suitable for gene transfer into a broad range of cell types from different mammalian species. Here we report the development and characterization of a derivative of RCAS that contains the *env* gene from an amphotropic MLV. The chimeric vector RCASBP-M(4070A) replicates efficiently in CEF and is able to transfer genes into cultured mammalian cells, in which it is replication defective.

* Corresponding author. Mailing address: ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, P.O. Box B, Frederick, MD 21702-1201. Phone: (301) 846-1619. Fax: (301) 846-6966.

MATERIALS AND METHODS

Cells. CEF derived from EV-0 chicken embryos (1) were maintained in Dulbecco modified Eagle medium (GIBCO BRL, Gaithersburg, Md.) supplemented with 5% fetal bovine serum, 5% newborn calf serum, 10% tryptose-phosphate broth (GIBCO BRL), 100 U of penicillin per ml, and 100 μg of streptomycin (Quality Biological, Inc., Gaithersburg, Md.) per ml. Dog D17 cells, human HeLa cervical carcinoma cells, and mouse NIH 3T3 cells were grown in Dulbecco modified Eagle medium with 10% calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Construction of plasmids. Plasmids were constructed by standard methods (44). The plasmid RSVgagpol was constructed as follows: plasmid DNA [RCASBP(A)] (38) was cleaved with *Sac*I and *Xba*I restriction endonucleases, and the fragment containing the complete *gag* coding sequence and the 3′ end of *pol* was isolated. In a separate reaction, RCASBP(A) was digested with *Xho*I. The *Xho*I ends were filled in by using T4 DNA polymerase and deoxynucleoside triphosphates, and the DNA was subsequently digested with *Xba*I. A fragment, *Xba*I-*Xho*I, containing the 3′ end of the *pol* open reading frame was isolated. The *Sac*I-*Xba*I and *Xba*I-*Xho*I fragments were ligated to *Sac*I- and *Eco*RV-cleaved pBluescript II KS(+) DNA. The resulting plasmid, pT7gagpol, contained the entire *gag-pol* region flanked by unique *Sac*I and *Cla*I sites. RCASBP(A) was digested with *Sac*I and *Cla*I to remove the *gag-pol-env* region, and the *Sac*I-*Cla*I fragment from pT7gagpol was inserted to generate plasmid RSVgagpol.

The *env* coding region of an amphotropic MLV was PCR amplified from a plasmid, pR4070A (34) (kindly provided by Alan Rein, ABL-Basic Research Program), by using the forward primer AMPH-F (AAAAGAGCTCGGCCGA CACCCAGAGTGGAC), which hybridizes immediately upstream of the *env* initiation codon, and the reverse primer AMPH-R (AAAAGAGCTCTCATG GCTCGTACTCTATGGGTT), which spans the *env* termination codon. *Sac*I sites were included at the 5′ end of each primer. The PCR product was cleaved with *Sac*I and inserted into the vector TFA-NEO (16), generating MLVenv, which can be used to express the amphotropic *env* gene under the transcriptional control of RSV long terminal repeats.

The recombinant retroviral vector RCASBP-M(4070A) was prepared as follows. An *Eco*RI fragment from RCASBP(A) that spans the 3′ end of the RSV *env* gene and the U3R segment of the 3′ long terminal repeat was subcloned into pUC19, giving rise to pUCenvRI. The termination codon for *env* was replaced with a *Not*I site by site-directed mutagenesis (11). Clones containing mutant plasmids with the *Not*I site were propagated in *Escherichia coli* BMH 71-18 mutS (Clontech, Palo Alto, Calif.) and selected by *Not*I cleavage, generating the plasmid pUCenvRINOT. To replace the *env* gene, RCASBP(A) was digested with *Kpn*I and *Cla*I and the *env*-containing fragment was removed. The *env* region containing the unique *Not*I site was introduced by ligating a *Kpn*I-*Eco*RI fragment of RCASBP(A) and the *Eco*RI-*Cla*I fragment from pUCenvRINOT with RCASBP(A) that had been cleaved with *Kpn*I and *Cla*I, generating the plasmid RCASBP(A)NOT.

Overlap extension (22) was used to construct a chimeric amphotropic *env* gene in which the sequence encoding the N-terminal signal peptide of MLV was replaced with the equivalent sequence from the RSV *env* gene. A fragment spanning the unique *Kpn*I site, the *env* splice acceptor site, and the signal peptide was PCR amplified from RCASBP(A) by using primers RSV-FOR (GGAC GAGGTTATGCCGCTGTG) and RSV-BACK (ACATTAAAGACCTGAT GGGGGCTAACATCAGCTCTTACCCCCGTAA). The fragment that begins with the codon for the first serine of the mature gp70 and spans the entire *env* open reading frame followed by the unique *Not*I site was amplified from pR4070A with the primers MLV-FOR (AGCCCCCATCAGGTCTTTAATGT) and MLV-BACK (AGCGGCCGCTCATGGCTCGTACTCTATGGGTT). These two PCR fragments were fused and amplified by PCR (22) with the primers RSV-FOR and MLV-BACK. The resulting PCR product, which contained a chimeric *env* gene, was cleaved with *Kpn*I and *Not*I. The *env* gene was removed from RCASBP(A)NOT by digestion with *Kpn*I and *Not*I, and the chimeric *Kpn*I-*Not*I fragment was inserted, generating the plasmid RCASBP-M(4070A). The sequence of the region containing the junction between RSV and MLV *env* sequences was confirmed by DNA sequencing using a Sequenase Sequencing Kit (U.S. Biochemicals, Cleveland, Ohio).

To construct RCASBP-M(4070A)NEO, the *Cla*I fragment containing the *neo* gene was isolated from RCASBP(A)NEO and cloned into the *Cla*I site of RCASBP(A)NOT. The chimeric *env* gene was transferred into this plasmid as a *Kpn*I-*Not*I fragment.

