The use of retroviral gene delivery for development of a bovine cytotoxic T cell assay system

by

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INTRODUCTION

Infection of cattle with viruses and other intracellular pathogens causes a great deal of harm to the animals and poses a serious economic burden to the cattle industry. The most common method to combat infection has been the development of vaccines, but in many cases vaccines are ineffective at preventing either infection or disease. The goal in most vaccine production is to elicit an immune response that mimics protective immune responses which animal develop after natural infection. For many viruses, especially those which cause persistent infections, a cellular immune response seems to be more important for protection from infection than humoral immune responses. In order to artificially produce a cellular immune response, it is usually necessary to vaccinate an animal using modified live or vectored viruses. A useful tool in development of live vaccines is the ability to measure what segments of the viral genome elicit the cellular immune responses that are associated with protection in animals infected with wild-type virus. This can allow researchers to design modified viruses which possess only the parts of the genome necessary for a protective response. Unfortunately, no widely applicable, effective system exists for measurement of acquired cellular immune responses in cattle.

The most important form of cellular immunity to viruses is generally mediated by class I MHC-restricted cytotoxic T lymphocytes, or CTL (5,7,40,88). An animal gains protective immunity when it possesses CTL which can recognize infected cells in the body and kill them, thereby stopping the virus life cycle. In animals such as mice or humans, measurements of CTL recognition are routinely performed by isolating CTL from the blood of an infected animal and measuring whether those CTL will specifically lyse infected target cells *in vitro*.

This method has been used to a small degree in cattle, but several technical obstacles prevent broad application of the technique. The greatest hurdle involves choice of the target cell. CTL are class I MHC-restricted, which is to say that they detect virus in a cell through the class I MHC antigen presentation pathway and will only lyse infected cells which have the same MHC genotype as they do. Because cattle are outbred, virtually no two animals have the same MHC genotype. So in order to measure CTL lysis of target cells in cattle, the CTL and the target cells must come from the same animal, and isolation of the target cell cannot result in the animal's death. Since many of the cells which are easily cultured reside in vital tissues, this greatly restricts the choice of a target cell. The target cell must also express a relatively high level of its class I MHC genes for viral antigens to be efficiently presented to the CTL, further limiting the choice of available cells. In addition, many viruses infect a limited range of cells or are difficult to culture *in vitro*, effectively eliminating all potential target cells for those viruses. Finally, many of the viruses which do infect cells *in vitro* lyse the cells they infect, creating a background level of lysis above which specific CTL lysis cannot be detected.

One method that holds promise in broadening the range of potential target cells is retroviral gene delivery. Retroviral vectors deliver genes to cells, but once the genes are delivered, no new viruses are made and no virus-induced lysis occurs. Also, retroviral vectors are available which will infect almost any dividing bovine cell. By using retroviral vectors to deliver individual genes from a bovine virus to target cells rather than trying to infect target cells with whole virus, the hurdles of limited virus host cell range, difficulty in culturing virus, and virus-induced lysis are circumvented. An added benefit of this method is that by using a panel of retroviral vectors which deliver different genes from the bovine virus, it would be possible to

specifically determine which genes elicit a CTL lysis, the information that is so potentially useful to vaccine development.

By using retroviral vectors to broaden the range of target cells available for use in a CTL assay, all that is then required of target cell is that they can be isolated without killing the animal donor, they must have a relatively high level of class I MHC expression, and they must be susceptible to infection with retroviral vectors. The first goal of this project was to choose a bovine target cell which meets these criteria. We then proposed to use bovine immunodeficiency virus (BIV) as a model virus from which to construct retroviral vectors and demonstrate expression of genes in target cells.

It is our hypothesis that retroviral vectors can be used to construct bovine CTL target cell lines, a technique which could be used for the study of cellular immunity to bovine viruses and other intracellular pathogens.

Specific Aims

1. Characterize and develop a bovine CTL target cell
2. Construct a retroviral vector for delivery of target genes to bovine cells
3. Use retroviral vectors to deliver genes to target cells and measure expression.

LITERATURE REVIEW

Cellular Immunity to Viruses

Infection by viruses and other intracellular pathogens interferes with the health and well-being of many animals. As long as the pathogens remain inside cells, they are inaccessible to soluble antibody molecules which do not penetrate host cell membranes. If antibodies were the only line of defense, the immune system would only be able to neutralize pathogens during the extracellular stages of their replication cycles. Therefore animals possess an additional mechanism to deal with intracellular infections. Nearly all host cells routinely present fragments of endogenously synthesized proteins on the cell surface complexed with class I major histocompatibility complex (MHC) molecules. In this way, the immune system can test for cells producing foreign proteins. Healthy cells present only host-derived antigens and are ignored by the immune system, but when an infected cell presents foreign peptide fragments on its surface, immune effector cells target the infected cell and lyse it (88). Cytotoxic T lymphocytes (CTL) are the effector cells of this immune mechanism.

Cytotoxic T cell lysis

CTL maturation. The precursors to $CD4^-$ $CD8^+$ T cells, or CTL, differentiate in the thymus. Part of the differentiation process involves rearrangement of the gene encoding the T cell receptor (TCR) which is used by mature CTL to detect class I MHC-foreign antigen complexes. This rearrangement of the TCR provides the cellular immune system with its ability to recognize an amazingly diverse range of foreign antigens. In the thymus, T cell precursors with newly rearranged TCR

undergo either negative or positive selection. Cells that bind MHC complexed with self antigens are eliminated, as are cells which do not bind any of the organism's class I MHC molecules. Cells that can bind the organism's class I MHC molecules but do not bind MHC-self antigen complexes are allowed to mature (70). Thus, CTL are produced which do not react to self and specifically react with foreign peptides bound to class I MHC.

Once CTL are released from the thymus, they migrate throughout the body in a non-activated state. When a CTL initially comes into contact with a foreign antigen-MHC complex, the CTL is stimulated to undergo cell division and activation (7). Division of CTL which recognize invading pathogens is important. Since the TCR differs between CTL, different cells will be able to recognize different antigen-MHC complexes. By dividing after recognizing a foreign antigen, the CTL increases the number of effector cells specific for infected target cells, and presumably increases the animal's capacity to outpace the infection. Activation of CTL is another important step. Activated CTL form granules containing serine esterases (granzymes) and perforin and upregulate expression of a ligand for the Fas receptor (FasL) on their surface (40). Both of these changes prime the CTL for target cell killing.

Target cell killing. When an activated CTL recognizes a foreign antigen on the surface of a target cell, it can kill the target cell by two different mechanisms: perforin secretion or Fas receptor binding (40). The major mechanism of target cell killing is perforin-induced lysis. Perforin is a glycoprotein which is expressed in T cells and natural killer (NK) cells, and when secreted, it can enter target cell membranes, multimerize, and form lytic pores. According to the granule exocytosis model of target cell killing, upon recognition of a foreign antigen, activated CTL release their perforin/granzyme granules onto the target cell membrane, where the

perforin forms pores. These pores then allow entry of water, ions, and the granzymes, which leads to death of the target cell. A second mechanism of target cell killing is Fas-mediated apoptosis. Many cell types express the Fas receptor, which upon crosslinking will signal the cell to undergo programmed cell death, or apoptosis. Activated CTL have a ligand for Fas (FasL) on their surface. When the TCR binds an MHC-antigen complex, the FasL molecules will bind and crosslink the target cell's Fas receptors, and thus begin the apoptotic cascade. For target cells which express Fas, the perforin-mediated and Fas-mediated killing pathway are used in conjunction; for cells which do not express Fas, the perforin pathway is used alone (40).

Antigen processing and presentation. In order for a CTL to lyse an infected cell, that target cell must first present pathogen-derived antigens on the cell surface. When infection of a cell occurs, a series of events occurs within the cell leading up to the presentation of class I MHC-foreign peptide complexes on the cell's surface. Animal cells contain a large multicatalytic proteolytic particle called the proteasome which cleaves cytosolic proteins into small polypeptide fragments, often after ubiquitination of the original protein. A transporter in the endoplasmic reticulum (ER) known as TAP (transporter associated with antigen presentation) is involved in assembling these polypeptide fragments with the two subunits of the class I MHC molecule in the lumen of the ER (88). The mature class I MHC complex is made up of a heavy chain, a light chain, and an 8 to 12 amino acid antigen peptide. The antigen peptide binds in a groove in the heavy chain. In mice and humans this groove is the site of most of the polymorphism among different alleles of class I MHC (60), and it is the site of TCR binding (9). The heavy and light chains are cotranslationally transferred to the lumen of the ER, where they form an unstable complex which can be stabilized by binding either the antigen peptide

(25) or the lumenal side of a TAP heterodimer. When a heavy and light chain are bound, TAP transports cytosolic peptides, 8 to 12 amino acids long, across the membrane of the ER forming a mature class I MHC complex, from which TAP then disassociates. The mature class I MHC-antigen complex is transported from the ER through the Golgi apparatus to the cell surface (88). At the cell surface, the complex is accessible for screening by CTL, and thus the immune system.

In vitro measurement of CTL lysis

In the standard method used to measure CTL lysis, an animal is challenged with virus, and lymphocytes are isolated from the animal and co-cultured *in vitro* with virus-infected target cells which have been intracellularly labeled. Lysis of target cells is measured as a function of release of the intracellular label.

Class I MHC restriction. In order for CTL to recognize target cells, the two populations of cells must share at least one class I MHC allele. Cattle have at least three class I MHC loci (1,2), and in order to measure the net lysis by all the CTL which recognize a virus, the CTL and target cells must be genetically identical at all class I MHC gene loci (26). The class I MHC restriction of CTL lysis means that in species of domestic livestock for which inbred lines do not exist, the most efficient method to measure CTL lysis is to isolate the CTL and the target cells from the same animal (6,10,11,26,30,50,51,53,54,78,81). In this way, both populations of cells are assured of being genetically identical, and thus matched at all class I MHC loci.

Choice of target cells. A critical decision in measurements of CTL lysis is the choice of target cells. The most obvious consideration is that the isolation procedure cannot kill the animal, and it must leave the animal healthy enough to respond normally to challenge with the virus. The cells which are isolated must

have a high enough level of MHC expression for efficient antigen presentation to take place (6,14). Though the specific level of class I MHC expression in a target cell necessary for CTL lysis has not been defined, higher expressing cells are desirable (6). The target cells must also be permissive to infection by virus or to delivery of viral genes.

Expression of viral genes. Another consideration in measurement of CTL lysis is how to express viral genes in target cells. CTL recognize peptides presented through the endogenous antigen presentation pathway, or the class I MHC pathway, so viral genes should be expressed endogenously in target cells. Sometimes the simplest way to do this is to infect the target cells with whole virus, and when it is possible, most CTL research examines lysis of virus-infected target cells (5,6,10,14,26,30,49,53,75,76). This method is not always possible, in that some viruses replicate at low or nonexistent titers in cultured cells which can obtained without killing the donor animal. For such viruses, an alternative method is to deliver viral genes to target cells via some vectored delivery system. This method is useful even for viruses which will infect cells *in vitro,* because many viruses which infect cultured cells lyse the cells they infect, and the viral lysis causes a background above which CTL lysis cannot be detected. Also, delivery of individual viral genes to target cells allows definition of the fine specificity of CTL to individual viral genes.

Assessment of Cell-Mediated Immunity

A variety of determinations of CTL lysis have been made in domestic animals using a number of technical variations on the basic measurement protocol outlined above. Still, a consistent and reliable method for testing this aspect of cell-

mediated immunity for a variety of viruses has not been developed. A review of the techniques that have succeeded, their strengths, and their limitations is important in illustrating what is technically possible and in determining what further steps need to be taken to develop a bovine CTL assay.

Humans and laboratory animals

The techniques used in the determination of CTL lysis in domestic animals have usually been pioneered in laboratory animals and humans.

Measurement of CTL responses in mice are greatly facilitated by the availability of inbred lines of mice. The MHC haplotypes are identical within inbred lines of mice and target cells from MHC-matched and MHC-mismatched lines can be used to show that the cytotoxicity is class I MHC restricted (11). Since target cells do not need to be isolated from the same animal as the CTL, cells like splenocytes can be used, even though their isolation kills the donor animal. Also, the cells can be transformed into cell lines, eliminating the difficulties associated with culture of primary cells. Because any cell which can be cultured *in vitro* can be used as a target cell, it is possible to perform a CTL assay with any virus that can be grown in cultured cells.

Other strategies have been used in laboratory animals that are not available in inbred lines. Autologous skin fibroblasts from rhesus monkeys have been used as target cells (48). The fibroblasts were infected *ex vivo* with a retroviral vector encoding HIV-1 ENV/REV cDNA, and monkeys were then immunized with the fibroblasts. $CD8^+$ CTL were found to lyse autologous fibroblasts infected with the retrovirus vector or with vaccinia virus vectors carrying the HIV genes. In rhesus monkeys, autologous peripheral blood monocytes (PBMC) can be transformed *in vitro* with herpesvirus, and the immortalized cells can then be infected with other

viruses and used as target cells (83). Monkeys were vaccinated with a retroviral vector carrying a hepatitis B virus (HBV) core-neomycin phosphotransferase (NEO) fusion gene. CTL from the monkeys lysed autologous PBMC infected with the retroviral vector. The specific epitope recognized by CTL from vaccinated monkeys was found by coating panels of PBMC with synthetic HBV peptide subunits spanning the length of the core protein, and then using the peptide-coated cells as target cells.

In humans, studies of HIV have primarily used Epstein-Barr virus (EBV)-transformed B cells as the target cells in CTL studies. Transformed target cells are often infected with either whole HIV virus or with vaccinia virus carrying individual HIV genes or segments of genes (5,11,51). Vaccinia virus constructs have allowed the localization of the CTL epitopes within the HIV genome. Though vaccinia virus is the most common gene delivery vehicle, a retroviral vector carrying HIV-1 Nef was successfully used in one study to measure a Nef-directed CTL response which was confirmed by lysis of cells infected with a vaccinia-Nef virus (69).

Domestic animals

Virus-specific CTL have been detected to vaccinia virus, maedi-visna virus (MVV), bovine respiratory syncytial virus (BRSV), Border disease virus, and bovine leukemia virus (BLV) in sheep (10,30,49,62,75,76,87), to caprine arthritic encephalitis virus (CAEV) in goats (50), to equine herpes virus type 1 and equine infectious anemia virus (EIAV) horses (12,53), to African swine fever virus (ASFV) in pigs (52,61), to feline immunodeficiency virus (FIV) in cats (26,78), to canine distemper virus in dogs (77), and to bovine herpes virus (BHV)-1, infectious bovine rhinotracheitis virus (IBRV), and parainfluenza type 3 (PI-3) virus in cattle (6,14,79). In addition, extensive work has been done to show class I MHC-restricted CTL

activity following infection with *Theileria parva*, an intracellular parasite (23,24,58). A variety of target cells and gene delivery mechanisms have been used to arrive at these results.

In goats infected with CAEV (50) and in sheep infected with MVV (10), autologous skin fibroblast lines were established from each animal. Skin cells were infected with virus and used to stimulate lymphocytes for one to two weeks, then ^{51}Cr-labeled skin cells were used as target cells. Autologous macrophage cultures were also established from sheep and infected with MVV (49). Autologous, infected target cells were lysed, but non-autologous or uninfected cells were not. Depletion of $CD8^+$ cells from lymphocyte pools resulted in an elimination of lysis.

Autologous skin fibroblasts of sheep infected with vaccinia virus were killed by CTL from infected sheep (62). Ovine testis cells infected with BRSV have also been used as target cells, and were lysed by autologous, $OvCD8^+$ CTL from BRSV-infected lambs. Similar strategies have been used in cattle. Autologous skin fibroblasts and testes cells were isolated from calves, infected with IBRV, and used as target cells. Autologous skin cells were lysed by *in vitro* stimulated CTL, but testes cells were not. This study indicated that bovine testes cells did not function well as target cells (14). In another study using bovine coronavirus, testes cells were also ineffective as target cells (41). In cattle, fibroblastoid cells isolated from gluteal muscle and infected with whole virus were able to show $CD8^+$ CTL lysis in calves infected with PI-3 virus between days six and nine post-infection (6).

Autologous cells infected with whole virus were also used to show genetically-restricted CTL activity against ASFV in swine (52), BHV-1 in cattle (79), equine herpes virus in ponies (12), and Border disease virus in sheep (87).

Methods of viral gene delivery other than infection with whole wild-type viruses have also been successfully used in horses, sheep, and cats. Gene delivery has not yet been used in CTL measurements of cattle.

In ponies infected with EIAV, autologous, virus-infected skin fibroblasts were not lysed by CTL when used as target cells. One kidney was isolated from each animal in the study, and autologous kidney cell cultures were established. Autologous EIAV-infected kidney cells were lysed by CTL from the infected ponies, indicating that although virus-specific CTL were present, the skin cells were ineffective as target cells. Removing $CD8^+$ cells from CTL removed the lytic activity. In the study, researchers mapped the specificity of the CTL using kidney cells infected with vaccinia viruses containing either an EIAV *gag*/5' *pol* gene segment or an *env* gene segment. Kidney cells infected with either recombinant vaccinia virus were lysed, indicating that the ponies possessed CTL recognizing epitopes in both regions of the EIAV genome.