To facilitate cloning into RCASBP-M(4070A), a silent point mutation (the substitution of a cytosine for the adenine in the third position of the Arg-623 codon) was introduced into the amphotropic *env* region by site-directed mutagenesis (11). This mutation eliminates a *Cla*I site in the *env* gene. The *Kpn*I-*Not*I fragment of RCASBP-M(4070A) was subcloned into pBluescript II KS(+) for mutagenesis. Mutant clones selected by *Cla*I cleavage were propagated in *E. coli* BMH 71-18 mutS. The *env* region in RCASBP-M(4070A) was replaced with the mutant *Kpn*I-*Not*I fragment, giving rise to vector RCASBP-MC(4070A).

To construct RCASBP-MC(4070A)Puro, the puromycin resistance gene (*pac*) was amplified by PCR from the plasmid pSVpac (10, 51) with the primers that appended *Nco*I and *Hin*dIII sites to the 5′ and 3′ ends of *pac*, respectively. The product was then cloned into the adapter plasmid Cla12Nco (23). The *pac* gene was isolated from the adapter construct as a *Cla*I fragment and introduced into RCASBP-MC(4070A). Recombinant clones were isolated in *E. coli* DH5α.

Transfection and preparation of virus particles. Calcium phosphate-mediated transfection of plasmid DNAs into CEF was performed according to standard procedures (18). Precipitates containing 10 μg of DNA per 100-mm-diameter plate were incubated with subconfluent CEF monolayers for 6 h at 37°C and then with medium containing 15% glycerol for 5 min at 37°O. Cells were washed twice with phosphate-buffered saline (PBS) and incubated in a growth medium for 24 h. Virus-containing culture fluid was then harvested. When necessary, transfected cells were passaged to allow the virus to spread through the culture.

To prepare virus particles, culture fluid was clarified by low-speed centrifugation and the virus was pelleted through a 15% sucrose cushion by centrifugation at 35,000 rpm for 1 h at 4°C using an SW41 rotor (Beckman, Fullerton, Calif.). The resulting pellet was resuspended in protein sample buffer and heated at 100°C for 4 min before being loaded to a gel.

Immunoblot analysis. Viral proteins were resolved by electrophoresis in a sodium dodecyl sulfate (SDS)–4 to 20% gradient polyacrylamide gel; 16% gels were used for the detection of the transmembrane protein p15E. After electrophoresis, proteins were electroblotted onto nitrocellulose membranes (BA85; Schleicher & Schuell, Keene, N.H.). The RSV matrix protein (MA) was detected by incubation with rabbit antiserum against p19 (generously provided by Volker Vogt, Cornell University). MLV envelope glycoproteins were detected with goat antiserum against gp70 and rabbit antiserum against p15E (kind gifts of Alan Rein). Protein bands were visualized by using an enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Heights, Ill.).

Virion RT assay. Determination of the virion-associated RT activity was performed as previously described (56). Briefly, virus particles were recovered from 1 ml of the culture fluid by centrifugation at 14,000 rpm (Eppendorf model 5415C; Brinkmann Instruments, Westbury, N.Y.) for 30 min at 4°C. Pellets were resuspended in RT buffer (50 mM Tris [pH 8.3], 60 mM NaCl, 12 mM $MgCl_2$, 20 mM dithiothreitol, 0.1% Nonidet P-40) containing oligo(dG) (5 μg/ml), poly(rC) (10 μg/ml), dGTP (10 μM), and 10 μCi of [α-^{32}P]dGTP (800 Ci/mmol; Amersham). Reaction mixtures were incubated at 37°C for 1 h. Nucleic acids were precipitated by addition of 1 ml of 10% trichloroacetic acid, collected on GF/C glass microfiber filters (Whatman, Hillsboro, Oreg.), and washed with a solution containing 10% trichloroacetic acid and 95% ethanol. Filters were dried and counted in a TriCarb 1500 Liquid Scintillation Analyzer (Packard).

Indirect immunofluorescence microscopy. CEF infected with RCASBP-M(4070A) were grown on glass slides. Cells were fixed with methanol, reacted with goat anti-gp70 antiserum (diluted 1:200 in PBS containing 1% bovine serum albumin), washed three times for 5 min with PBS containing 0.1% Triton X-100, and incubated with fluorescein isothiocyanate-conjugated rabbit anti-goat secondary antibody. Photomicroscopy was performed with a Nikon Microphot-FXA microscope (Nikon, Inc., Melville, N.Y.).

Titration of RCASBP-M(4070A)NEO and RCASBP-MC(4070A)Puro on mammalian cells. To prepare virus stocks, CEF transfected with plasmids containing the viral genome were passaged to allow spread of the virus. At different cell passages, culture fluid was harvested and filtered through a 0.45-μm-pore-size membrane. Host cells were plated on 60-mm-diameter plates (5×10^5 cells per plate) and grown overnight. Cells were infected with serial dilutions of the retroviral vector stocks in the presence of Polybrene (10 μg/ml) for 24 h, trypsinized, and plated in a selective medium containing G418 (400 μg/ml; GIBCO BRL) or puromycin (2.5 μg/ml; Sigma). Ten days later, colonies that developed from resistant cells were fixed with methanol, stained with Giemsa stain, and counted. In some experiments, individual colonies were isolated and expanded into cell lines for further analysis.

Southern blot analysis of genomic DNA. Genomic DNA was isolated as described previously (28). Southern hybridization was performed according to standard methods with a nonradioactive digoxigenin-labeled probe (Genius System; Boehringer Mannheim, Indianapolis, Ind.). Bands were detected by using the Lumi-Phos 530 reagent (Boehringer Mannheim) according to the manufacturer's recommendations.