In sheep infected with BLV, autologous PBMC were used as target cells without addition of exogenous virus. Cells were stimulated by PHA blast and incubated overnight with 20-amino-acid-long subunits of the BLV gp51 protein. Cells were then labeled with ^{51}Cr and were found to be lysed by autologous CTL. Using different combinations of peptide subunits, a ten-amino acid subunit of the gp51 protein was found to be the target for $CD8^+$ CTL.

In cats vaccinated with inactivated FIV virus and infected with live FIV, autologous skin fibroblasts infected with FIV were lysed by $CD8^+$ CTL. Researchers used autologous fibroblasts infected with recombinant vaccinia virus carrying either FIV gag or FIV env to determine if epitopes in either of the two genes were recognized, and were able to show different levels of CTL activity between the two groups of cats (26).

Finally, inbred lines of pigs, known as NIH miniature swine, have been bred which are homozygous at class I MHC loci. A nine-amino-acid-long CTL epitope to classical swine fever virus (CSFV) was mapped in these pigs, and the target cells used were nonautologous transformed kidney cells taken from other MHC-matched syngeneic animals. The epitope was found by using target cells infected with recombinant vaccinia viruses carrying different portions of the CSFV genome (61).

Skin fibroblasts, macrophages, PBMC, testes cells, gluteal muscle cells have been infected with wild-type virus and used as target cells. Many of these cells were easy to isolate and culture, but in each case the choice of target cell was only applicable to the particular virus being studied because that virus was able to infect the target cells and many other viruses would not be able to infect them. Autologous PBMC have been coated with synthetic viral peptide subunits and used as CTL target cells. This method was able to quickly map CTL specificities to a short amino acid segment, but it is prohibitive because of the cost of synthesizing the peptides. Skin fibroblasts, transformed B cells, and kidney cells infected with recombinant vaccinia viruses carrying individual viral genes were used as CTL target cells. Vaccinia virus was able to infect a variety of cell types, and by carrying individual genes it can map CTL responses to specific regions of a viral genome. The main drawback of vaccinia virus vectors is that they are live replicating viruses and can themselves lyse infected target cells. Retroviral gene delivery has also been used with skin fibroblasts and transformed B cells to measure CTL specificities. The retroviral vectors had the advantages of vectored delivery, but did not lyse infected cells. The main drawback to the retroviral vectors was a lower level of gene expression than with live virus or vaccinia vectored viral genes.

Relationship to disease

A major reason for the interest in cellular immunity is the role it plays in controlling disease. There is evidence that for many intracellular pathogens, CTL are sufficient to control disease and, for some, CTL are necessary.

One of the most striking examples of the ability of CTL to control disease comes from research in cattle, not with a virus, but with the intracellular protozoan, *Theileria parva*. During part of its life cycle, this parasite lives inside B cells. It transforms the B cells and stimulates them to divide, which makes it relatively easy to obtain cultures of infected autologous B cells from cattle. A study was performed in which pairs identical twin cattle from split embryos were infected with a lethal strain of *T. parva* after one animal had been immunized against infection and the other had not. The immunized animals were protected against the infection and their naive twins began to sicken and die. Immunized animals were previously shown to possess class I MHC-restricted $CD8^+$ lymphocytes capable of lysing autologous infected cells (58). $CD8^+$ lymphocytes from the immunized animals were transferred to their dying twins, and within several days, the animals which received the transferred lymphocytes were able to control the infection and recovered (54). This study clearly showed that class I MHC-restricted CTL can be sufficient in conferring protective immunity against an intracellular pathogen. This is supported by a second study in which cattle immunized against one strain of *T. parva* were challenged with another strain. In all cases, animals which developed class I MHC-restricted CTL cross-reactive against both strains were protected, and animals which possessed CTL against only the immunizing strain were susceptible to heterologous challenge (81).

The importance of CTL in clearance of virus infection has been shown using knockout mice and adoptive lymphocyte transfer studies (13,32,36,43,46,64,66,74).

It appears that antibodies are the immune system's primary mechanism of protection against fast replicating, cytopathic viruses. These viruses enter cells and replicate to high enough levels that lysis only serves to release the viruses from cells. On the other hand, CTL are necessary in the control of slow replicating and noncytopathic viruses. CTL responses are quick enough to mount a response to slow replicating viruses before their replication is complete, and noncytopathic viruses would continue to produce virus from infected cells indefinitely if the cells were not lysed (40,89).

In humans, a strong case has been made for the control of HIV infection by virus-specific CTL. In primary HIV-1 infection, patients which mount a strong CTL response to HIV the gp160 subunit of the env gene show a faster reduction of viremia and virus core antigenemia and a slower progression to disease than patients with a weak CTL response (11). It appears that the first effective antiviral immune response following HIV infection is mediated by $CD8^+$ T cells (72). In the blood and lymph nodes of SIV_{mac}-experimentally infected rhesus monkeys, virus-specific CTL were detected before any virus-specific antibodies, and containment of acute viremia and antigenemia occurred about a week before neutralizing antibodies reached significant titers (67). High levels of HIV-specific CTL in humans are linked to lower viral load and slower progression to AIDS in both cross-sectional (68) and longitudinal (15,44,55,86) disease studies. CTL to the HIV Gag, Pol, and Env genes appear to be most effective at controlling infection (68). In summary, CTL appear to control initial HIV infection, high levels of CTL correlate with nonprogression, and the loss of CTL control coincides with progression to AIDS. CTL immunity seems to be very important in controlling HIV infection.

In domestic animals, there is less compelling evidence of viral control by CTL, mostly because of the difficulties in measuring CTL lysis. A portion of cats

vaccinated with inactivated feline immunodeficiency virus (FIV) were protected against subsequent challenge with homologous FIV. The development of protective immunity in these cats was strongly correlated with the development of a class I MHC-restricted Env-specific CTL response (26). Sheep vaccinated with a recombinant vaccinia virus containing the gp51 portion of the bovine leukemia virus (BLV) env gene were protected from infection of BLV, and infected sheep were able to suppress further BLV replication after vaccination. There was no correlation between neutralizing antibodies and protection (59). In fact, protection conferred by the recombinant vaccine was correlated with a decrease in anti-gp51 antibodies (31,63). In unprotected animals, antibody levels to gp51 increased for up to 16 months post-challenge and were ineffective in controlling virus titers (31). On the other hand, protection did correlate with the development of a $CD8^+$ CTL response against a conserved region of the BLV gp51 (30). These data provide evidence in support of control of viral infection by CTL in domestic animals.

A necessary role in the control of virus infection has been shown for CTL in mice, and evidence supports the same role in humans, sheep, and cats. Conclusive evidence has been found to support the controlling role played by bovine CTL during *T. parva* infection. It is reasonable to infer that bovine CTL play a necessary part in controlling infection by some viruses, but determining which viruses are controlled CTL will require direct measurements of the CTL responses each virus elicits.

Retroviral Gene Delivery

One of the main components of a CTL assay is the method by which viral proteins are presented on the target cell surface. In some cases the simplest

method is to infect target cells with wild-type virus, but because of virus-induced lysis or an inability to infect cultured cells, a vectored gene delivery system is sometimes a more practical method of introducing viral genes into target cells. Several gene delivery systems have been successfully used in CTL assays. Vaccinia virus delivery is the most commonly used system, but retroviral vectors have also been used and they bring several advantages to a CTL assay system.

Construction

In retroviral gene delivery, cultured cell lines are used to produce hybrid virions which possess all the structural elements necessary for infection, but none of the genetic information to produce more viruses. The virions carry RNA transcribed from a synthetic plasmid into which any gene seven kilobases or smaller can be inserted. Thus, individual genes from any source, including a different virus, can be delivered by retroviral vectors.

In order to achieve retroviral gene delivery, two components are necessary: a packaging cell line and a transfer vector. A packaging cell line contains the genes of a retrovirus and thus produces the structural proteins which can self-assemble into virus particles. Wild-type retroviruses contain a packaging signal which directs their incorporation into virions, but the retroviral genes in the packaging cell have had their packaging signal deleted. Thus, the virions that the packaging cell produces are empty and contain no genetic information. A transfer vector is a DNA plasmid which contains a retroviral packaging signal, an antibiotic resistance marker, and a site which can carry any inserted gene. When a packaging cell line is transfected with a transfer vector, the packaging signal directs the retroviral structural proteins to incorporate the transfer vector into virions. A packaging cell

line transfected with a transfer vector is called a vector-producer cell line since it produces retroviral vectors which will deliver the inserted gene upon infection.

Advantages

There are some benefits that are conferred by using retroviral vectors to deliver genes to target cells. Three important benefits are stable expression in infected cells, a wide range of cell types permissive to infection, and the absence of virus-induced lysis in infected cells.

In comparison to other methods of gene delivery, expression of retrovirus-delivered genes is stable. After delivery by some other methods, genes are maintained in the cytoplasm or in endosomes and can be lost during rounds of cell division. Retroviruses integrate the genes they carry directly into the host cell chromosomes, and thus become a permanent feature of cells' genetic repertoire. Because the gene delivery is stable, a stock of retroviral vector-transduced target cells can be used for repeated assays of CTL activity without the variability in infection rates and expression levels caused by a different round of gene delivery for each CTL assay.

The retroviruses used in gene delivery have a wide host cell range. The retroviral *env* gene expressed by the packaging cell determines which cells are permissive to infection by the retroviral vector. Packaging cells are available with *env* genes which infect almost every mammalian cell which has been tested. This allows much more freedom in the choice of a target cell.

Another major advantage to retroviral vectors is that they do not induce lysis in infected cells. After a defined period of time, many wild-type viruses cause lysis of infected cells, either through a breakdown in the cells' synthetic pathways or by expression of lytic viral genes. This virus-induced lysis can be a large source of

background during an assay meant to measure CTL lysis. In order to minimize the background, the assay must be performed after expression of viral genes and before cell lysis, a time-consuming optimization process for which a good compromise time cannot always be found. Since they do not induce lysis, retroviral vectors avoid the whole problem.

Disadvantages

Retroviral vector-mediated gene delivery has some drawbacks that can make it difficult to use. One common problem that occurs in vector-producer cell lines is recombination of the transfer vector. Transfer vectors are maintained in vector-producer cell lines using a selectable marker on the plasmid. Retroviruses can undergo cycles of transcription of viral RNA and reverse transcription back into the host genome, and occasionally a recombination occurs with the inserted gene deleted and the selectable marker remaining. These recombinants possess an advantage over vector-producer cells with unaltered transfer vectors because the antibiotic resistance gene allows them to survive selection, but the energy saved by loss of the inserted gene may let them divide faster. The advantage is even greater if the inserted gene's product is somewhat toxic. Another problem that occurs in both packaging cells and infected cells is inactivation of one of the promoters in the retroviral vector. In this situation, the gene inserted into the transfer vector is partially silenced. A low level of transcription and thus antibiotic resistance may be maintained, but levels of the inserted gene's product will fall to undetectable levels. A third rather minor problem is a small number of restriction enzyme sites in the multiple cloning site of the available transfer vectors. This problem may soon be resolved with the availability of better plasmids, but does create difficulties at present.

Bovine Virus Model

In order to test a bovine CTL assay system, it is necessary to chose a bovine virus which is likely to elicit a CTL response. Viruses which are noncytopathic *in vivo* or replicate slowly are more likely to be controlled by CTL in mice. Members of the lentivirus family, HIV, SIV_{mac}, FIV, EIAV, CAEV, and MVV, have all been shown to elicit CTL responses during infection. Thus a good candidate for a model bovine virus to test a CTL assay is the lentivirus, bovine immunodeficiency virus (BIV).

Bovine immunodeficiency virus

BIV is a member of the retrovirus family and lentivirus subfamily of viruses. Retroviruses are diploid RNA viruses which undergo reverse transcription and integration into the host cell genome after entering a cell. The integrated viral genome is known as the provirus. The proviral genome structure of a simple retrovirus is shown in Figure 1. Long terminal repeats (LTRs) lie at both ends of the genome, with the 5' LTR acting as the promoter for the rest of the viral genome and the 3' LTR acting as a polyadenylation site. Three open reading frames between the LTRs encode the *gag*, *pol*, and *env* genes. The *gag* gene product, or the Gag polyprotein, is proteolytically cleaved into three proteins: matrix (MA), capsid (CA),

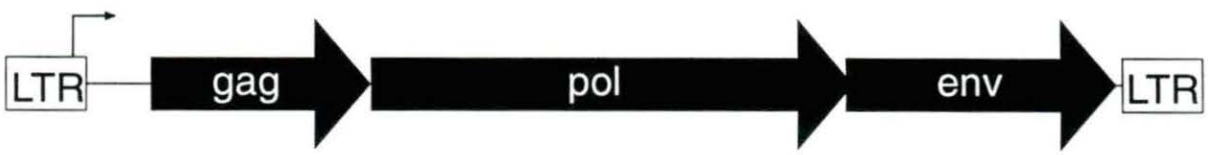

Figure 1. Proviral genome of a simple retrovirus (Moloney murine leukemia virus). The large arrows represent genes. The small arrow indicates that the LTR acts as the promoter for the provirus.

and nucleocapsid (NC). The BIV CA protein is a 26 kDa molecule which assembles into the hydrophobic virion core. It is the most abundant protein in the virion. The MA and NC proteins are 16 kDa and 15 kDa in size, respectively, and are responsible for association of the virion core with the cell membrane and the viral RNA genome, respectively. The Pol protein is cleaved to form viral proteinase (PR), reverse transcriptase (RT) and integrase (IN) enzymes. The PR protein cleaves the Gag and Pol polyproteins, the RT enzyme uses viral genomic RNA as a template for synthesizing double-stranded DNA, and the IN protein integrates the viral DNA into the host cell chromosome. The *env* gene product is a glycoprotein which is cleaved by a host cell protease into a surface glycoprotein (SU) and a transmembrane glycoprotein (TM). The SU protein is responsible for binding a receptor on the host cell plasma membrane, and the TM protein anchors the Env complex in the viral envelope and induces fusion of the viral envelope with the host cell membrane (39).

Retroviruses are divided into the oncovirus, lentivirus, and spumavirus families. Lentiviruses are non-oncogenic viruses characterized by genomic organization, virus morphology, and in some cases by a slow, progressive disease that eventually leads to wasting and death. In Latin, *lenti* means slow, thus the name lentivirus. The lentiviral genome differs from that of simple retroviruses with an additional three to six accessory proteins. Two accessory proteins, Tat and Rev, temporally regulate the expression of viral genes. After reverse transcription and integration, the lentiviral provirus produces a full length RNA which is multiply spliced to form *tat* and *rev* RNAs. Tat upregulates transcription, and Rev facilitates the nuclear export of singly spliced and unspliced RNAs from which Gag, Pol, and Env can be translated. This regulation pattern allows proviruses to lie essentially dormant inside cells for long periods of time after infection (39).

Some common aspects of pathology are shared by different lentiviruses. During primary infection, the virus undergoes rapid replication and proliferation in lymphocytes or macrophages. This initial proliferative stage is usually controlled after several days or weeks by the immune system, but the infection is not cleared and detectable virus replication persists. In HIV or maedi/visna virus (MVV) of sheep for example, initial productive infection is followed by an asymptomatic incubation period of months to years. During the incubation period, viruses continue to replicate and the continuous onslaught on the cells of the immune system may result in a chronic wasting syndrome which can eventually lead to cachexia and death.

The animal from which BIV was originally isolated showed persistent lymphocytosis, lymphoid hyperplasia, and perivascular cuffing in the brain (84). Experimental infection with BIV was shown to cause follicular hyperplasia of lymph nodes and a transient lymphocytosis in infected calves (16). BIV infection elicits detectable antibody responses. Antibodies to subunits of the Gag (CA) and Env (Env8 portion of TM) were detected in cattle 4 weeks post-infection. By 40 weeks post-infection, serum antibody levels to the CA protein had gradually disappeared in 7 out of 8 cattle (37). In HIV infection, loss of Gag-specific antibodies is associated with disease progression to AIDS (3,8,18,33,47), but BIV-infected cattle remained healthy during and after loss of Gag antibodies. The cattle remained infected and circulating virus could be isolated from the peripheral blood of all animals, but none developed clinical disease. This suggested that antibodies to Gag were not a major factor in controlling BIV in experimentally-infected cattle. It is possible that virus neutralizing antibodies are responsible for controlling virus replication. However, studies in our laboratory have shown an inverse relationship between neutralizing antibody titer and frequency of virus recovery: animals with

higher neutralizing titers had a higher frequency of virus isolation (17). Together, these data suggest that the humoral immune system plays only a minor, or supporting, role in control of BIV replication *in vivo*. This observation, in conjunction with the detection of CTL in every other lentiviral infection that has been tested, makes BIV-infected cattle good candidates for models to use in a CTL assay.

MATERIALS AND METHODS[1]

Development of a Bovine CTL Target Cell

Culture of cells

The primary cell cultures used in this research were derived from bovine fetal testes (BOTEST), fetal turbinate (BOTUR), fetal skin (BOSK), fetal tongue (BOTONG), embryonic kidney (EBKp), fetal lung (FBL), adult pulmonary artery endothelium (CPAE, ATCC #CCL-209), and adult peripheral blood adherent cells (PBAC). Immortal cultured cell lines used were SV40-transformed bovine macrophages (BOMAC, kindly provided by Judy Stabel) (20), bovine adrenal medullar endothelial cells (EJG, ATCC #CRL-8659), Madin-Darby bovine kidney epithelial cells (MDBK, ATCC #CCL-22), mouse fibroblasts (NIH/3T3, ATCC #CRL-1658), human cervical carcinoma epithelial cells (HeLa, ATCC #CCL-2), and adenovirus-transformed human kidney cells (293 cells, a subpopulation derived from ATCC #CRL-1573 kindly provided by Tom Hope). Packaging cell lines used were PA317 (ATCC #CRL-9078) and PG13 (ATCC #CRL-10686). The vector-producer cell line PG13/LNc8 (ATCC #CRL-10685) was also used.

All cells except CPAE were cultured in DMEM† with 10% FBS. CPAE were cultured in DMEM with 20% FBS. All cells except 293 cells were passed by trypsinizing cells for one to five minutes, then inactivating the trypsin by addition of media containing serum and transferring cells to new flasks. The 293 cells were passed by removing the old media, vigorously washing cells off of their adherent

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[1] The recipes for most of the solutions listed in this chapter are located in Appendix A, and all such solutions are marked with a † symbol the first time they are mentioned in the text. The expanded forms of many abbreviations can be found in Appendix B.

surface using fresh media, and placing cells in new flasks which had been treated for at least three hours at 4°C with 0.1% gelatin.

Isolation of primary cells

Cells were cultured from different tissue by several different techniques by Lawrence Elsken's laboratory at NVSL (Ames, Iowa) and Janice Miller's laboratory at NADC (Ames, Iowa). Bovine testes, bovine tongue, bovine turbinate, and bovine kidney cells were isolated from their respective tissues in fetal calves at NVSL. Cells were released from the tissue by trypsinization, grown in MEM with Earle's salts, and then frozen in liquid nitrogen as BOTEST, BOTONG, BOTUR, and EBKp cultures. The membrane was removed from fetal bovine lung, cells were released by trypsinization, grown in culture, and frozen in liquid nitrogen as FBL cultures at NADC. Cells from bovine fetal skin explants were grown in culture in Lawrence Elsken's laboratory, and frozen in liquid nitrogen as BOSK cultures.

PBAC were isolated from cultures of adherent peripheral blood mononuclear cells (PBMC). Blood was drawn from cattle by jugular venipuncture using either EDTA or ACD as an anticoagulant. The blood was centrifuged at 400 x g for 30 minutes to separate plasma and red blood cells (RBC). The buffy coat at the interface was collected. Two volumes of RBC Lysis buffer† were added, the mixture incubated for about 1 minute, and one volume of Restoring buffer† was added. Cells were pelleted at 150 x g for 10 minutes, and the cell pellet resuspended in PBS. Two volumes of RBC Lysis buffer were added for one minute, then one volume of restoring buffer was added. Cells were again pelleted for 10 minutes at 150 x g and resuspended in PBS. Cells were seeded in tissue culture flasks for 24 to 48 hours, then non-adherent cells were removed by washing the cultures with HBSS†, and media was replaced. The media was changed weekly. After three

weeks, a much larger cell type with prominent cytoplasmic striations when viewed under phase could be seen to be outgrowing monocytes in some cultures. These cells, termed PBAC, were trypsinized and passed. After three passages, PBAC were frozen in liquid nitrogen.

Measurement of class I MHC expression

Cell cultures were grown to confluency in 75 cm^2 flasks. Cells were trypsinized with STV and passed into two or three 75 cm^2 flasks, so all cells would be dividing. The next day, media was removed from the sub-confluent cultures and replaced with PBS containing 0.5 m*M* EDTA and cells were scraped off the flask after five to ten minutes. Duplicate flasks were pooled and each cell type was seeded in wells of a 96-well plates. The plates were centrifuged briefly to pellet the cells, the supernatant was removed and cells were washed once in FACS buffer†. Cells were again pelleted and thoroughly resuspended in 50 µl of FACS buffer or 50 µl of VMRD monoclonal antibody H58A (specific for the heavy chain of bovine class I MHC) diluted 1:200 in FACS buffer. Cells were incubated for 20 minutes on ice. After primary antibody incubation, cells were pelleted and washed in FACS buffer three times. Cell pellets were resuspended in either 50 µl FACS buffer or 50 µl FITC-conjugated goat antibody to mouse IgG diluted 1:200 in FACS buffer. Cells were incubated 20 minutes on ice in the dark in secondary antibody. After incubation, the cells were pelleted and washed in FACS buffer three times. Cells were finally resuspended in 500 µl FACS buffer and their level of fluorescence measured in a Coulter Epics XL-MCL flow cytometer. Cells left untreated with antibodies and cells treated with only the secondary antibody were used as controls. Staining and FACS analysis was performed on two separate occasions with all cell types analyzed on both occasions. The non-specific reactivity of the

secondary antibody for each cell type was assessed as the difference between the mean fluorescence (mean fluorescent channels) of the secondary only-labeled cells and the unstained controls. The MHC-specific fluorescence is calculated as the difference between the mean fluorescence of class I MHC-labeled cells and the mean fluorescence of secondary only-labeled cells.

Titering of PG13/LNc8 retrovirus

The PG13 cell line produces a pseudotyped retrovirus with Moloney murine leukemia virus (MoMLV) core proteins and gibbon ape leukemia virus (GaLV) envelope glycoproteins. The PG13/LNc8 cell line contains the LN transfer vector, which has a gene for neomycin resistance between two MoMLV LTRs. The retroviruses produced by the PG13/LNc8 cell line deliver the neomycin resistance gene to cells (56).

A virus stock was made from PG13/LNc8 supernatant. Two 75 cm^2 flasks of PG13/LNc8 cells were grown to confluency. The old media in each flask was discarded and replaced with 10 ml of fresh media. Thirty-two hours later, the supernatant from the two flasks was collected and pooled. The pooled media was clarified for 10 minutes at 1800 x g, and aliquots were stored at -80°C.

To test for susceptibility to infection with PG13 recombinant virus, cells were passed into 6-well plates to produce approximately 40% confluency after one day of growth ($2.5x10^4$ to $1.5x10^5$ cells per well). The day after passage, media was replaced with 1 ml of LNc8 virus stock at 10-fold serial dilutions in media with 8 μg/ml polybrene. Dilutions were made from 10^0 to 10^{-4} ml virus stock per ml media. After 28 to 36 hours, the virus stock was replaced with normal media containing 1.5 mg/ml G418 (active) to select for neomycin-resistant cells. Between 5 to 10 days of G418 selection were sufficient to kill uninfected control cells and for

infected cells to form resistant colonies. At that time, colonies were fixed and stained by incubating cells for 5 minutes in 0.1% methylene blue in methanol/water (1:1 vol./vol.). Blue colonies were counted and the number of neomycin-resistant colony-forming units (cfu) per ml of virus stock was calculated. Virus titrations were performed for each cell culture at least twice.

Immunocytochemistry (ICC)

Cultured cells were tested for various proteins by ICC. Cells were washed once with TNFBS[†], then fixed with ice-cold methanol for five minutes. Methanol was diluted 1:1 with TNFBS and removed, and cells were washed twice with TNFBS. Cells were incubated for thirty minutes with the primary antibody at room temperature with rocking, and washed three times with TNFBS. The HRP-conjugated secondary antibody was added, and cells were incubated for thirty minutes at room temperature with rocking. The secondary antibody was removed and cells were washed three times with TN. HRP substrate was added, and cells were incubated for nineteen minutes in the dark at room temperature. The HRP substrate was removed, and cells were washed with tap water and allowed to dry. Cells were examined by brightfield microscopy for reddish-brown staining, indicating a positive reaction.

Immunofluorescence assay (IFA)

Cultured cells were tested for various proteins by IFA. Cells were washed once with TNFBS, then fixed with either ice-cold methanol for five minutes or with formaldehyde/Triton X-100 as follows. Cells were fixed with 3.7% formaldehyde in PBS for 20 minutes at room temperature. Formaldehyde was removed, and cells were washed twice with TNFBS. In some cases, cells were permeabilized with

0.5% Triton X-100 for 15 minutes at room temperature. Triton X-100 was removed, and cells were washed three times with TNFBS. Cells were incubated in primary antibody for thirty minutes at room temperature with rocking. Primary antibody was removed, and cells were washed three times with TNFBS. The FITC-conjugated secondary antibody was added to cells and incubated in the dark for thirty minutes at room temperature with rocking. The secondary antibody was removed and cells were washed three times with TN. Cells were examined by fluorescent microscopy while in the TN buffer. If necessary, cells were stored in a light-proof box at 4°C for later examination.

DiI-Ac-LDL uptake

Some cells were tested for the presence of the "scavenger cell pathway" by measuring uptake of DiI-Ac-LDL. Ac-LDL is taken into cells via the pathway, and the DiI label accumulates in cholesterol deposits in the cytoplasm (85). The media from cells was replaced with normal media containing 5 μg/ml DiI-Ac-LDL, and cells were incubated at 37°C and 7% CO_2 for one hour. DiI-Ac-LDL media was then removed, and cells washed several times with probe-free media. Cells were visualized by fluorescent microscopy using standard rhodamine excitation: emission filters. Uptake was indicated by bright reddish-orange deposits of the dye in the cytoplasm of cells. Quantitation of the fluorescence was carried out by FACS analysis. Cell were trypsinized with STV†, then the trypsin was inactivated by adding normal media containing serum. Cells were pelleted by centrifugation, the supernatant removed, and cells resuspended in FACS buffer. The fluorescence of DiI-labeled cells and unlabeled control cells was analyzed on a Coulter Epics XL-MCL flow cytometer. The level of fluorescence was analyzed as the mean

fluorescence of labeled cells minus the mean fluorescence of unlabeled cells. DiI-Ac-LDL uptake measurements were performed in triplicate.

Lymphocyte binding assay

A characteristic of high endothelial cells is their ability to bind freshly isolated lymphocytes. Several cell cultures were tested for lymphocyte binding. Bovine peripheral blood mononuclear cells (PBMC) were obtained by a buffy coat isolation of blood in the same manner as for PBAC isolations. Approximately 3 x 10^6 PBMC were added per well cultured cells and incubated for 2 hours at 37°C and 7% CO_2. After incubation, the slides were immersed in PBS, and the chamber dividers removed. Cells were washed three to four times to remove unbound lymphocytes by rocking the PBS bath and changing the PBS after one to two minutes. Cells were fixed by in 3.7% formaldehyde in PBS for 20 minutes, and bound lymphocytes were detected by IFA using an anti-CD45 mouse monoclonal antibody (VMRD), and a FITC-conjugated goat anti-mouse secondary antibody. The number of lymphocytes bound to 100-110 cultured cells was counted, and the number of lymphocytes bound per cultured cell was calculated. Only lymphocytes which had spread out upon a cultured cell were counted; lymphocytes which remained rounded were not counted because many rounded lymphocytes were also non-specifically bound to the bottom of the flask.

Construction of Retroviral Vectors

Polymerase chain reaction (PCR)

Briefly, 5 to 25 ng of template DNA were put in 100 µl of solution with final concentrations of 1X PCR Buffer II (Perkin-Elmer), 1.5 m*M* $MgCl_2$, 200 µ*M* each of

deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytosine triphosphate, and deoxythymidine triphosphate (dNTPs), 1 μ*M* of each primer, and 2.5 units of *Taq* polymerase. Each reaction mixture was incubated at 94°C for 2 minutes, 50°C for 1 minute, and 72°C for 6 minutes, then for 30 cycles of 94°C, 50°C, and 72°C each for 1 minute, and one final cycle at 94°C and 50°C for 1 minute and 72°C for 4 minutes, after which reactions were cooled to 5°C.

The size and number of PCR products were assessed by agarose gel electrophoresis. PCR products were electrophoresed through 1% agarose gels in TAE buffer†. The gel was stained in 1.5 μg/ml ethidium bromide for 10 to 15 minutes and destained in water for 10 to 15 minutes. DNA bands were visualized by placing stained gels on an ultraviolet lamp.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA samples were analyzed with RT-PCR similarly to PCR analysis, but with a preceding DNase treatment and reverse transcription step. Each 20 μl DNase reaction contained 5 m*M* $MgCl_2$, 1X PCR Buffer II, 1 m*M* of each dNTP, 2.5 μ*M* random DNA hexamers, 20 U of RNase Inhibitor, 1 U of RNase-free DNase I, and up to 1 μg of total cellular RNA. Each reaction mixture was incubated at 37°C for 30 minutes, 75°C for 5 minutes, and then cooled to 4°C. Keeping the reaction tubes on ice, 50 U (1 μl) of MLV reverse transcriptase was added to each reaction mixture. Reverse transcription was carried out at 42°C for 45 minutes, 99°C for 5 minutes, and 5°C for at least 5 minutes. The reaction volume was then brought up to 100 μl and final concentration of reagents adjusted to 1X PCR Buffer II, 2 m*M* $MgCl_2$, 200 μ*M* of each dNTP, 1 μ*M* of each primer, and 2.5 units of *AmpliTaq* polymerase. PCR amplification temperatures and times were the same as for PCR.

RT-PCR products were analyzed by agarose gel electrophoresis and ethidium bromide/UV visualization.

Cloning of PCR products into bacterial plasmids

Taq PCR products possess an overhanging adenosine at each of their 3' ends. These overhangs were used to clone PCR products into three different bacterial plasmids, pCRII, pGEM-T, and pCR3. The pCRII vector (Invitrogen) is a bacterial plasmid linearized with a cut in the middle of a betagalactoside (ß-gal) gene and which possesses 5' thymidine triphosphate overhangs on each terminus. Ligating a PCR product into the plasmid will disrupt the ß-gal gene and transformed bacterial colonies will be yellowish-white; plasmids which self-ligate without a PCR product will maintain a functioning ß-gal gene and will produce blue colonies. PCR products were also ligated into pGEM-T, a ß-gal disruption plasmid sold by Promega which is similar to pCRII. The pCR3 vector is linearized downstream of a cytomegalovirus (CMV) promoter and allows expression of ligated PCR products in eukaryotic cells. In a 10 µl reaction, 1 to 2 µl of PCR product were combined with 1 µl 10X ligation buffer (supplied by manufacturer), 50 µg linearized pCRII, pGEM-T, or pCR3 vector, and 4 units of T4 DNA ligase. The ligation reaction was carried out overnight at 15°C.

Transformation of *E. coli*

Competent *E. coli* were transformed with bacterial plasmids using a method by Hanahan (34). A culture of DH5α-strain *E. coli* cells was grown overnight in NZY broth†; 1.5 ml was inoculated into 25 ml of SOB† and grown for 2 to 2.5 hours. Cells were pelleted by centrifugation at 4200xg, resuspended in 8 ml TFB† and incubated on ice for 10 to 15 minutes. Bacteria were pelleted again and

resuspended in 2 ml TFB. Seventy µl of DMF were added, cells were chilled on ice for 5 minutes, 4 µl of ß-mercaptoethanol added, cells iced for 10 minutes, 70 µl DMF added, and cells iced once more for 5 minutes. In ice-cold Falcon 2059 tubes, 210 µl of the bacterial cell suspension were added to 1-2 µl of ligated DNA and 8-9 µl of SOB, and the mixture was incubated on ice for 30 min. Cells were heat shocked at 42°C for 90 seconds, chilled on ice for 90 seconds, and then 0.8 ml of SOC† were added. The cells were incubated at 37°C with 225 rpm shaking for one hour. Bacteria were spread on YT-carbenicillin plates† and grown overnight at 37°C. All plasmids used in this study possessed a gene for ampicillin resistance, and the carbenicillin antibiotic in the plates selects bacteria transformed with plasmid. When plasmids contained the ß-gal gene, YT-carb plates were pre-treated with 25 µl of 40 mg/ml X-Gal and 8 µl of 100 m*M* IPTG to produce blue and white colonies.

Plasmid isolation preparations

Quantities of plasmid were obtained from transformed *E. coli* by growing large quantities of the bacteria, separating plasmid DNA from chromosomal DNA, and then removing protein. One bacterial colony was used to inoculate 10 ml of Super Broth† containing 100 µg/ml ampicillin, and incubated overnight at 37°C and 225 rpm shaking. Bacteria were pelleted in 3 ml aliquots by centrifugation and the supernatant removed. Bacterial pellets were resuspended in 200 µl IH Buffer 1†. Bacteria were lysed by adding 300 µl of a 0.2 *N* NaOH/1% SDS solution and incubating on ice for 5 minutes. Lysis was halted and chromosomal DNA precipitated by adding 300 µl of 3.0 *M* potassium acetate (pH 4.8) and icing for 5 minutes. Cellular debris was pelleted by a 10 minute centrifugation at 15,000xg and discarded. The supernatant was added to 2 µl of 10 mg/ml RNase A and

incubated at 37°C for 20 minutes. Protein was extracted from the supernatant solution three times by adding 400 µl chloroform, vortexing 30 seconds, centrifuging for 2 minutes, and collecting the top, aqueous phase. Plasmid DNA was precipitated by adding an equal volume of isopropanol and centrifuging at 15,000xg for 15 minutes. The DNA pellet was washed with 500 µl of 70% ethanol and centrifuged for 2 minutes. The ethanol supernatant was discarded, the DNA pellet dissolved in water, and traces of ethanol removed by 3 minutes of vacuum centrifugation. Plasmid DNA was again precipitated by adding 8 µl of 4 *M* NaCl and 40 µl of 13% PEG_{8000}, incubating on ice for 30 minutes, and centrifuging for 20 minutes at 4°C. The supernatant was removed, the pellet of purified plasmid DNA was rinsed with 70% ethanol and resuspended in 23 µl 0.1X TE[†], and ethanol traces were evaporated by vacuum centrifugation.