Cloning of RCASBP-M(4070A)NEO provirus. Low-molecular-weight DNA was isolated from CEF infected with RCASBP-M(4070A)NEO at passage 3 (see Results) by the Hirt extraction procedure (21). The low-molecular-weight DNA was cleaved with the restriction endonuclease *Sac*I, ligated with *Sac*I-cleaved vector λZAP Express (Stratagene, La Jolla, Calif.), and packaged by using GigaPack II Gold packaging extract (Stratagene). A library of 4.7×10^6 independent clones was screened by hybridization with a ^{32}P-labeled amphotropic *env*-specific probe (*Sal*I-*Cla*I fragment derived from pR4070A). Positive phage clones were converted into plasmids by coinfection with ExAssist Helper phage (Stratagene). RCASBP-M(4070A)NEO proviral DNA in the resulting plasmid clones was analyzed by cleavage with restriction endonucleases. To construct RCASBP-M2(4070A)Puro, DNA fragments containing the $(gag\text{-}pol)^{RSV}$-env^{MLV} region were derived from the plasmid clones by digestion with *Sac*I and *Not*I and ligated with the long terminal repeat-Puro-long terminal repeat cassette that was obtained by *Sac*I-*Not*I cleavage of RCASBP-MC(4070A)Puro. To facilitate cloning into RCASBP-M2(4070A), the *Cla*I site in the amphotropic *env* gene of RCASBP-M2(4070A)Puro8 (see Results) was eliminated by introducing a silent point mutation by site-directed mutagenesis [the procedure is described above for the construction of RCASBP-MC(4070A)]. The resulting vector was designated RCASBP-M2C(4070A)Puro.

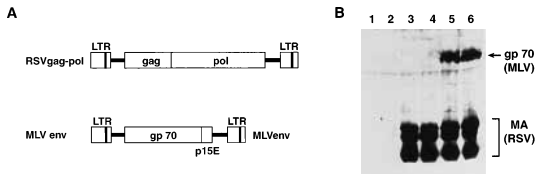

FIG. 1. Assembly of pseudotyped virus particles upon coexpression of RSV Gag and amphotropic MLV Env proteins in CEF. (A) Schematic of the structures of the DNA constructs RSVgagpol and MLVenv. LTR, long terminal repeat. (B) Immunoblot analysis of proteins present in virions. Plasmids MLV-env (lanes 1 and 2) and RSVgagpol (lanes 3 and 4) were transfected into CEF separately and together (lanes 5 and 6). At 24 h after transfection, virus particles were recovered from the culture fluid by ultracentrifugation and proteins were resolved in SDS–4 to 20% gradient polyacrylamide gels and analyzed by immunoblotting with antibodies against RSV p19 (MA) and MLV gp70 (SU).

Sequencing of *env* gene. The *env* gene sequence of the cloned RCASBP-M(4070A)NEO proviruses was determined by cycle sequencing of both strands using a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.). Sequencing reactions were analyzed with an Automated 373A DNA Sequencer (Applied Biosystems). The complete sequence of the *env* gene was assembled by using Sequencer software (Gene Codes Corporation, Ann Arbor, Mich.).

RESULTS

The amphotropic MLV Env protein efficiently assembles with RSV Gag proteins. To investigate the ability of RSV Gag and MLV Env to assemble, we used a complementation assay in which the RSV Gag and MLV Env proteins were transiently expressed in CEF. The plasmids RSVgagpol, which contains the *gag-pol* open reading frame derived from the retroviral vector RCASBP(A) (38), and MLVenv, which carries the region encoding the signal peptide, surface glycoprotein gp70, and transmembrane protein p15E of an amphotropic MLV (clone 4070A) (Fig. 1A), were introduced into CEF by transfection both separately and together. Twenty-four hours after transfection, the culture medium was harvested and the viral particles were recovered. The viral proteins were fractionated and analyzed by immunoblotting with antibodies against RSV p19 (MA) and MLV gp70 (surface glycoprotein [SU]). The glycoproteins expressed from the MLVenv plasmid are anchored in the membranes of CEF and can be detected by immunoblotting of the membrane fraction (data not shown). The MLV Env glycoproteins are not secreted into the medium in the absence of Gag (Fig. 1B). Transfection of the RSVgagpol plasmid results in the synthesis of capsid proteins that assemble into virus-like particles (lanes 3 and 4). Since RSV MA is phosphorylated at several positions, the additional bands detected by the anti-p19 antiserum may represent proteins with different degrees of phosphorylation (6).

Cotransfection of RSVgagpol and MLVenv resulted in the coexpression of the RSV capsid and MLV envelope proteins in the same cell. Immunoblotting analysis showed that both RSV Gag and MLV Env were present in the particles produced upon cotransfection (lanes 5 and 6), suggesting that the viral particles do contain the MLV Env glycoprotein.

The recombinant retroviral vector RCASBP-M(4070A) replicates efficiently in avian cells. Given the apparent efficiency with which MLV Env assembles with RSV Gag, a recombinant viral genome in which the *env* gene of the parental avian virus was replaced with a gene encoding the gp70 and p15E of an amphotropic MLV was constructed. To ensure the efficient intracellular transport of the Env precursor and signal peptide cleavage in avian cells, we constructed a chimeric *env* gene in which the sequence encoding the mature MLV gp70 was fused to the sequence encoding the RSV Env signal peptide. The retroviral vector RCASBP(A) was modified by replacing the *env* gene stop codon with a unique *Not*I cleavage site. RSV *env* coding sequences were removed and the chimeric envelope coding region was inserted, generating the recombinant retroviral vector RCASBP-M(4070A) (Fig. 2A). To test the expression of the chimeric *env* gene in the context of an RSV genome and the production of avian virus particles with MLV surface glycoproteins, CEF were transfected with RCASBP-M(4070A) DNA or with pR4070A, the molecular clone of the amphotropic MLV from which the MLV *env* gene was derived (Fig. 2B, lanes 3 and 4). Twenty-four hours after transfection, cell culture medium was harvested and viral particles were recovered. As can be seen in Fig. 2B, CEF transfected with RCASBP-M(4070A) generate particles that contain the MLV surface glycoprotein (lanes 1 and 2). The incorporation of gp70 into particles produced by RCASBP-M(4070A) appeared to be approximately as efficient as the incorporation of gp70 into MLV virions (compare lanes 1 and 2 with lanes 3 and 4).