In some cases, plasmid preparation was performed using an affinity column DNA purification kit. Bacteria were lysed under alkaline conditions, then returned to normal pH, and the plasmid-bearing supernatant was removed from the precipitate of cellular debris and added to an affinity column containing an anion-exchange resin (manufactured by Qiagen). RNA, proteins, and other low molecular weight contaminants were separated from DNA using different molarity salt solution to wash the column. DNA was precipitated from solution by adding 0.7 volumes of isopropanol and centrifuging at 15,000xg for 30 minutes. The supernatant was discarded and the pellet washed with 70% ethanol and resuspended in 0.1X TE.

Subcloning of DNA fragments between plasmids

DNA fragments were sometimes transferred from a source plasmid into a destination plasmid. Fragments were excised from the source plasmid by restriction enzyme digestion using at least 5 units of enzyme per microgram of

plasmid. Restriction enzyme digestion was carried as per manufacturer instructions, usually in a 1X buffer (supplied by the manufacturer) at 37°C for 2-3 hours. All subcloning performed in this research made use of two different restriction enzyme sites on either side of the excised fragment. The entire digestion reaction was then electrophoresed in a 1% agarose gel at a low voltage overnight. The gel was stained in 1.5 μg/ml ethidium bromide and visualized on a UV lamp. The band containing the desired fragment was excised from the gel, and a 2.5% low-melting-temperature agarose containing 0.2-0.4 μg/ml ethidium bromide was cast around the excised piece of 1% agarose. The DNA was electrophoresed out of the 1% agarose in the low-melting-point agarose, and the new DNA band excised from the gel. The DNA was isolated from the agarose using a Wizard DNA Purification kit (Promega). The gel fragment was melted at 70°C, then 1 ml of Wizard Prep resin was added and mixed gently for 20 seconds. The slurry was pushed through a Wizard Minicolumn with a syringe, washed with 2 ml of 80% isopropanol, and the DNA eluted with 50 μl 1X TE. The restriction enzyme DNA fragment was stored at -20°C until ready for ligation.

The destination plasmid was prepared by digesting with one of the two restriction enzymes. Complete linearization was verified by agarose gel electrophoresis. The DNA was then separated from the restriction enzyme by adding an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), vortexing for 30 seconds, and centrifuging at 15,000 x g for 5 minutes. The top, aqueous layer was transferred to a clean tube and 0.1 volumes of 3 *M* sodium acetate (pH 5.2) and 2.5 volumes of 95% ethanol were added. The DNA was precipitated overnight at -20°C, then pelleted for 15 minutes at 15,000 x g and 0°C. The pellet was washed with 70% ethanol, and redissolved in 0.1X TE. The DNA was then similarly digested with the second restriction enzyme and purified by

phenol/chloroform extraction follow by ethanol precipitation. Finally, the 5' terminal phosphate groups were removed from the linearized destination plasmid to prevent self-ligation. A reaction of the DNA, 1X dephosphorylation buffer, and 1 U calf alkaline phosphatase was incubated at 37°C for 30 minutes. A solution of 0.2 *M* EDTA was added to final concentration of 5 m*M* and the mixture incubated at 65°C for one hour. The reaction was allowed to cool, and the DNA was extracted with an equal volume of phenol. The top phase was transferred to a clean tube and the DNA again extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The top, aqueous phase was transferred to a clean tube, and the DNA was precipitated overnight with sodium acetate and ethanol, washed with 70% ethanol, and resuspended in 1X TE.

The destination plasmid was then ligated with the excised DNA fragment. A 20 µl solution with 15-30 fmol of the destination plasmid, 45-90 fmol of the DNA fragment, 1X ligase buffer (supplied by manufacturer), and 1 U of T4 DNA ligase was incubated overnight at 14°C. One microliter of 0.5 *M* EDTA was added to inactivate the ligase, and the DNA was used to transform competent *E. coli*. A ligation reaction without the DNA fragment was used as a control to test for excessive self-ligation of the destination plasmid. Transformed bacteria were tested for the DNA fragment by colony blot hybridization, or if the amount of self-ligation was very low, plasmid was immediately isolated from colonies and tested by restriction enzyme digestion/agarose gel electrophoresis.

Southern blot hybridization

For a more sequence-specific analysis, DNA electrophoresed through an agarose gel was often transferred to a nylon membrane. The gel was agitated for 30 minutes in Southern I Buffer[†] twice. The DNA was then blotted from the gel onto

a Hybond-N nylon membrane overnight using Alkaline Transfer Buffer† as the wicking solution. The membrane was air dried, and its DNA crosslinked for 5 minutes on a UV lamp. Membranes were hybridized with radioactive DNA probes.

DNA probes labeled with ^{32}P were made by random primed synthesis. Briefly, a 20 µl reaction mixture with 20 ng of template DNA, 1X hexanucleotide reaction buffer (Boehringer-Mannheim), 25 µ*M* each of dATP, dCTP, and dGTP, 20 µCi α-^{32}P-dTTP (200-800 Ci/mmol), and 0.5 U Klenow DNA polymerase was incubated at 37°C for 30 minutes. Then, 78 µl of 1X TE and 2 µl of 0.2 *M* EDTA were added, and 1 µl of the mixture was dotted onto each of two DE81 filters. The probe was incubated in boiling water for five to ten minutes and iced for five to ten minutes. The percent incorporation of the radioactive dTTP into the probe was determined by washing one of the two DE81 filters three times for five minutes in 0.35 *M* Na_2HPO_4 buffer, and dividing the radioactive counts per minute (cpm) of the washed filter by the unwashed filter. No probes with percent incorporations below 60% were used, and thus all probes had a specific activity of at least 5×10^8 dpm/µg. Before addition of the probe, DNA membranes were prehybridized for at least one hour at 65°C in 50 ml of DNA pre-hybridization solution. DNA pre-hybridization solution consisted of 3X SSPE, 5X Denhardt's reagent†, 0.1% SDS, and 100 µg/ml denatured, sheared salmon sperm DNA. After pre-hybridization, the radiolabeled DNA probe was added to 5-10 ml of the pre-hybridization solution, the remainder of the pre-hybridization solution was discarded, and the DNA membrane was incubated in the radioactive solution overnight at 65°C. After the overnight hybridization, the membrane was rinsed three times in 2X SSPE + 0.1% SDS, and incubated for thirty minutes at 65°C in 2X SSPE + 0.1% SDS. Another set of three rinses and thirty minute incubation was performed in 0.1X SSPE + 0.1% SDS. Finally, the membrane was rinsed two times in 2X SSPE + 0.1% SDS and exposed

Kodak X-Omat X-ray film for various lengths of time to generate autoradiographs of the DNA.

A radioactive probe was sometimes stripped from a membrane so that the membrane could be hybridized with a different probe. This involved pouring boiling 0.1% SDS over the membrane, and letting it cool to room temperature. Two or three SDS treatments were usually sufficient to remove all radioactivity from membranes.

Bacterial colony blot hybridization

An agar plate of bacterial colonies was sometimes blotted onto nylon filters in order to screen the colonies containing specific DNA sequences. A circular Hybond-N nylon membrane was laid on each agar plate, then removed. Membranes were laid upon Whatman paper soaked with 0.5 *M* NaOH for 5 minutes, air dried for 5 minutes, and again laid on 0.5 *M* NaOH Whatman paper for 5 minutes. Membranes were rinsed three times for 5 minutes in 2X SSPE† + 0.2% SDS, air dried, and their DNA crosslinked for 5 minutes on a UV lamp. Membranes were hybridized with radioactive DNA probes in the same manner as in Southern blot hybridization.

Calcium phosphate transfection of cultured cells with DNA plasmids

Cells were passed the day before transfection to give 30-80% confluency. The media was changed about an hour before DNA was added. A 250 µl reaction mixture of DNA (up to 15 µg) and 250 m*M* $CaCl_2$ was added dropwise to 250 µl 2X HBS† with low speed vortexing. The mixture was allowed to incubate for 15-20 minutes until a fine white precipitate was visible in suspension. The DNA

precipitate was added dropwise to the cultured cells, and allowed to incubate for three hours at 37°C and 7% CO_2. The media was then removed, the cells washed with HBSS + 2% FBS, and 15% glycerol in HBS was added to for 2 minutes. The HBS-glycerol was removed, the cells washed once more in HBSS + 2% FBS, and cells were refed with fresh media.

Analysis of Gene Delivery and Expression

Isolation of RNA from cultured cells

RNA was extracted from cells using a kit from Stratagene. Approximately 1 ml of a guanidinium thiocyanate/ß-mercaptoethanol solution was added per 75 cm^2 of growth area for thirty seconds with rocking to dissolve cells and inactivate RNases. To 10 ml of dissolved cells, 1 ml of 2 *M* sodium acetate (pH 4.0) was added and gently mixed, 10 ml of water-saturated phenol (pH 5.5) was added and gently mixed, and 2 ml of chloroform:isoamyl alcohol (49:1) was added and vigorously mixed. The solution was incubated on ice for 15 minutes, then centrifuged at 10,000 x g for 20 minutes at 4°C. The upper aqueous phase was transferred to a clean tube, mixed with an equal volume of isopropanol, the RNA precipitated at -20°C for at least one hour, and again centrifuged at 10,000 x g for 20 minutes at 4°C. The supernatant was discarded and the RNA pellet dissolved in 3 ml of guanidinium thiocyanate/ß-mercaptoethanol solution. Three ml of isopropanol were added, and the RNA was again precipitated at -20°C for at least one hour. The RNA was pelleted at 10,000 x g for 10 minutes at 4°C and the pellet washed with 70% ethanol. The pellet was dissolved in 60 µl of DEPC $H_2O^{\dagger}$ and stored at -70°C.

Northern blot hybridization

RNA to be analyzed by Northern blotting was mixed with an equal volume of Ambion's Gel Loading Buffer II† and denatured at 80°C for 5 minutes. The denatured RNA was electrophoresed in a 1.2% agarose/formaldehyde gel† at 50 V (5 V/cm) for about 3 hours. The gel was agitated for 10 minutes in DEPC H_2O twice, and the RNA visualized on a UV lamp. The gel was agitated for 20 minutes in 0.05 *N* NaOH, rinsed briefly in DEPC H_2O twice, and agitated for 45 minutes in 20X SSC†. RNA was blotted from the gel to a Hybond-N nylon membrane overnight with 20X SSC as the wicking solution. The membrane was air dried, and its RNA crosslinked for 5 minutes on a UV lamp. Membranes were hybridized with radioactive DNA probes.

Hybridization of radiolabeled DNA probes to RNA membranes was almost identical to Southern blot hybridization. The DNA probe was made with 100 ng of template DNA rather than 50 ng, and after addition of 1X TE and EDTA, radiolabeled probes were centrifuged through G-50 Sephadex columns (Boehringer-Mannheim) to remove unincorporated nucleotides. After centrifugation, the DNA probe was boiled five to ten minutes, iced five to ten minutes, then added to 5-10 ml of pre-hybridization solution. RNA pre-hybridization solution consisted of 6X SSPE, 2X Denhardt's reagent, 0.1% SDS, and 100 µg/ml denatured, sheared salmon sperm DNA. The remainder of the hybridization procedure was identical.

Dot blot hybridization

RNA was applied directly to nylon membranes using a dot blotting apparatus. The holes in the apparatus were 3-4 mm in diameter. An RNA sample (or a DNA control) was diluted to 15 µl, and 30 µl formamide, 12 µl 37% formaldehyde, and 3

µl 20X SSC were added. The RNA was denatured at 68°C for 15 minutes and incubated on ice for 5 minutes. Then, 120 µl of 20X SSC were added, and samples were kept on ice until added to the dot blot apparatus. A Hybond-N nylon membrane pre-wet with 10X SSC was placed in the dot blot apparatus and samples were added into the wells of the apparatus. Samples were drawn onto the membranes by applying a vacuum to the apparatus, and wells were washed three times with 10X SSC. The vacuum was allowed to dry the membrane for 5 minutes, then the apparatus was disassembled and the membrane allowed to air dry. The nucleic acid on the membrane was crosslinked for 5 minutes on a UV lamp. Membranes were hybridized with radioactive DNA probes as with Northern blot hybridization.

Western blot hybridization

Protein from cultured cells was sometimes analyzed by Western blotting. Media was removed from cells, and cells were incubated several minutes in STV or PBS containing 5 m*M* EDTA until cells came loose from the culture flask. The cell suspension was centrifuged for 2 minutes at 14,000 rpm, and the supernatant removed. The cell pellet was dissolved in 2X Western sample buffer[†], and the solution was pipetted repeatedly to break up the viscous chromosomal DNA. Dissolved cell pellets were stored at -20°C until they were used.

When all samples were ready, a 12% polyacrylamide/SDS running gel was made by making a solution of 375 m*M* Tris-HCl, pH 8.8, 0.1% SDS, and 12% acrylamide/bis. The solution was degassed under vacuum with stirring for 15 minutes, and 5×10^{-3} parts 10% APS and 5×10^{-4} parts TEMED were added. The acrylamide solution was poured between two glass plates, overlaid with a ribbon of water, and allowed to polymerize. The running gel was overlaid with a 4%

polyacrylamide/SDS stacking gel. A solution of 125 m*M* Tris-HCl, pH 6.8, 0.1% SDS, and 4% acrylamide/bis was degassed under vacuum with stirring for 15 minutes, and 5 x 10^{-3} parts of 10% APS and 1 x 10^{-3} parts TEMED were added. The 4% acrylamide solution was overlaid on the 12% gel, a well comb was added, and the 4% gel was allowed to polymerize. The gel apparatus was placed in Western running buffer†. Samples were heated to 95°C for 10 minutes and loaded into the wells of the polyacrylamide gel. Gels were electrophoresed at 200 volts for 45 minutes.

After electrophoresis, gels were removed from the apparatus and soaked for 15 minutes in Western transfer buffer† with shaking. A PVDF membrane was prepared by soaking 30 seconds in 100% methanol, then 10 minutes in Western transfer buffer. Proteins were electrophoresed from each polyacrylamide gel onto a PVDF membrane at 100 volts for one-and-a-half to two hours with a cooling bar. Gels were stained with 0.1% Coomassie Brilliant Blue R250 in 40% methanol and 10% acetic acid overnight to verify that proteins ran normally during the first electrophoresis. PVDF membranes were washed three times for 5 minutes in TTBS†, and soaked overnight with 5% dehydrated milk in TTBS.

Membranes were washed three times for five minutes in TTBS, incubated for one to two hours in the primary antibody, and again washed three times in TTBS for five minutes. The PVDF membranes were then incubated in a TTBS solution containing 60 nCi/ml ^{125}I-labeled Protein G (8-12 mCi/mg) for one to two hours, then washed three times for five minutes in TTBS, and exposed Kodak X-Omat X-ray film for various lengths of time to generate autoradiographs of the protein.

RESULTS

Identification of Potential Target Cells

Our first step in developing a CTL assay was choice of a target cell. Three basic qualities necessary in a target cell for a bovine CTL assay using retroviral gene delivery are the ability to isolate the cell without killing the animal, a relatively high level of class I MHC expression, and susceptibility to retroviral gene delivery. We examined primary cell cultures from a variety of tissues in cattle. The class I MHC expression and susceptibility to retroviral gene delivery was compared among different cultures. An adherent cell culture derived from peripheral blood (PBAC) exhibited the best combination of these two characteristics, and were further characterized to give an indication of their origin.

Characterization of primary cells

A number of different primary bovine cell cultures were isolated by Lawrence Elsken's lab at NVSL, Janice Miller's lab at NADC, and by our lab, and these cell cultures were chosen as candidate cells for use in a CTL assay. Initially, cells were examined for morphology and growth rate (Table 1). Primary cell cultures are usually a mixture of different cell types, but the cell morphologies that predominated in each culture were compared with the morphologies of known cell types. When confluent cell cultures are viewed by phase microscopy, a cobblestone pattern is indicative of an epithelial or endothelial origin, whereas bipolar arrays of cells which appear to form whorls when viewed with the naked eye are typical of fibroblasts (29). The BOTONG, BOTUR, BOSK, and FBL had a clear fibroblastic morphology. They formed tightly packed bipolar arrays when

Table 1. Primary cell cultures

Cell name	Tissue source	Morphology/ Growth Rate	Likely cell type
BOSK	Fetal bovine skin	Bipolar arrays, macroscopic whorls High growth rate	fibroblast
BOTONG	Fetal bovine tongue	Bipolar arrays, macroscopic whorls High growth rate	fibroblast
BOTUR	Fetal bovine turbinate	Bipolar arrays, macroscopic whorls High growth rate	fibroblast
FBL	Fetal bovine lung (outer membrane)	Bipolar arrays, macroscopic whorls High growth rate	fibroblast
BOTEST	Fetal bovine testicle	Bipolar arrays, some large and irregular High growth rate	fibroblast, epithelial, endothelial
EBKp	Embryonic bovine kidney	Cobblestone Low growth rate	epithelial, endothelial
PBAC 79A	Bovine peripheral blood adherent cell	Large and irregular, some cobblestone Low growth rate	epithelial, endothelial
PBAC 79B	Bovine peripheral blood adherent cell	Cobblestone Moderate growth rate	epithelial, endothelial
PBAC 342	Bovine peripheral blood adherent cell	Bipolar arrays or cobblestone High growth rate	fibroblast, epithelial, endothelial

viewed under phase microscopy, and the whorls formed by cultured fibroblasts were visible to the naked eye. BOTONG, BOTUR, BOSK, and FBL cells all divided quickly in culture. EBKp did not often grow into tightly packed cell cultures, but usually left space between cells at confluence. Where cell-cell contact occurred, EBKp formed cobblestone patterns, indicating an epithelial or endothelial origin. EBKp were very regular in size and were round with a centered nucleus, much like a fried egg, and grew more slowly in culture. BOTEST cells had a more heterologous morphology and formed both bipolar arrays and arrays of a loose cobblestone pattern with very large irregularly shaped cells interspersed in the culture. Whorls were not visible in BOTEST cultures, and so they somewhat resembled fibroblasts and epithelial and endothelial cells. PBAC 79B cells had a cobblestone appearance at confluence and divided quickly, thus appearing to be epithelial or endothelial. PBAC 79A were much larger cells which did not pack tightly at confluence, were rather irregularly shaped, and divided more slowly. They occasionally formed cobblestone arrays at confluence, and tentatively appeared to be epithelial or endothelial. PBAC 342 divided quickly and formed both cobblestone and bipolar arrays at confluence, but no macroscopic whorls were visible, so PBAC 342 could also have been fibroblasts, epithelial or endothelial cells.

Morphologies give only a partial indication of cell type, whereas biochemical indicators can provide much more certainty in cell type determinations. To better determine cell type, the different cell cultures were tested for expression of the intermediate filaments, vimentin and cytokeratin. Vimentin has been shown to be present in cells of mesenchymal origin such as fibroblasts, macrophages, and endothelial cells (22,28,42,65), whereas cytokeratin has been shown to be confined to epithelial cells (27,42,80). MDBK are a bovine epithelial cell line, and

HeLa are a human epithelial line. Both cell lines should contain cytokeratin but not vimentin. EJG and CPAE on the other hand are bovine endothelial cells, and should contain vimentin but not cytokeratin. ICC was performed for each intermediate filament, then the number of stained and unstained cells were counted out of at least 500 cells, and the percent positive was calculated. The results are summarized in Table 2.

Table 2. Cytokeratin and vimentin staining

Cell name	Cell type	Percentage of Cells Stained Positive		Deduced cell type
		Cytokeratin	Vimentin	
HeLa	epithelial	40	100	N/A[a]
MDBK	epithelial	2	100	N/A
EJG	endothelial	0	100	N/A
CPAE	endothelial	0	100	N/A
BOMAC	macrophage	100	100	N/A
BOSK	primary	0	100	fibroblast
BOTONG	primary	0	100	fibroblast
BOTUR	primary	0	100	fibroblast
FBL	primary	0	100	fibroblast
BOTEST	primary	2	100	epithelial
EBKp	primary	1	100	epithelial
PBAC 79A	primary	0	100	unknown
PBAC 79B	primary	0	100	unknown
PBAC 342	primary	0	100	unknown

[a]N/A = not applicable

Surprisingly, every cell line stained positive for vimentin, including the non-mesenchymal MDBK and HeLa cells. The studies which found vimentin only in mesenchymal cells were performed mostly with *in situ* staining of tissue samples, so it is possible that expression of vimentin was gained by *in vitro* culture of the

epithelial cells. In any case, vimentin's presence in all of the primary cell cultures was not highly meaningful. Of the primary cell cultures, only BOTEST and EBKp reacted with the cytokeratin antibody. The percent of cells positive for cytokeratin was low, but comparable to that found in the bovine epithelial cell line, MDBK. Thus, BOTEST and EBKp cultures contained epithelial cells. The lack of cytokeratin in the BOTONG, BOTUR, BOSK, and FBL cultures supported the morphologic evidence that they contained predominantly fibroblasts. The lack of cytokeratin staining ruled out the presence of epithelial cells in the PBAC cultures. The PBAC were isolated from blood collected by venipuncture, and since they all formed cobblestone arrays to some degree, an endothelial origin seemed likely, but not enough information was available to make that conclusion with much certainty.

Class I MHC expression

The different cell cultures were analyzed by FACS analysis for their relative levels of class I MHC expression. A typical histogram set generated by FACS analysis of class I MHC expression is shown in Figure 2. Expression of class I MHC was demonstrated by the shift of the shaded histogram (MHC-labeled cells) to the right of the unshaded one (unlabeled cells), and for the histogram set shown in Figure 2, both EBKp and PBAC 79A expressed class I MHC. The relative level of MHC fluorescence of each group of cells was calculated by subtracting the mean fluorescence of the unlabeled cells from the mean fluorescence of the labeled cells (Figure 3). Although the two sets of data yielded slightly different absolute numbers for most of the cell cultures, the relative relationships between the different cell cultures remained largely identical.

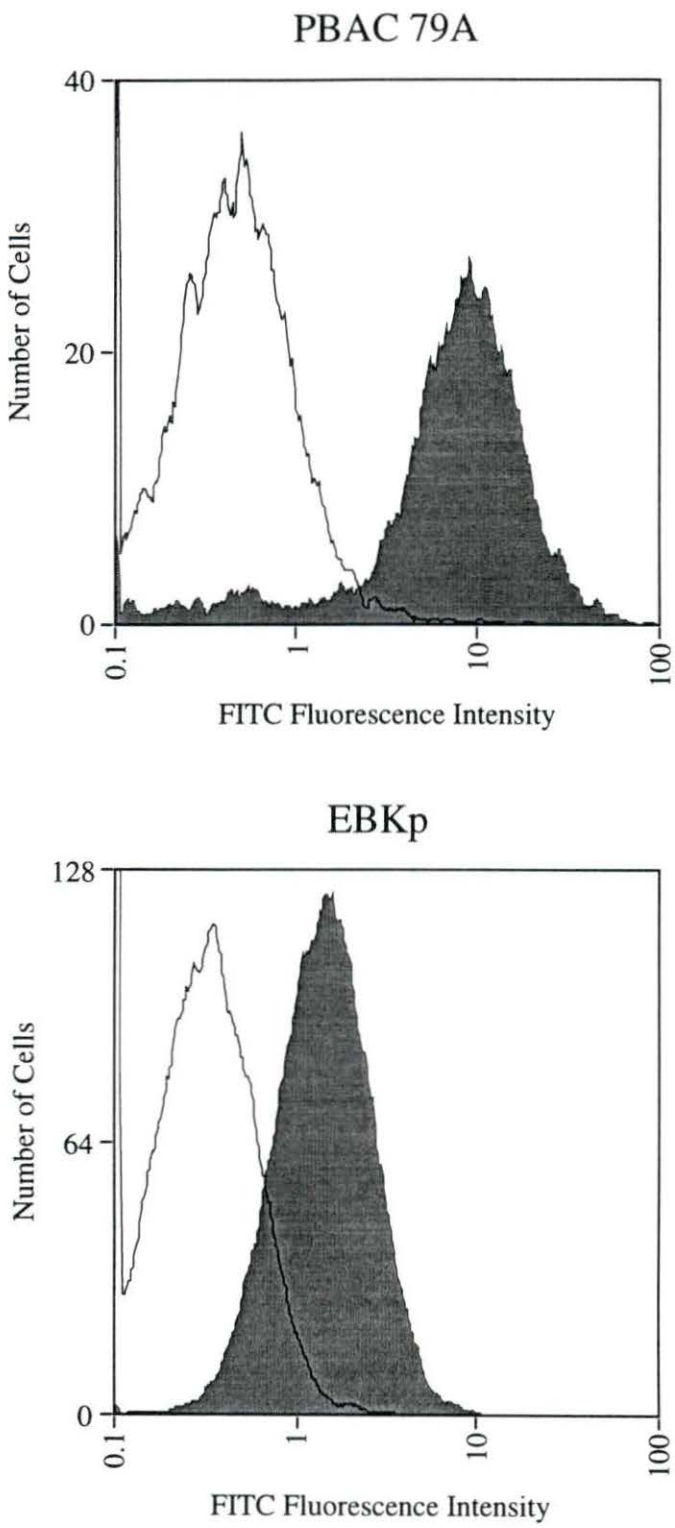

Figure 2. FACS histograms of class I MHC expression. Cells were labeled with an anti-class I MHC antibody and a secondary fluorescent antibody (shaded histogram) or the secondary antibody alone (unshaded histogram).

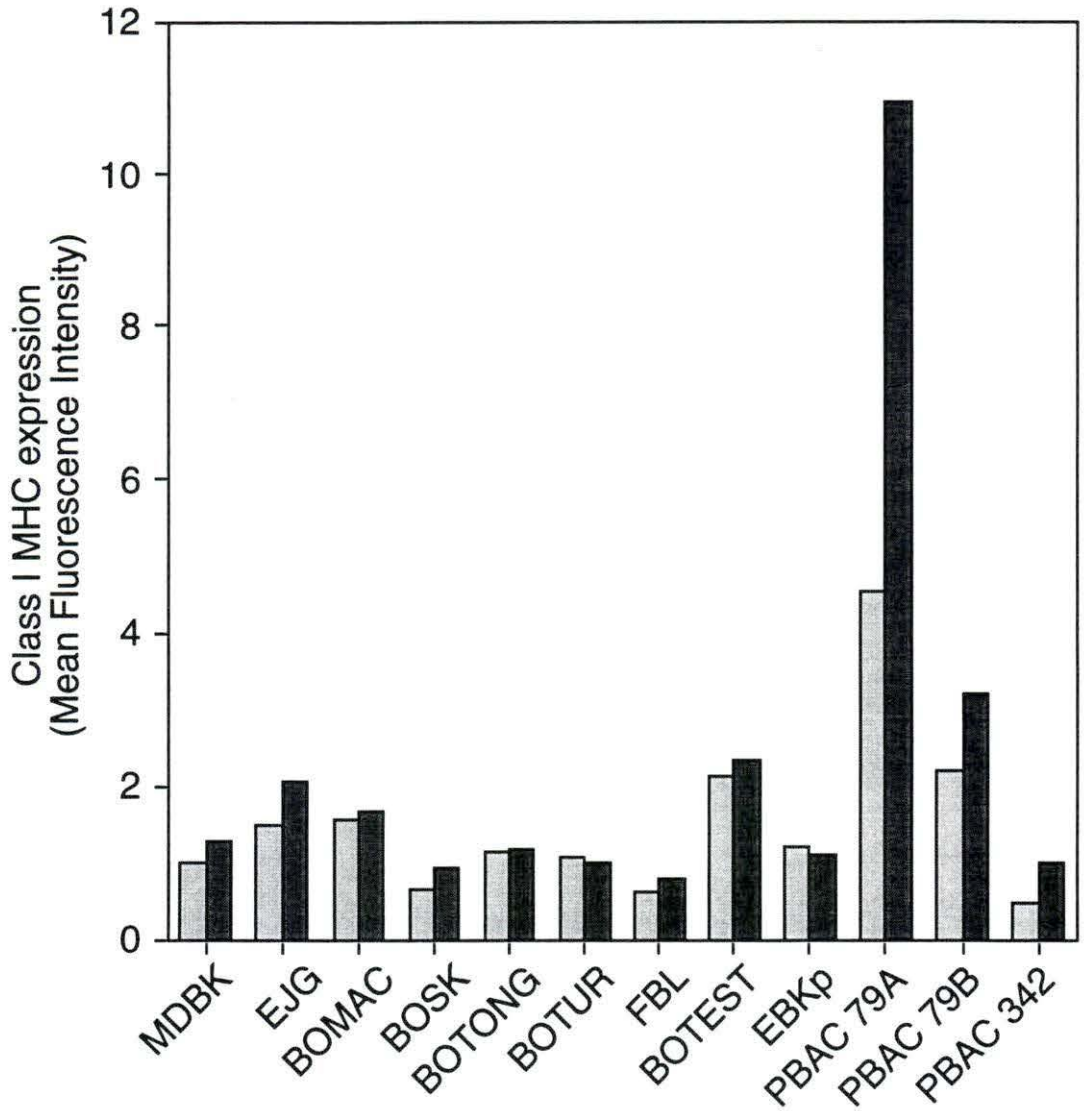

Figure 3. Level of class I MHC expression. Class I MHC expression was calculated as the mean fluorescence of cells analyzed by FACS analysis with an antibody to class I MHC minus the mean fluorescence of cells labeled with the secondary antibody only. Two FACS analyses of all cells were performed, and the data for each analysis is shown in a different shade of gray.

Of the primary cell cultures, BOSK, BOTONG, BOTUR, FBL all had a relatively low level of class I MHC expression. These cell all bore a strong morphological resemblance to fibroblasts, and this data is consistent with previous work showing that bovine fibroblasts have a rather low level of class I MHC and do not function well as a CTL target cells (14). The EBKp also had a relatively low level of class I MHC expression, whereas the BOTEST had a high level. Thus the presence of epithelial cells in a cell culture did not necessarily correlate with higher or lower levels of class I MHC expression.

The level of class I MHC expression varied widely among the PBAC isolates. PBAC 79A expressed class I MHC far higher than any other cell type tested in this study; PBAC 79B had the second highest level of expression, though it did not exceed the other cell cultures by such a large amount; and PBAC 342 had one of the lowest relative levels seen in this study. Therefore, the differences seen in morphology and growth rate of the PBAC cultures extended to the biochemical level and indicated that they may not have been the same cell type. The higher levels of expression seen in BOTEST, PBAC 79A, and PBAC 79B made them the promising candidates as CTL target cells, based simply on class I MHC expression.

Susceptibility to retroviral gene delivery

For cells to function in a CTL assay using retroviral gene delivery, they need to be highly susceptible to infection by the retroviral vectors. So in addition to examining class I MHC expression, potential target cells were evaluated for their susceptibility to retroviral gene delivery (Figure 4). All cells were susceptible to infection with retroviral vectors produced by PG13 vector-producer cells, in accordance with previous results for bovine cells (56), but infection titers varied by several orders of magnitude. Cells with titers between 10^3 and 10^4 would be

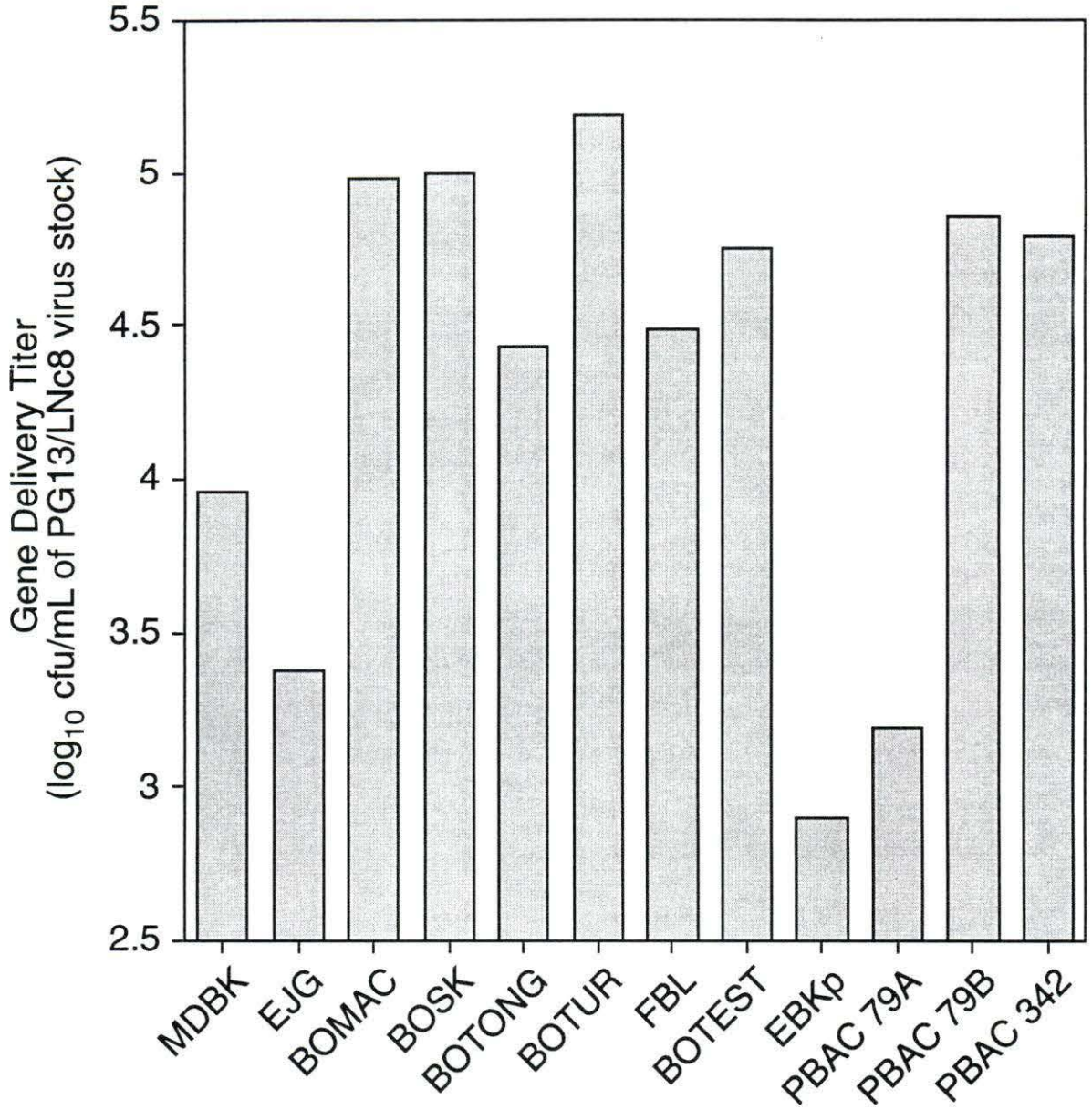

Figure 4. Susceptibility to retroviral gene delivery. Gene delivery was measured in duplicate as the number of neomycin-resistant colonies after infection with 10-fold serial dilutions of PG13/LNc8 retroviral vector. Numbers shown are the average of duplicate experiments. Individual titers varied by less than 0.5 logs from the mean in all cells.

minimally suitable as target cells with a 1 to 10% infection rate, and cells with titers higher than 10^4 would be much more suitable with infection rates above 10%. Titers below 10^3 indicated that cells would be very unsuitable for a CTL assay using retroviral gene delivery.