We next measured the ability of RCASBP-M(4070A) to replicate in CEF. Cells were transfected with RCASBP-M(4070A) DNA and passaged six times to allow the virus to spread. At passages 1, 4, and 6, a small number of cells were plated separately and virus spread was assayed by the staining of CEF with antibodies to gp70. As shown in Fig. 3A, only a small number of positively stained cells were seen initially (passage 1). This number increased significantly by passage 4, and virtually all cells were infected and expressed gp70 at passage 6. To monitor the production of virus particles, cell culture medium was harvested and particles were recovered at each passage. The proteins in the particles were analyzed by immunoblotting with antibodies against p19 and gp70. The amount of viral proteins increased significantly by passage 4 and reached a high level by passage 6 (Fig. 3B). These data indicate that RCASBP-M(4070A) was able to spread in the CEF culture; however, the initial rate of spreading was significantly slower than that of the parental vector RCASBP(A).

Gene transfer to mammalian cells by RCASBP-M(4070A). To determine the ability of RCASBP-M(4070A) particles to infect and transfer genes into mammalian cells, the *neo* marker gene was introduced into RCASBP-M(4070A) to generate vector RCASBP-M(4070A)NEO (Fig. 4A). Viral stocks that were generated 24 h after transfection of the RCASBP-M(4070A) NEO DNA into the CEF were titered on the murine NIH 3T3 and human HeLa cells. The virus obtained by the transient expression of RCASBP-M(4070A)NEO in CEF had a titer of

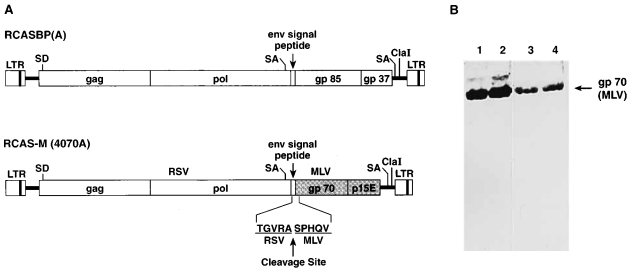

FIG. 2. Formation of pseudotyped virions by the recombinant retroviral vector RCASBP-M(4070A). (A) Schematic of the structures of the parental vector RCASBP(A) (38) and recombinant vector RCASBP-M(4070A). SD, splice donor; SA, splice acceptor; LTR, long terminal repeat. (B) Immunoblot detection of amphotropic MLV gp70 incorporated into RCASBP-M(4070A) particles. CEF were transfected with RCASBP-M(4070A) (lanes 1 and 2) and pR4070A (34) (lanes 3 and 4). At 24 h after transfection, particles were recovered from the culture fluid by ultracentrifugation, proteins were fractionated in SDS–4 to 20% gradient polyacrylamide gels, and gp70 was detected by immunoblotting with antibodies against gp70.