All the fibroblastic cells, BOSK, BOTONG, BOTUR, and FBL, had titers well above 10^4, with BOSK and BOTUR showing titers of approximately 10^5, indicating that nearly 100% of the cells were being infected. The titer of slightly above 10^5 in BOTUR is probably because these cells divide very quickly and grew to more than 10^5 cells per well in the first few hours of the infection. The EBKp had a titer below 10^3, whereas the BOTEST showed a titer of above $10^{4.5}$, again demonstrating that although both cultures contained epithelial cells, their suitability as target cells was very different.

The susceptibility of the PBAC isolates also varied immensely. With a titer of about $10^{3.2}$, only about 15% of PBAC 79A cells were infected by the retroviral vector. The PBAC 79B and PBAC 342 both were highly susceptible to infection with titers above $10^{4.5}$. Interestingly, the susceptibility to retroviral gene delivery correlated with the speed at which the cell cultures grew (Table 1), which is reasonable since simple retroviruses only infect dividing cells. The correlation is useful because it implies that growth rate can provide a method for approximating the titer which can be expected from different cell isolates.

PBAC are most suitable

To be useful as target cells, isolates need to have both high class I MHC and high susceptibility to retroviral gene delivery. Examining both criteria simultaneously (Figure 5) gave an indication of the target cell potential of each of the primary cell cultures. EBKp cells had a low level of class I MHC expression and

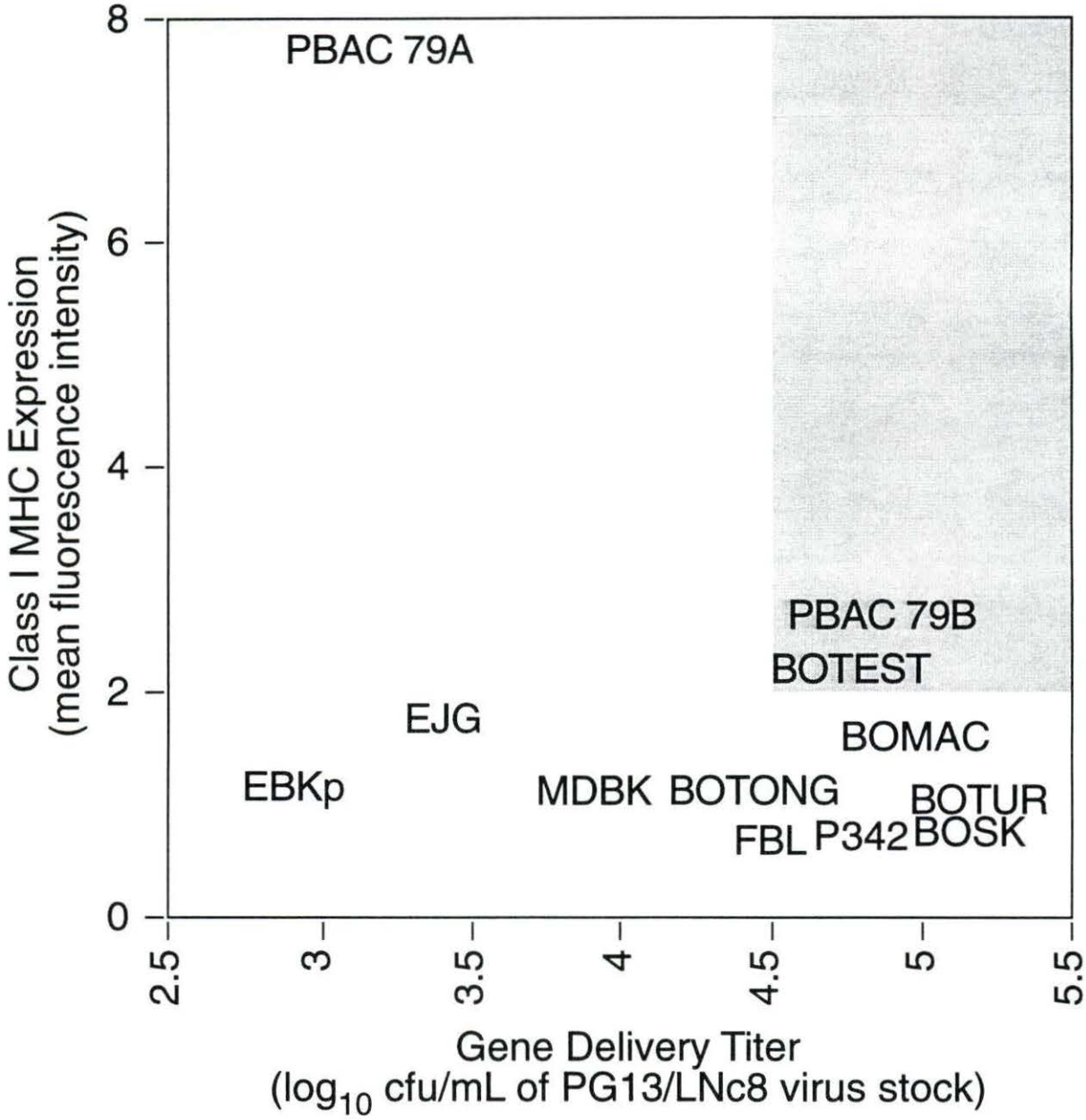

Figure 5. Target cell potential. This figure contains data from both Figure 3 and Figure 4. The name of each cell type is placed on the graph at its corresponding position for class I MHC expression and gene deliviery titer. P342 refers to PBAC 342 cells. The shaded region highlights the cells with the high combination of class I MHC expression and susceptibility to retroviral gene delivery. The highlighted cells are those most suitable as CTL target cells.

a low gene delivery titer and would be quite unsuitable as CTL target cells. Though PBAC 79A expressed a high level of class I MHC, they were not very susceptible to retroviral gene delivery, and thus would not make very good CTL target cells. Conversely, BOSK, BOTONG, BOTUR, FBL, and PBAC 342 all had high gene delivery titers, but low class I MHC expression; they would also make poor CTL target cells. Peripheral blood isolate 79B (PBAC 79B) and bovine testes cells (BOTEST) had the highest combination of class I MHC expression and gene delivery titer, and they would thus be most useful as potential target cells in a CTL assay using retroviral gene delivery.

We chose to pursue further study with the PBAC cells rather than the BOTEST cells for several reasons. Isolation of PBAC requires far less surgical manipulation of the animal donor than do testes cell isolations, a CTL assay using testes cells would restrict researchers to studies of male cattle, and previous results indicate that bovine testes cells do not function well as target cells (14).

PBAC cell type characterization

Different PBAC isolates demonstrated noticeable differences in morphology, growth rates, susceptibility to retroviral gene delivery, and class I MHC expression (Tables 1 and 2, Figures 3 through 5). The cells were isolated as overgrowths of PBMC cultures, and there was some uncertainty about their origin. So, further characterizations were performed on the different PBAC isolates to try to provide guidelines as to which PBAC phenotype correlated with high potential as CTL target cells.

None of the PBAC isolates contained epithelial cells, as evidenced by the absence of cytokeratin. They were isolated by collecting peripheral blood mononuclear cells (PBMC) as buffy coats from peripheral blood and selecting for

adherent cells. Because buffy coats contain primarily white blood cells, all PBAC isolates were tested for expression of CD45, a leukocyte marker. Fresh PBMC had very bright staining for CD45 on their surface by IFA, but PBAC showed no staining (data not shown). Thus, PBAC were not leukocytes.

Typical endothelial cell morphology is a cobblestone appearance at confluence. Some of the PBAC exhibited cobblestone morphology (Table 1), so we tested the cells for two biochemical characteristics of endothelial cells: expression of von Willebrand factor and uptake of DiI-Ac-LDL. Von Willebrand factor is produced exclusively by vascular endothelium. Ac-LDL is taken up via the "scavenger cell pathway" present in macrophages and most vascular endothelial cells (45,85). When cells take up the fluorescently tagged form of Ac-LDL, known as DiI-Ac-LDL, the fluorescent DiI tag accumulates in cytoplasmic deposits, causing a punctate fluorescence when viewed under the microscope. Bovine pulmonary artery endothelial cells (CPAE) stained strongly positive for von Willebrand factor by IFA (data not shown), but all PBAC isolates were negative (data not shown), and thus were not vascular endothelial cells. PBAC were incubated with DiI-Ac-LDL and their fluorescence measured by FACS analysis. Though all PBAC isolates took up far less DiI-Ac-LDL than CPAE positive control cells, their levels of uptake differed (Figure 6). PBAC 79B cells took up significantly more DiI-Ac-LDL than the MDBK epithelial cell control or the NIH/3T3 fibroblast control. PBAC 79A and 342 took up more than NIH/3T3 cells, but less than MDBK. Thus, whereas as the vascular endothelial cell control, CPAE, had a high level of DiI-Ac-LDL uptake, PBAC 79B had a moderate level of uptake, and PBAC 79A and 342 had low levels of uptake.

The absence of von Willebrand factor and moderate level of DiI-Ac-LDL uptake shown by PBAC 79B were consistent with the cell characteristics reported for

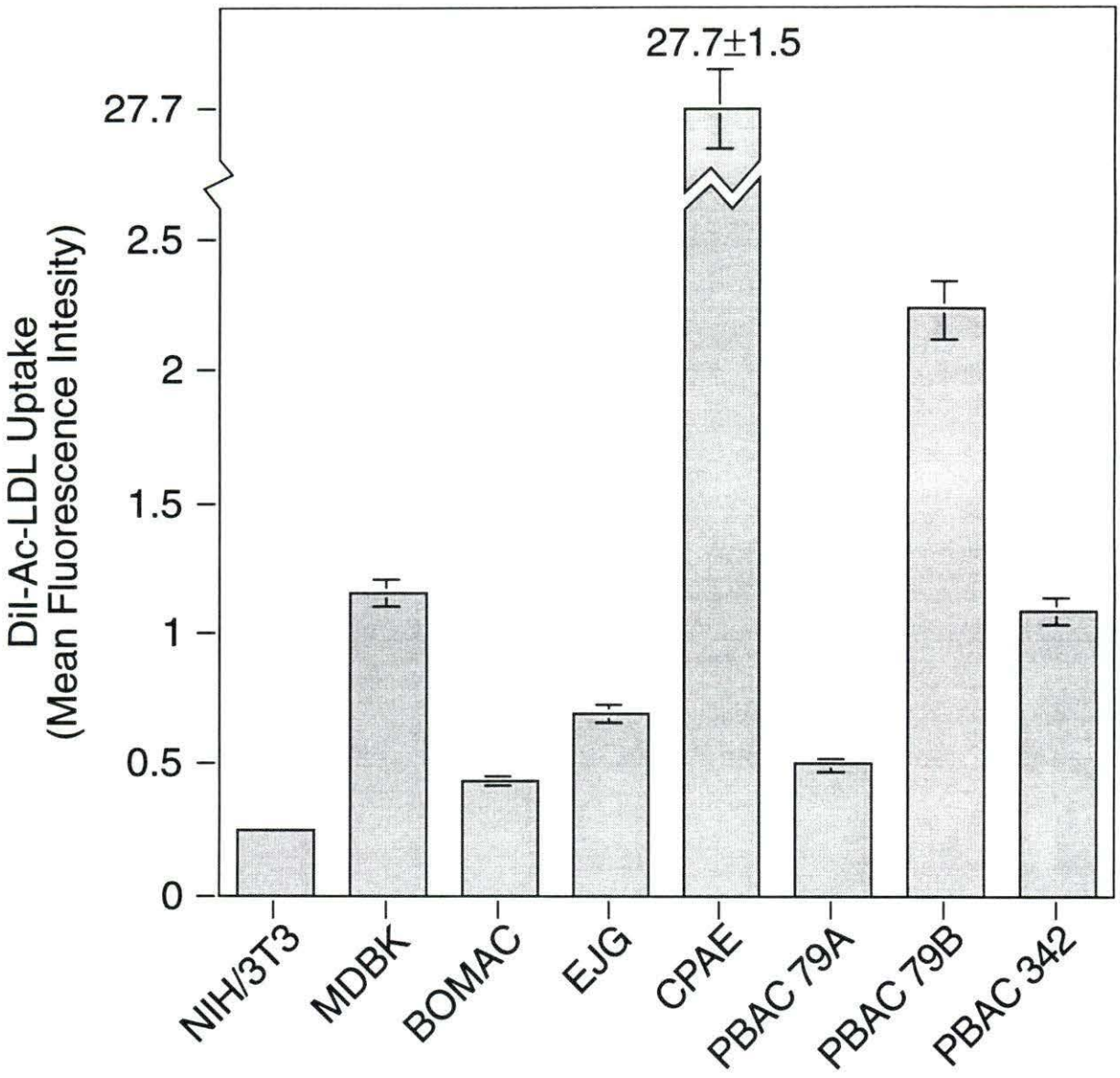

Figure 6. DiI-Ac-LDL uptake. Cells were incubated with normal media containing 5 μg/ml DiI-Ac-LDL, and uptake of the fluorescent compound was measured by FACS analysis. Values shown are the average of triplicate measurements, and error bars indicate standard deviation.

cultured high endothelial cells (45,71). High endothelial cells are involved in lymphocyte trafficking *in vivo*, and freshly isolated lymphocytes bind high endothelial cells *in vitro* (45,71). To test whether the PBAC isolates were high endothelial cells, a lymphocyte binding assay was performed, and the number of lymphocytes bound per cell was calculated (Figure 7). Again, differences were observed among the various PBAC isolates. The level of lymphocyte binding in PBAC 79A and 79B was consistent with reported results for high endothelial cells, but in PBAC 342 lymphocyte binding was at a level much lower than that found in high endothelial cells.

Based on their cobblestone morphology, lack of von Willebrand factor, moderate level of DiI-Ac-LDL uptake, and level of lymphocyte binding, PBAC 79B appear to be high endothelial cells. PBAC 79A was isolated from the same sample of blood as 79B, and they differed from PBAC 79B mostly in their growth rate and level of DiI-Ac-LDL uptake. *In vitro*, vascular endothelial cells can lose the scavenging cell pathway, and their levels of DiI-Ac-LDL uptake will fall. PBAC 79A were probably high endothelial cells, but in course of being grown in culture their growth rate may have slowed and they may have lost their ability to take up moderate levels of DiI-Ac-LDL. PBAC 342, on the other hand, did not bind lymphocytes efficiently, they sometimes had a fibroblastic bipolar morphology (Table 1), and their target cell potential closely resembled that of the other fibroblastic primary cells (Figure 5). The most likely explanation is that the PBAC 342 culture contained mostly fibroblasts. The appearance of sectors of cobblestone morphology may have been due to high endothelial cells which were later overgrown by fibroblasts.