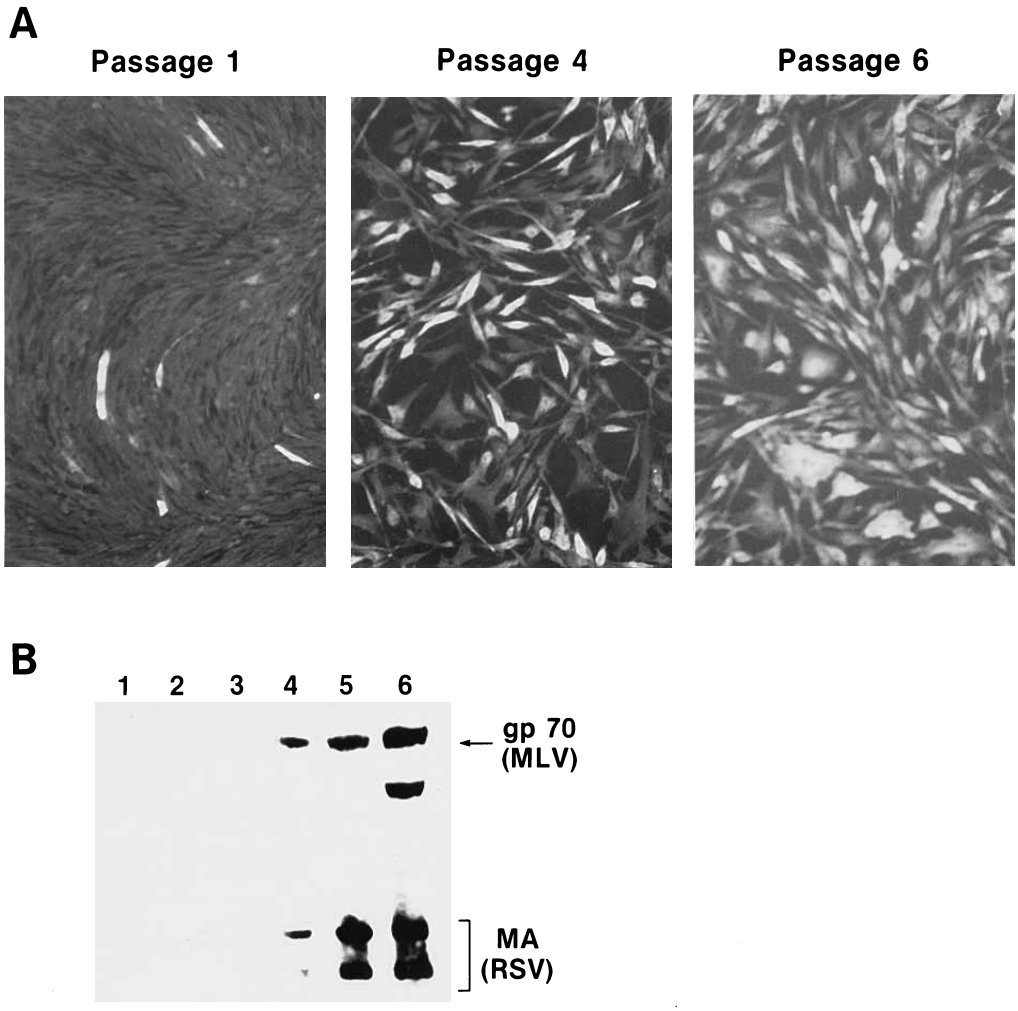

FIG. 3. Replication of RCASBP-M(4070A) in CEF. Cells were transfected with RCASBP-M(4070A) plasmid DNA and passaged every other day. (A) Immunofluorescence analysis. Cells were fixed with methanol, incubated with goat anti-gp70 serum, washed, and stained with fluorescein-isothiocyanate-conjugated rabbit anti-goat secondary antibody. (B) Immunoblot analysis of viral proteins. At each passage of the transfected CEF, virus particles were recovered from the culture fluid by ultracentrifugation and proteins were fractionated in SDS–4 to 20% gradient polyacrylamide gels and analyzed by immunoblotting with antibodies against RSV p19 and MLV gp70. Lane numbers correspond to passage numbers.

2×10^3 to 3×10^3 CFU/ml on both cell lines. Genomic DNA isolated from the G418-resistant clones was analyzed by Southern blot hybridization. As shown in Fig. 4B and C, the majority of the G418-resistant clones derived from NIH 3T3 and HeLa cells contained provirus that was structurally indistinguishable from RCASBP-M(4070A)NEO.

The transfected CEF were passaged, and the resulting virus was used to infect both fresh CEF and mammalian cells. By immunoblotting analysis of the viral particles collected at each passage, we found that the virus replicated reasonably efficiently in CEF (data not shown); however, the ability to transfer Neo^r into NIH 3T3 cells decreased relatively rapidly (Table 1). To determine whether genetic instability of the *neo* gene resulted in low virus titers, we constructed molecular clones of RCASBP-M(4070A)NEO proviruses. Sequencing of the proviral DNA showed that a number of proviruses suffered deletions that involved the splice acceptor site, the initiation codon, and the 5′ end of the *neo* gene (data not shown).

Because the initial rate of spread of the virus in CEF was slow, we looked for mutations in the cloned proviral DNA. By sequencing of the *env* regions from individual clones, we found a rather limited spectrum of mutations located primarily in the central and C-terminal regions of the gp70 coding sequence (Fig. 5). In all of the clones, two adjacent cytosines in the region encoding gp70 were mutated to A and T, resulting in the substitution of Ile for Pro at position 242 (Pro242Ile). We sequenced the same region of the *env* gene in the parental plasmid RCASBP-M(4070A)NEO and found that the proline codon was present at position 242, thus confirming that the Pro242Ile mutation had been selected during the passage of the vector on CEF and had not occurred in the course of the vector construction. In addition to this mutation, other substitutions, including Lys308Gln (clones env1 and env9), Ala310Gly (clones env3 and env6), and Met446Leu (clones env6 and env9), were found. Some clones had additional amino acid changes which were seen only in those clones. In addition, in the clone env3, the sequence encoding the C-terminal end of the gp70 and almost all of the p15E was deleted.

We also examined the propagation of RCASBP-M(4070A) Puro, which carries the marker gene *pac* and confers resistance to puromycin (10, 51). CEF were transfected with RCASBP-M (4070A)Puro and passaged six times. The titer of the resulting

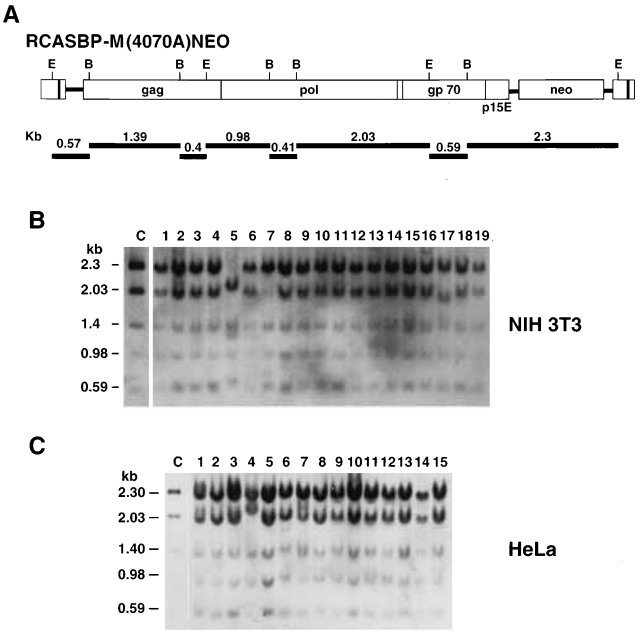

FIG. 4. Infection of mammalian cells by the vector RCASBP-M(4070A)NEO. (A) Schematic of the structure of RCASBP-M(4070A)NEO. Restriction endonuclease recognition sites are indicated as follows: E, *Eco*RI; and B, *Bam*HI. (B and C) Detection of the RCASBP-M(4070A)NEO provirus in the genomic DNA of infected NIH 3T3 (B) and HeLa (C) cells by Southern blot hybridization. Cells were infected with RCASBP-M(4070A)NEO, and G418-resistant clones were selected. Their genomic DNA was digested with *Eco*RI and *Bam*HI. Fragments were resolved in agarose gel, transferred onto charged nylon membrane, and hybridized with digoxigenin-labeled *Eco*RI-digested RCASBP-M(4070A)NEO DNA. Membranes were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody, and bands were detected by using Lumi-Phos 530 reagent. Lane C shows fragments derived by cleavage of plasmid RCASBP-M(4070A) DNA with *Eco*RI and *Bam*HI.

virus was determined on dog D17 cells, as well as on NIH 3T3 and HeLa cells.