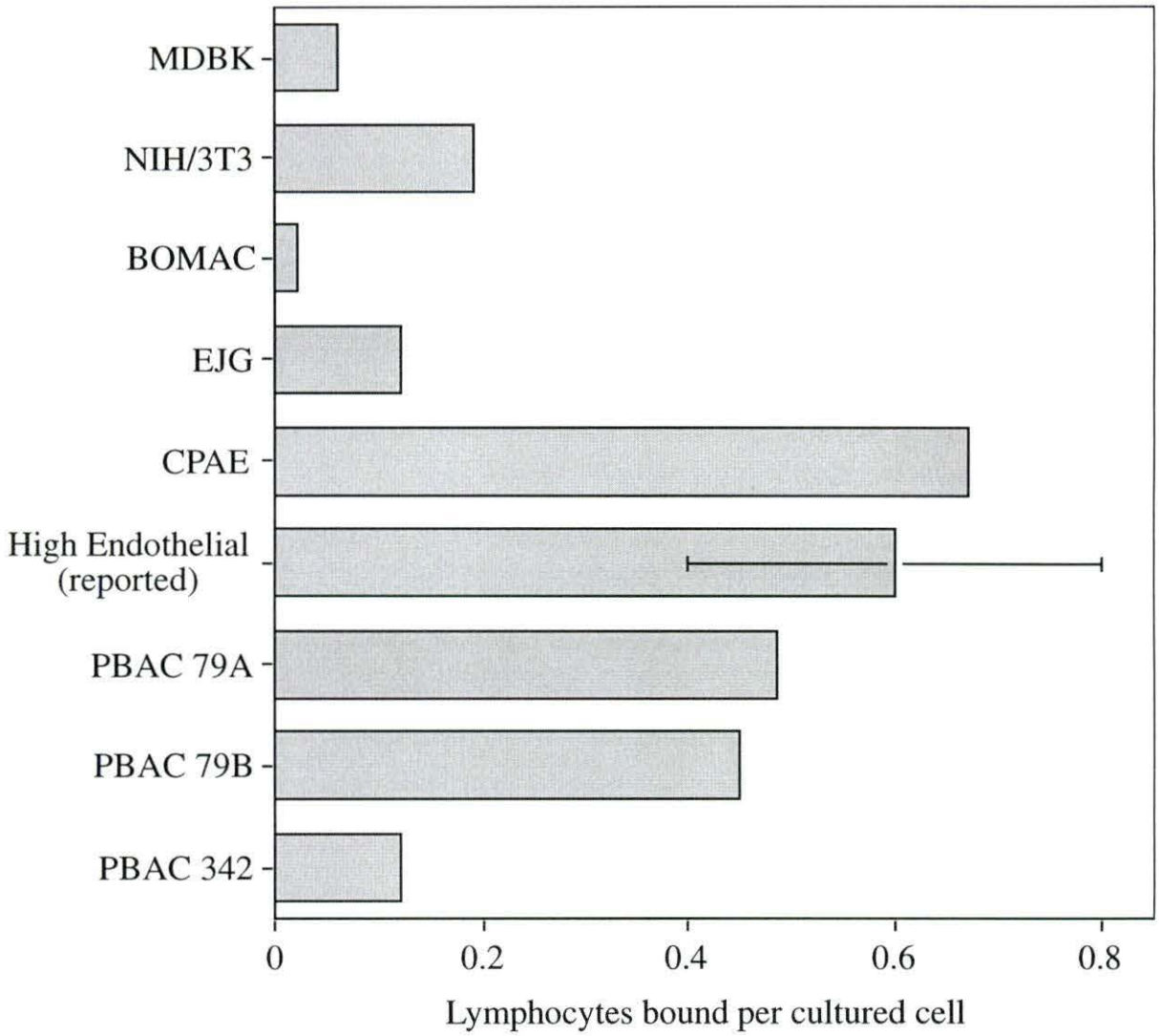

Figure 7. Lymphocyte binding assay. Adherent cells were co-cultured with fresh lymphocytes (PBMC) for 2 hours, non-adherent cells were washed off, and cells were fixed and stained with CD45. The number of lymphocytes per adherent cultured cells was counted, and the ratio is given in the graph. The ratio of lymphocytes which bound high endothelial cells has been previously determined (45), and it is displayed with the reported standard error.

Summary

PBAC 79B cells were the isolate most suitable as a CTL target cell, as determined by high class I MHC expression and a high susceptibility to retroviral gene delivery. They appeared to be high endothelial cells, based on a cobblestone morphology, a lack of von Willebrand factor expression, a moderate level of DiI-Ac-LDL uptake, and a lymphocyte binding greater than 0.4 lymphocytes per cell. High endothelial cells are normally found in lymph nodes, but they have been previously isolated from bovine peripheral blood adherent cultures (71), just as the PBAC were. The less suitable PBAC 79A isolate also probably contained high endothelial cells, but its slow growth rate made it less susceptible to gene delivery, and thus less suitable as a CTL target cell. PBAC 342 cells did not appear to be high endothelial cells and had a low level of class I MHC expression, making them very unsuitable as CTL target cells. In conclusion, high endothelial cells isolated from peripheral blood are the most promising candidates for target cells in a CTL assay which uses retroviral gene delivery.

Production of Retroviral Vectors for Delivery of Bovine Genes

With a target cell chosen, the next step in developing a CTL assay was production of a retroviral vector which could deliver genes cloned from a bovine virus into the target cells. The model bovine virus used in this study was BIV. Production of the retroviral vector involved construction of transfer vector plasmids carrying BIV genes, transfection of packaging cells with the plasmids to make vector producer cells, and collection of retroviral vectors from the supernatant of vector producer cells. Target cells were then infected with the recombinant retroviral vector and tested for expression of the BIV genes.

BIV CA gene delivery and protein production

The CA subunit of the BIV *gag* gene was chosen to be used in transfer vector construction (Figure 8) and gene delivery to target cells for several reasons. Monoclonal and polyclonal antibodies reactive to the CA protein were available. BIV-infected cattle develop humoral immune responses to the CA protein (4), and CTL specific to the FIV CA protein have been detected in FIV-infected cats by a CTL assay which used retroviral gene delivery (78).

LNCX/CA construction and packaging cell assembly. The LNCX/CA transfer vector was made by first PCR amplifying the BIV CA gene from the pBacHisC/gag3 plasmid with primers carrying restriction enzyme sites and then ligating the PCR product into the easily manipulated intermediate plasmid, pCRII. The pBacHisC/gag3 plasmid was used as a source because it had previously been used to produce a baculovirus-gag3 fusion protein which could be detected by Western blot hybridization with the CA-specific monoclonal antibody, indicating a preservation of the open reading frame and the sequence of the monoclonal binding site in the CA gene. Intermediate plasmids containing the correct insert were detected using colony blot hybridization. Bacterial colonies carrying the CA gene were grown in quantity, and the pCRII/CA plasmid was isolated. Plasmids were screened by restriction enzyme digestion and fragment sizes visualized by agarose gel electrophoresis. Intermediate plasmids with the expected banding pattern were sequenced to verify that the BIV gene was intact. The BIV gene was then subcloned into the pLXSN transfer vector using the restriction enzyme sites added by the PCR primers. Correct construction of the transfer vector was also verified using colony blot hybridization, restriction enzyme analysis, and DNA sequencing.

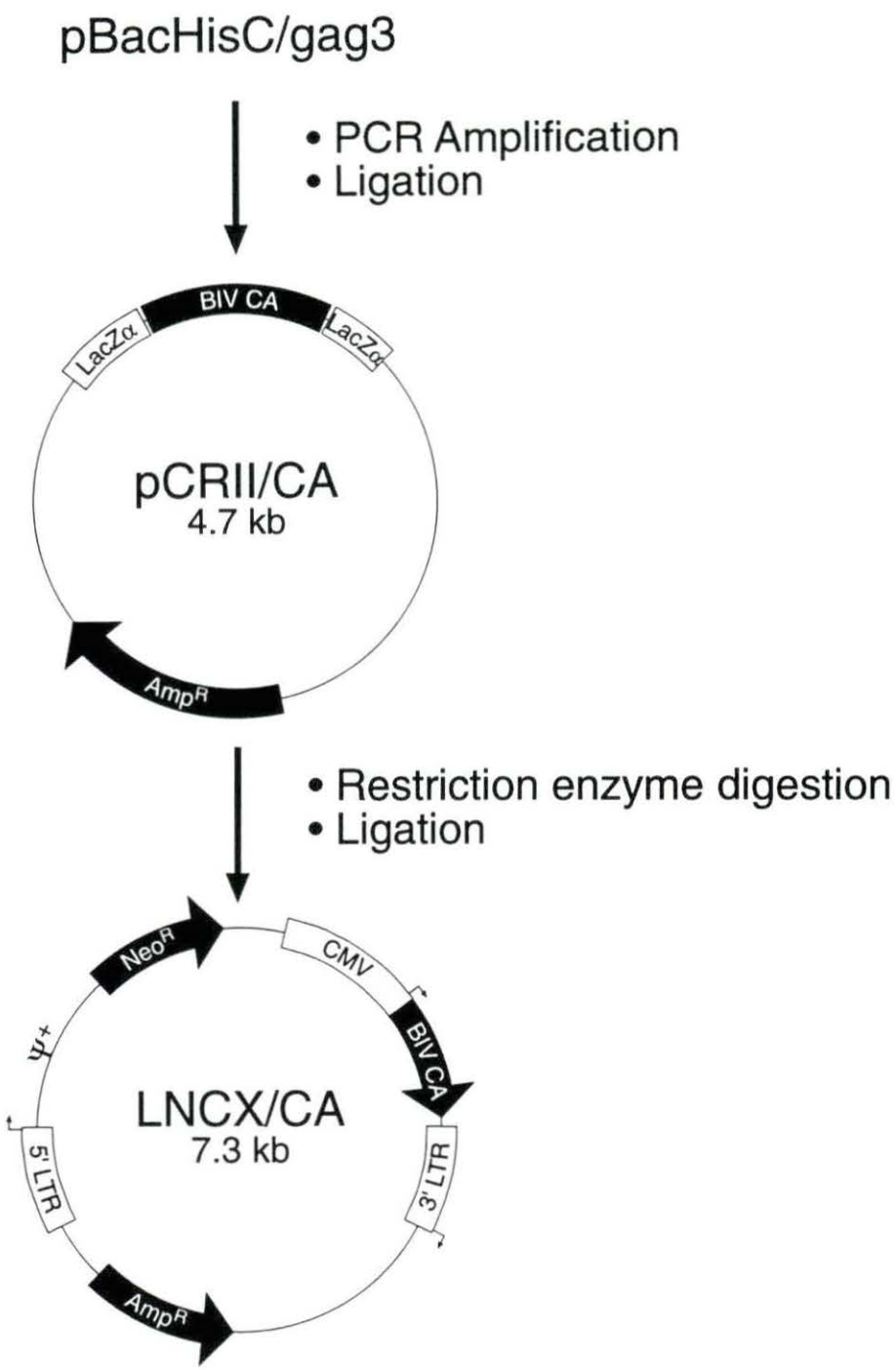

Figure 8. LNCX/CA transfer vector construction. The BIV CA gene segment was amplified from the baculovirus expression plasmid, pBacHisC/gag3, which has been shown to produce BIV CA protein detectable by monoclonal antibody (data not shown), and ligated in the pCRII plasmid. After correct amplification and ligation were verified, the CA gene fragments was excised from the intermediate plasmid by restriction enzyme digestion and ligated into LNCX.

Packaging cell transfection, gene delivery, and testing of expression. The LNCX/CA plasmid was used to transfect PG13 packaging cells, and stable transfectants were selected for neomycin resistance (neo^R) with G418. Neo^R colonies formed, confirming successful transfection of packaging cells. Supernatant from neo^R PG13--LNCX/CA cells presumably contained recombinant CA retroviral vector, and the supernatant was used to infect bovine FBL cells. Upon selection with G418, neo^R colonies formed. These results confirmed both production of retroviral vector and delivery of vector-borne genes into bovine cells. In order to verify that the BIV CA protein was being produced, neo^R cells were fixed with methanol and stained by ICC using a CA-specific monoclonal antibody. Neither PG13--LNCX/CA cells nor the neo^R FBL cells visibly reacted with the antibody, suggesting that no CA protein was being produced after transfection or gene delivery. The absence of detectable protein could have been the result of any of several complications. In packaging cell lines, transfer vectors can undergo recombination to delete the inserted gene, in this case the CA gene. The promoter controlling the inserted gene can also become silenced. Both events block protein production from the inserted gene. When protein expression is blocked in a majority of cells, one way to find the unblocked minority is to examine clonal populations of cells.

Clonal isolation of packaging cells and testing of expression. Clonal populations are the result of a single integration of the transfer vector in the host cell chromosome. Cells with an integration site that has a high probability of recombination or gene silencing will lose protein production within the first few rounds of cell division, and the entire colony will be negative. Cells with a more stable integration event will produce colonies with at least a portion of positive cells. By examining clonally-derived colonies, positive cells are concentrated

together and more easily identified as being above background than they would be when mixed together in a pool of cells. PG13--LNCX/CA cells were taken from the pool of cells generated by the first transfection, seeded at limiting dilutions, allowed to form colonies, and tested for CA protein by ICC. Several hundred colonies were examined, but all were negative for protein.

It was possible that the packaging cells which had eliminated expression of the CA protein gained a selective advantage over those which maintained expression. The non-expressors could have outdivided the cells expressing the CA protein, eliminating the expressors before the cells were divided into clonal populations. To remove the bias that may have been produced by growing cells in a pool, another round of transfection and selection of PG13 and PA317 cells (a similar packaging line) was performed and colonies were grown up at limiting dilutions during the initial neomycin selection. Cell colonies were tested by ICC, but again all populations were negative for protein.

Alternative transfer vectors

It was not possible to detect protein produced by the LNCX/CA vector after transfection into packaging cells, gene delivery in bovine cells, or clonal selection of transfected packaging cells. One likely explanation was that the LNCX/CA vector had been somehow improperly constructed. For example, in constructing the vector, artificial start and stop codons had been introduced in the CA gene during PCR amplification. At that time, no consideration was given to the translation initiation context of the artificially introduced start codon, and the non-consensus initiation context may have led to a large reduction in the level at which transfer vector RNA was transcribed. Other unknown construction errors may also

have taken place, and for this reason several other transfer vectors were constructed.

LNCX constructs. Several other LNCX transfer vectors were made using the different segments of the BIV genome (Figures 9 and 10). The gag3 portion of BIV was used because it is exactly the same part of *gag* which was originally used in a fusion protein to detect anti-Gag humoral immune responses (4), and so has been shown to react with Gag-specific polyclonal and monoclonal antibodies. The entire *gag* gene was used to construct LNCX/gag because it contains the natural translation initiation and termination sites, so mRNA containing it should be as efficiently translated as mRNA from wild-type BIV virus. As shown in Figure 9, the plasmid p61-3E which was used as the source for *gag* in LNCX/gag had also been used as the source of gag3 in pBacHisC/gag3, and thus the source gene should be able to react with the available Gag-specific antibodies. BIV-infected cattle develop antibodies to the protein product of a portion of the *env* gene, known as Env8 (19), so sera from these cattle can be used to detect Env8. The Env8-specific antibodies were detected using an *E. coli* TrpE-Env8 fusion protein produced by the pTrpE/env8 plasmid, so this plasmid was used as the source of the env8 in LNCX/env8. Construction of the alternative LNCX transfer vectors was performed similarly to construction of LNCX/CA.

Direct transfection, which has been reported to increase the frequency of transfer vector rearrangement and recombination (57), was used to introduce LNCX/CA transfer vector into PG13 packaging cells. Rather than being directly transfected into PG13 cells, the LNCX/gag3, LNCX/gag, and LNCX/env8 transfer vectors were instead transiently transfected into the PA317 packaging cell line, and recombinant retroviral vectors in the supernatant from the PA317 were used to infect PG13 packaging cells. This technique reportedly results in a much higher

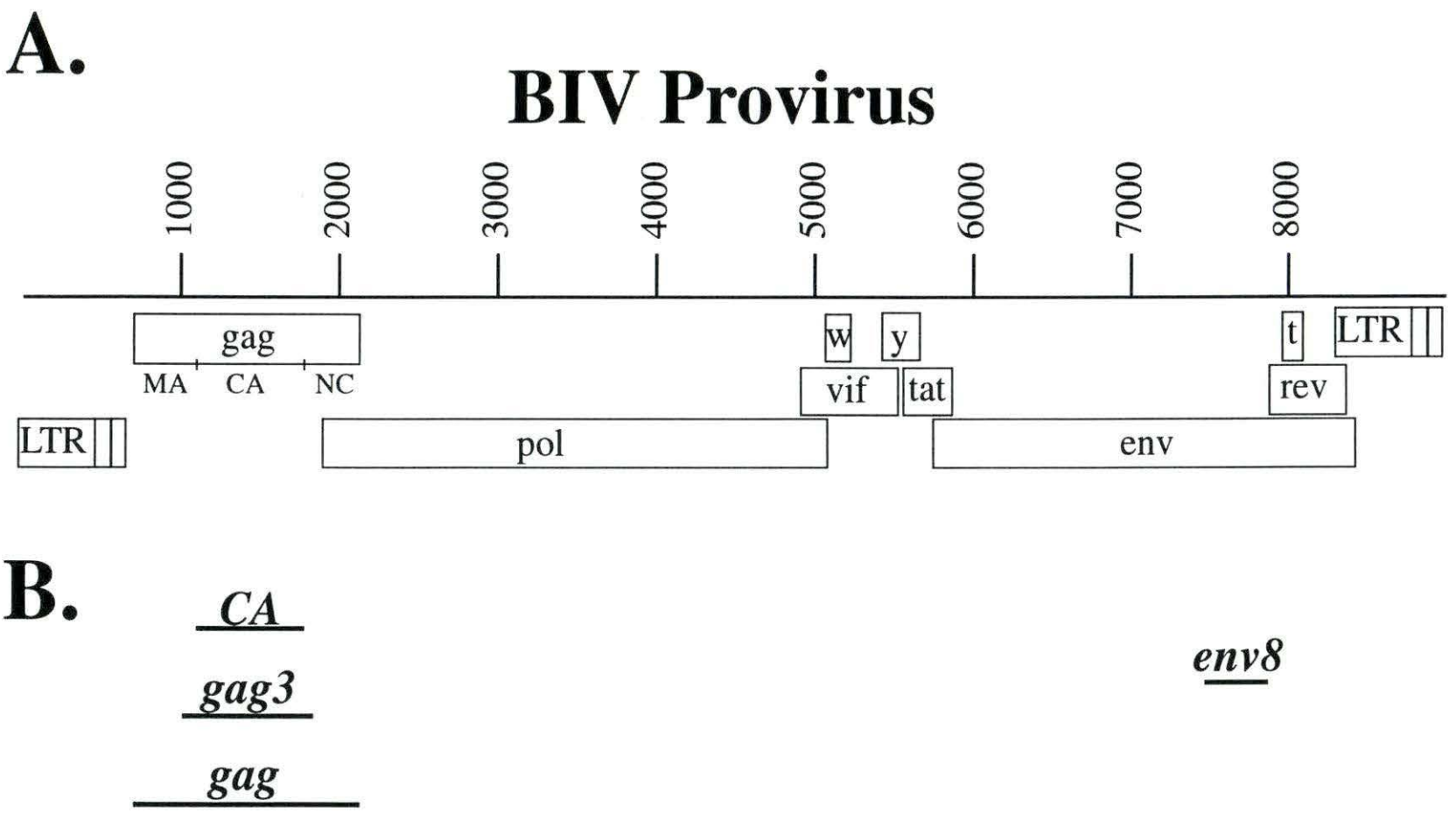

Figure 9. BIV genes used in transfer vector construction. A. Map of the provirus genomic organization of BIV. B. DNA segments which were amplified for cloning into transfer vectors, aligned with the location in the BIV provirus from which they are taken.