As shown in Table 2, the titer of RCASBP-M(4070A)Puro produced at passage 6 was several orders of magnitude higher than that of RCASBP-M(4070A)NEO produced at a similar passage (Table 1). The titer of RCASBP-M(4070A)Puro on D17 cells was approximately 1 order of magnitude higher than the titer on NIH 3T3 or HeLa cells.

The transmembrane protein p15E is correctly processed in RCASBP-M(4070A) virions. In the murine leukemia viruses, the Env precursor is initially cleaved by a cellular protease into gp70 (SU) and p15E (TM). After the virus particle is released from the cell, the viral protease removes the 16 C-terminal residues from the cytoplasmic domain of p15E, yielding the mature p15E and p2E (40). This cleavage activates the membrane fusion capability of the Env protein and is essential for viral infectivity. Virions produced by cells infected with RCASBP-M(4070A) do not contain the MLV protease. However, the chimeric virus is infectious, suggesting that pre-p15E is properly cleaved. We prepared viral particles of RCASBP-M(4070A) and analyzed proteins by immunoblotting using antibodies against p15E (Fig. 6). The transmembrane protein pre-p15E appears to be processed with approximately the same efficiency in RCASBP-M(4070A) particles as in wild-type MLV virions (compare lanes 1 and 3).

RCASBP-M2(4070A) is a fast-replicating and stable vector. After transfection of RCASBP-M(4070A) into CEF, three to four cell passages were required before detectable amounts of

TABLE 1. Titer of RCASBP-M(4070A)NEO on NIH 3T3 cells[a]

Time or passage	Virus titer (CFU/ml)[b]
24 h posttransfection	2.6×10^3
Passage 1	10^1
Passage 2	10^1
Passage 3	5×10^2

[a] Vector DNA was transfected into CEF, and the cells were passaged to allow the virus to spread. The virus generated at passage 6 was used to infect fresh CEF. Three virus passages on CEF were made. At each passage, the titer of a vector stock was determined on NIH 3T3 cells.

[b] Values are averages of two independent determinations.

the virus were produced and five to six passages were required for the entire culture to be infected (Fig. 3). We detected genetic changes in the viral genome during this period of initial slow replication, which could help the chimeric virus to grow more efficiently. To construct an "adapted" version of the vector, we replaced the $(gag\text{-}pol)^{RSV}\text{-}env^{MLV}$ region in RCASBP-M(4070A)Puro with the $(gag\text{-}pol)^{RSV}\text{-}env^{MLV}$ regions derived from clones env1, env6, env8, and env9 of RCASBP-M(4070A)NEO. The resulting vectors, designated RCASBP-M2(4070A)Puro1, -6, -8, and -9, were transfected into CEF, and the cells were passaged to generate virus. At each passage, the production of virus particles was quantified by determining the level of virion-associated RT activity and the number of virus particles able to infect mammalian cells was determined by titration on D17 cells.

As shown in Fig. 7A, the initial rates of replication of the adapted vectors were much faster than those of the parental vector. Virus production was detectable at passage 1 and reached a maximum at passages 3 to 4, indicating a rate of replication which was similar to the initial rates of replication of RCASBP(A). In this experiment, the production of the parental vector (env wt) could not be detected until passage 6 (data not shown). The adapted RCASBP-M2(4070A)Puro vectors were infectious for mammalian cells, exhibiting significant titers on D17 cells (Fig. 7B). The fastest-replicating virus (clone env8) reached the maximum titer on D17 cells by passage 2 (compare Fig. 7A and B).

To facilitate cloning into RCASBP-M2(4070A), the *Cla*I site in the *env* gene of RCASBP-M2(4070A)Puro (derived from clone env8) was eliminated. The DNA of the resulting vector,

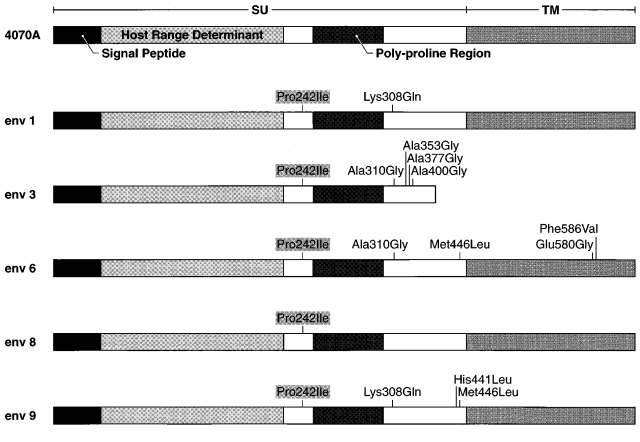

FIG. 5. Mutations in the amphotropic *env* gene of RCASBP-M(4070A)NEO. CEF were infected with RCASBP-M(4070A)NEO after three passages of the virus. Full-length clones of the viral DNA were derived from the library of a low-molecular-weight DNA which was extracted from infected CEF. Env genes of clones env1, -3, -6, -8, and -9 were sequenced. The mutation Pro242Ile results from the substitution of the Ile codon ATC for the Pro codon CCC.

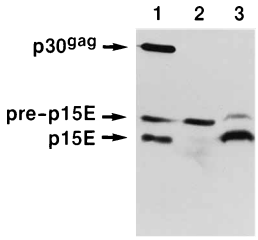

FIG. 6. Proteolytic processing of the pre-p15E in RCASBP-M(4070A) virions. Virus particles produced by infected CEF were recovered from the culture fluid by ultracentrifugation. Virion-associated transmembrane protein was analyzed by immunoblotting with antibodies against p15E. Lane 1, wild-type amphotropic MLV (control); lane 2, protease-defective MLV; lane 3, RCASBP-M(4070A). Note that anti-p15E antibodies cross-react with the MLV $p30^{gag}$ protein (40) (lane 1).

RCASBP-M2C(4070A)Puro, was transfected into CEF and the cells were passaged. At each passage, the number of virus particles able to infect mammalian cells was quantified by titration on D17 cells. As can be seen in Fig. 7C, RCASBP-M2C(4070A)Puro replicated efficiently in CEF and the titer of a virus stock produced at each passage was the same as the titer of the RCASBP-M2(4070A)Puro virus from which it was derived.

Mammalian cells infected with RCASBP-M2(4070A) do not generate infectious particles and do not synthesize viral proteins. To determine whether infected mammalian cells produce any infectious virus particles, we infected D17 cells with the RCASBP-M2(4070A)Puro vector stock. Cell clones infected with the vector were obtained by puromycin selection. About 100 puromycin-resistant clones were pooled and grown together. Fresh D17 cells were infected with 10 ml of the culture medium harvested from a confluent monolayer of vector-containing cells. At 48 h postinfection, the medium was replaced with one containing puromycin. The D17 cells were grown in the presence of puromycin for 14 days. No puromycin-resistant colonies were detected, indicating that less than 1 infectious particle per 10 ml was present in the culture medium of the vector-containing cells.

To show that the vector-containing D17 cells did not produce particles that were infectious for avian cells, CEF were infected with 10 ml of the culture medium harvested from the vector-containing D17 cells. The infected CEF were passaged six times. At passage 6, the culture medium was harvested and subjected to ultracentrifugation. The pellet and the lysate of the CEF were analyzed for the presence of viral proteins by immunoblotting with antibodies against RSV p19 and MLV gp70. No viral proteins were detected either in culture medium or in the lysate of infected CEF (data not shown), indicating that no infectious particles were present in 10 ml of the culture medium harvested from vector-containing D17 cells.

DISCUSSION

Most strains of RSV, and vectors derived from these strains, are not able to penetrate mammalian cells. There are exceptions; for example, the subgroup D Schmidt-Ruppin strain can infect mammalian cells with low efficiency (26). Attempts to develop RSV-based vectors with broader host ranges have been made. Recently, a recombinant RSV-based vector expressing the influenza virus hemagglutinin was found to form hemagglutinin-containing particles and to infect mammalian cells (13). However, the efficiency of infection was low and the ability of the vector to replicate was not demonstrated. In another study, the putative receptor-binding domain of the subgroup A RSV Env protein gp85 (SU) was replaced with a peptide known to interact with the cellular integrin receptor (48). Viral particles coated with the modified Env were infectious for mammalian cells. However, the titer of this vector also appeared to be quite low.

Retroviruses are relatively promiscuous in their ability to incorporate foreign glycoproteins. A number of retroviruses have been shown to form pseudotyped particles when a second glycoprotein is expressed in the cell (29, 46, 53, 54). Pseudotypes are formed between RSV and MLV (54). RSV particles infectious for mammalian cells are produced following infection of cultured cells with both viruses. However, when both RSV and MLV Env proteins are expressed, the MLV Env protein does not appear to compete efficiently with RSV Env for incorporation into RSV virions (54).

The mechanisms of capsid and envelope association are still obscure. In a retroviral particle, there may be some specific interactions between the cytoplasmic domain of the envelope transmembrane protein (TM) and the matrix protein (MA) of the capsid. Gebhardt et al. (17) showed that the MA and TM proteins in RSV particles could be chemically cross-linked, indicating a close physical association between these proteins in the virion. Introduction of termination codons into the TM cytoplasmic domain caused a loss of infectivity and impaired incorporation of Env in human immunodeficiency virus (14, 58) and MLV (19). However, RSV particles in which TM lacks its cytoplasmic domain remain fully infectious (37) and the ability of retroviruses to form pseudotypes and incorporate nonviral membrane glycoproteins (57) suggests that envelope glycoproteins can be incorporated into virions relatively nonspecifically.

The amphotropic MLV envelope can associate efficiently with an RSV virion if a competing RSV envelope is not present. RCASBP-M(4070A) particles appear to contain approximately the same amount of gp70 as do amphotropic MLV particles, and they can infect mammalian cells efficiently. Initially, the chimeric retrovirus replicates at a rate considerably lower than that of the parental vector RCASBP(A). However, after a number of passages on CEF, the virus infects these cells efficiently and spreads quickly throughout the culture. As might be expected from this result, we found that rapidly growing viruses contain a mutation, Pro242Ile, in the *env* gene. This mutation appears to be critically important for efficient replication, since it is present in all of the clones that we derived from passaged RCASBP-M(4070A)NEO virus. Furthermore, this mutation alone is sufficient to dramatically increase the efficiency of virus replication, since it is the only mutation found in the *env* gene of clone env8, the virus that replicates most efficiently in CEF and produces the highest titers on mammalian cells. The replacement of Pro-242 with an isoleucine residue could indicate an important structural change in the gp70, since proline residues are often located at the points of β-turns. This change could reflect the adjustment necessary for the gp70 to recognize both avian and mammalian types of the amphotropic receptor or, alternatively, could enhance the interaction between the RSV capsid protein and the Env protein from the amphotropic MLV. It is also possible that the mutation affects the structure and/or function of viral RNA.

TABLE 2. Titer of RCASBP-M(4070A)Puro on mammalian cells

Species of cells	Cell line	Titer (CFU/ml)[a]
Mouse	NIH 3T3	7.1×10^4
Dog	D17	7×10^5
Human	HeLa	3.3×10^4

[a] Values are averages of two independent determinations.

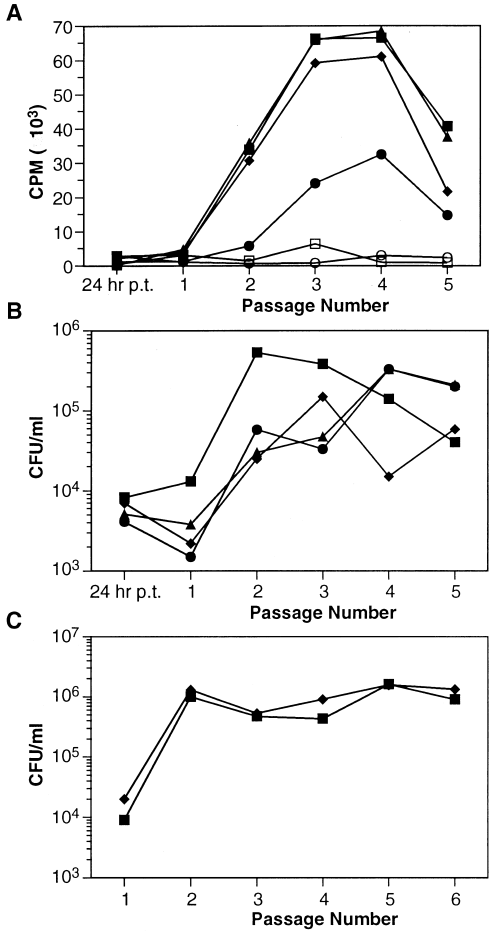

FIG. 7. Replication of RCASBP-M2(4070A)Puro in CEF. (A) RT assay. CEF were transfected with plasmid DNA and passaged. At 24 h after transfection and at each passage, virus particles were recovered from the culture fluid by centrifugation and quantified by determination of the level of RT activity in 1 ml of culture fluid. □, mock; ○, env (wt); ▲, env1; ◆, env6; ■, env8; ●, env9. p.t., posttransfection. (B) Titers on D17 cells. Cells were infected with serial 10-fold dilutions of virus-containing culture fluid harvested at each passage. Resistant clones were selected in the medium containing puromycin. Pac^r colonies were stained with Giemsa stain and counted. ▲, env1; ◆, env6; ■, env8; ●, env9. (C) Titers of RCASBP-M2(4070A)Puro8 (■) and RCASBP-M2C(4070A)Puro (◆) on D17 cells. Values are averages of two independent determinations.

The mammalian cellular receptor for amphotropic MLV has recently been cloned and characterized (31, 50). However, the gene for the avian amphotropic receptor has not been unambiguously identified (50). Passaging of RCASBP-M(4070A) through CEF could potentially select for the viral variants that have increased affinity for the avian amphotropic receptor. Interestingly, no mutations were seen either in the N-terminal half of gp70, the region that appears to determine host range (33, 34), or in the hypervariable proline-rich region. If the alteration in gp70 increases the affinity of the protein for the avian receptor, it does not compromise the ability of the virus to infect mammalian cells.

In our initial experiments, we used retroviral vectors containing the *neo* gene as a marker; however, this gene was relatively unstable in the context of the amphotropic *env* gene. We obtained much better results with the *pac* gene, which encodes resistance to puromycin. We believe that the difference in stability between these selectable markers is not a function of the vector but rather a measure of the relative cytotoxicities of these markers for the host cell. The alternative argument that there is a much greater tendency for the *neo* insert to rearrange seems unlikely. Facile rearrangements would imply that there are a few preferred sites for rearrangement in the *neo* insert. We looked at a few *neo* deletions; all were different.

One step in the maturation of MLV virions that is critical for particle infectivity is the proteolytic cleavage by the virus-encoded protease of the pre-p15E glycoprotein, resulting in the formation of the mature transmembrane glycoprotein, p15E (40). Our observations show that although MLV protease is not present, the processing of p15E in RCASBP-M(4070A) chimeric virus particles appears to be approximately as efficient as that in wild-type MLV virions, suggesting that either RSV protease or some protease derived from avian cells can effectively process pre-p15E. The short p2E tail of pre-p15E inhibits the fusogenic activity of the MLV p15E until after the virus particles have left the cell (40). If pre-p15E is cleaved by the cellular protease before being incorporated into the virion, fusogenic activity is induced. A premature cleavage of this type could explain the formation of syncytia that is induced by RCASBP-M(4070A) in CEF (data not shown). Experiments are now in progress to establish which protease is involved in the maturation of the transmembrane glycoprotein in RCASB P-M(4070A) particles.

In clinical applications of retroviral gene transfer, both the titer and the safety of the vector are of critical importance. The titer of the RCASBP-M2C(4070A)Puro vector exceeds 10^6 CFU/ml. This titer can be obtained by two to three cell passages after transfection of CEF. Not only is the vector simple to use and capable of producing high titers, but it should also be quite safe. RSV-infected mammalian cells do not produce infectious viral particles, and the vector cannot spread in the mammalian host. The inability of RSV to efficiently assemble, export, and process the Gag precursor protein in mammalian cells (32, 52) or to export a full-length genomic RNA into the cytoplasm (3, 27, 32, 39) appears to prevent the production of infectious virus particles. We did not find any infectious viral particles in the dog D17 cells containing RCASBP-M2(4070A)Puro provirus. Since it is inherently defective in mammalian cells, the RSV-based vector RCASBP-M2C (4070A) appears to have the safety features required for such sensitive applications as human gene therapy.

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