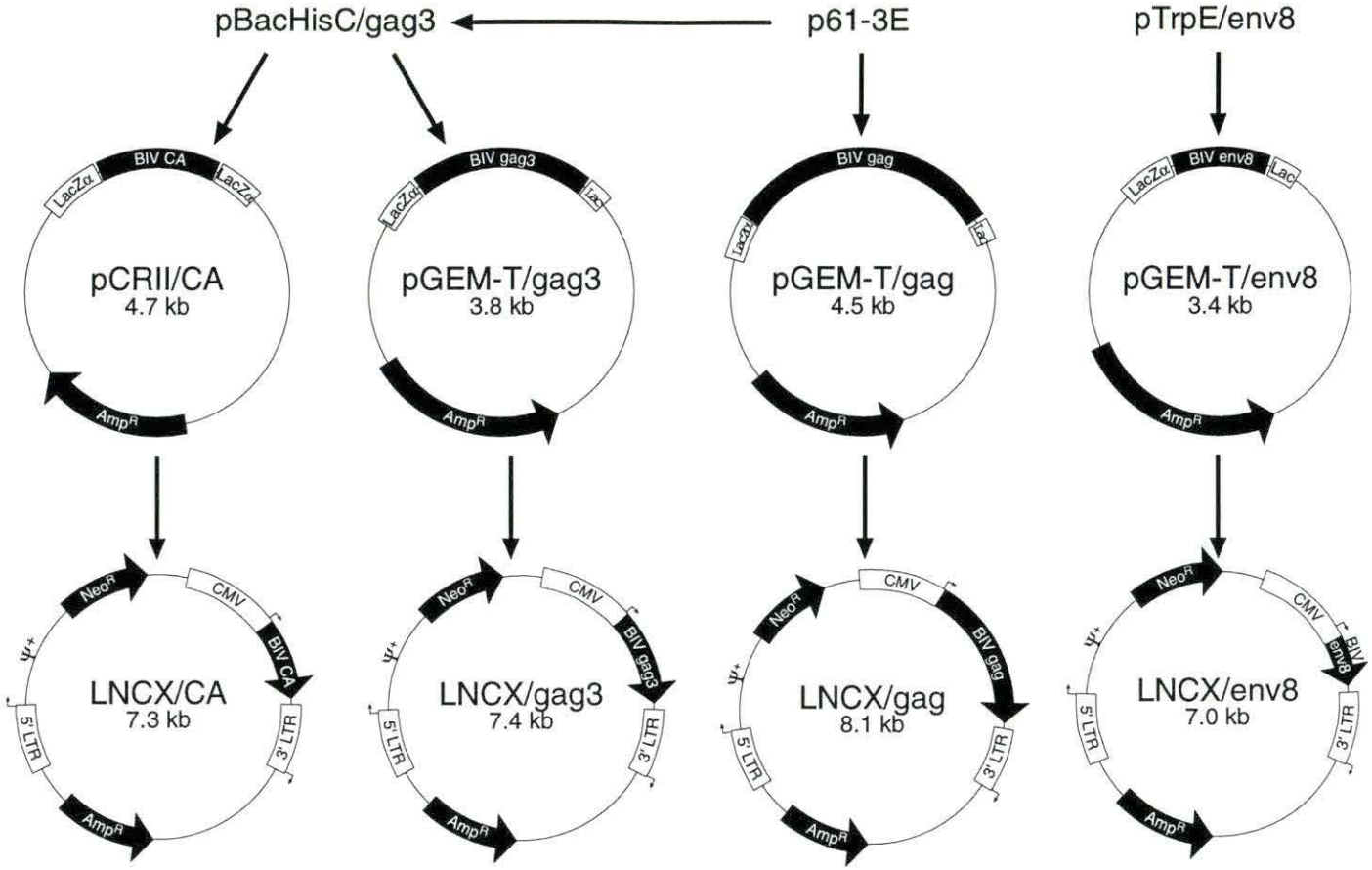

Figure 10. LNCX transfer vector construction. Several different-sized segments of the BIV *gag* gene and one segment of the BIV *env* gene were used to construct transfer vectors in a manner similar to LNCX/CA construction. The *gag3* insert was amplified from pBacHisC/gag3, and the *gag* insert was amplified from p61-3E, a pUC19 plasmid containing the 5' half of the BIV genome which had been used to construct the pBacHisC/gag3 plasmid. The *env8* insert was taken from the pATH/env8 plasmid which produces an *E. coli* TrpE-env8 fusion protein that reacts with sera from BIV-infected cattle (19). BIV DNA fragments were PCR amplified from the plasmids listed in the top row and ligated in intermediate plasmids. When correct amplification was verified, BIV gene fragments were subcloned into LNCX.

proportion of unrearranged transfer vector integrated in the packaging cells' nuclei (57). Serial dilutions of infected PG13 packaging cells were passed and selected for neomycin resistance. ICC was performed on the colonies using a Gag-specific monoclonal antibody for neo^R PG13 cells with the LNCX/gag3 or LNCX gag, and using a polyclonal calf serum previously demonstrated to react with Env8 protein (4) for cells with LNCX/env8. No reaction was seen with any of the colonies containing LNCX/gag3 or LNCX/gag. Small differences in antibody reactivity were seen among colonies containing the LNCX/env8 vector, however the level of background staining of uninfected PG13 control cells with the Env8-specific polyclonal sera was much higher than with the monoclonal, and sera did not react with any colonies to a greater degree than with the uninfected controls. No protein expression was detected in clonal populations of PG13 packaging cells with LNCX/gag3, LNCX/gag, or LNCX/env8. Because of the different sources of the BIV genes and the different steps necessary in the construction of these vectors, it did not appear that the lack of expression was merely a result of faulty plasmid construction.

LXSN constructs. Another possible explanation for the lack of expression is some unknown inactivating interaction between the BIV lentiviral genes and the retroviral elements in the LNCX plasmid and the packaging cell. There is anecdotal evidence that lentiviral genes are spliced out of LNCX in cells (35,73). The LXSN transfer vector has been successfully used to express the CA protein of feline immunodeficiency virus (FIV), another of the lentiviruses (78). The pLXSN plasmid was used to construct the LXSN/gag and LXSN/gag3 transfer vector plasmids (Figure 11). In order to eliminate any interaction between the retroviral genes in the packaging cell and the transfer vector, plasmids were transiently transfected into 293 cells, an easily transfected human kidney cell line. After two

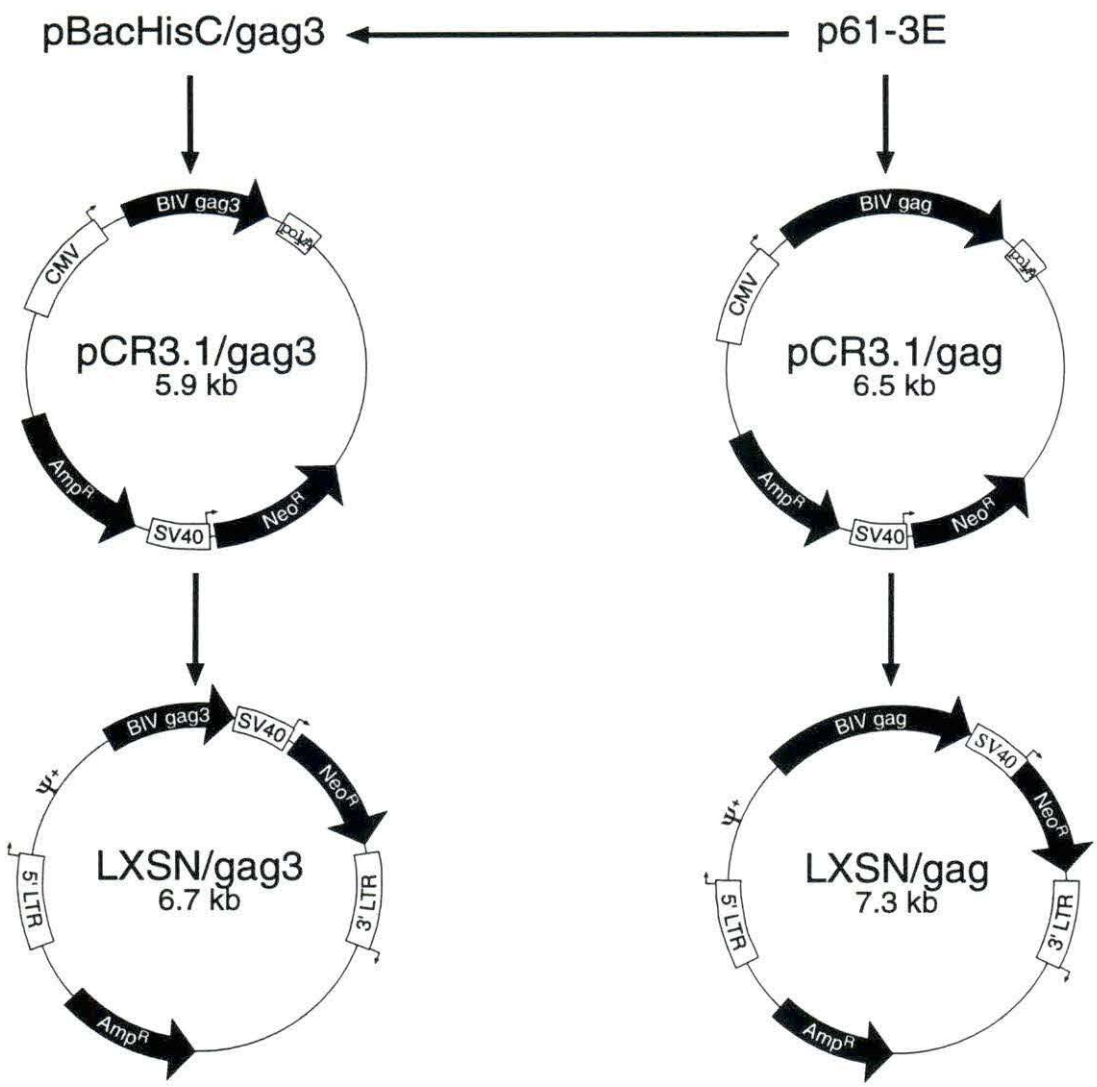

Figure 11. LXSN transfer vector construction. As with the LNCX vectors, BIV DNA segments were amplified from source plasmids, ligated in intermediate plasmids, and subcloned from the intermediate plasmid into LXSN.

days, 293 cells with each transfer vector were tested for protein by ICC using either methanol or formaldehyde fixation and either the CA-specific monoclonal antibody or a Gag-specific rabbit polyclonal. No specific protein expression was seen in cells with either LXSN/gag or LXSN/gag3 using any combination of fixative and antibody. The lack of expression seen with the LNCX-based transfer vectors was not due solely to an interaction of the BIV lentiviral genes with the retroviral genes in the LNCX plasmid or the PG13 packaging cells.

Eukaryotic expression constructs. A possible reason that no protein was detected is that the sequence of the BIV genes may have been faulty. This possibility seemed unlikely since sequencing of the DNA revealed no stop codons or frameshifts. The amino acid sequence appeared to contain two amino acid changes from the previously reported BIV sequence, but these were present in the pBacHisC/gag3 source plasmid as well, and protein produced by that plasmid reacted with the CA-specific monoclonal antibody.

We did not have a *gag*-containing plasmid that we could transfect into PG13 or 293 cells to use as a positive control for ICC. Therefore, the problem may not have been a lack of expression, but instead merely a lack of detection. For this reason, we constructed gag and gag3 eukaryotic expression plasmids. The intermediate plasmid used in construction of the LXSN transfer vectors was pCR3.1, a eukaryotic expression vector which uses the CMV promoter (Figure 11), and the pCR3.1/gag3 and pCR3.1/gag were tested for Gag expression. One plasmid clone of pCR3.1/gag3 and twenty different plasmid clones of pCR3.1/gag were transfected into 293 cells. Four sets of 293 cells transfected with different pCR3.1/gag plasmid clones were selected for neomycin resistance for three weeks before they were tested for Gag protein, and the remainder of transfected 293 cells were tested for protein two days after transfection. No cells tested positive by ICC

using any combination of methanol or formaldehyde fixation and CA-specific monoclonal or Gag-specific rabbit polyclonal antibody. These results indicated that either some problem had arisen in the nucleotide sequences of the segments of the gag genes that we used, or detection of Gag by ICC, though adequate to detect cells infected with wild-type BIV virus, was not sufficient to detect Gag protein produced by plasmids.

Western blotting. Since Gag protein was not detectable by ICC, Western blot hybridization was used as an alternative method to try to detect Gag protein in transfected cells. Lysates were collected from 293 cells transiently transfected with the LNCX/CA, LNCX/gag3, LNCX/gag, LXSN/gag3, LXSN/gag, or pCR3.1/gag3 plasmids. Cell lysates were also taken from the sixteen sets of 293 cells transiently transfected with different pCR3.1/gag plasmid clones and the four sets of neo^R 293 cells stably transfected with pCR3.1/gag plasmid clones. The lysates were analyzed by Western blot using a CA-specific monoclonal antibody (Figure 12). Purified BIV virus was used as a positive control. A strong reaction was detected to the 26 kDa CA protein in BIV-infected cells, but no other proteins were detected. Although protein was detectable in virus isolated from BIV-infected cells, the CA protein is the most prevalent component of virions, so by concentrating the virus, the CA protein was concentrated as well. The lack of protein in cells transfected with the different gag plasmids could have been due to a non-functional form of the gag gene in the plasmids, but this seems unlikely since Gag protein was readily detectable by both ICC and Western blot in insect cells transfected with the pBacHisC/gag3 plasmid. An alternate possibility is that the Gag-specific antibodies were not sensitive enough to detect the amount of Gag protein produced in cell transfected with the LNCX, LXSN, or pCR3.1 plasmids.

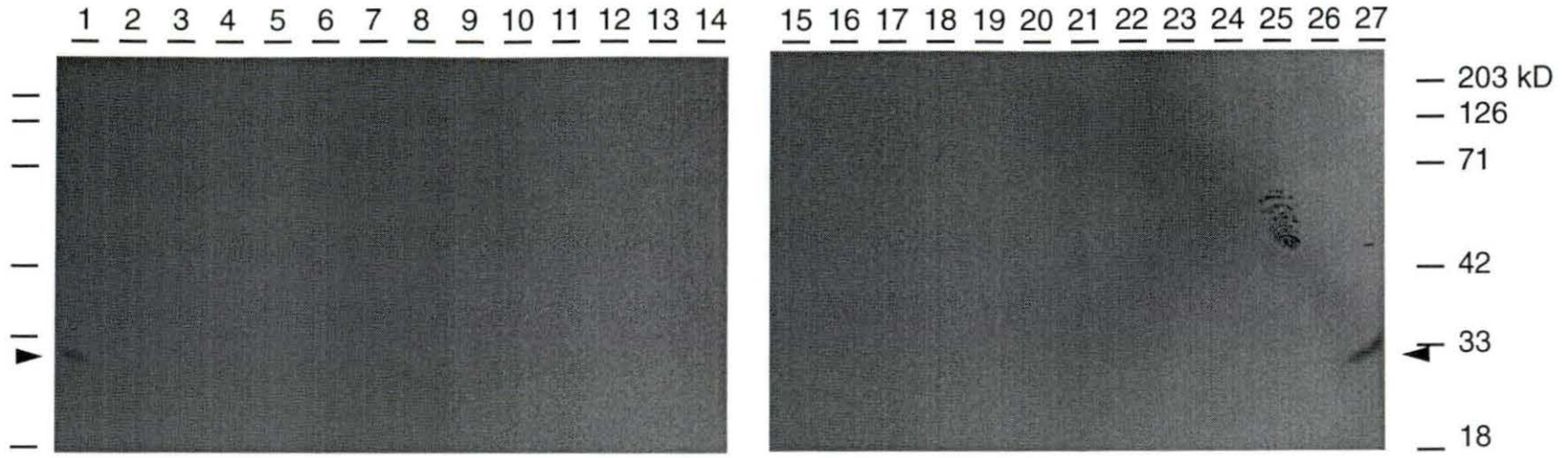

Figure 12. Western blots of cells transfected with transfer vectors. Lanes 1 and 27 contain protein purified from BIV-infected cells. Lane 2 contains the lysate of untransfected 293 cells. Lanes 3-13, 15-22, and 26 contain the lysates of 293 cells transiently transfected with different gag-containing plasmids as follows: 3 with LNCX/CA, 4-5 with LNCX/gag3, 6 with LNCX/gag, 7-8 with pCR3.1/gag, 10 with LXSN/gag, 11 with LXSN/gag3, and 9, 12-13, 15-22, and 26 with pCR3.1/gag. Lanes 14 and 23-25 contain lysates of 293 cells transfected with pCR3.1/gag and selected for neomycin resistance for four weeks with G418. Western blotting was performed using a monoclonal antibody to BIV CA and ^{125}I-labeled Protein G. The bands indicated by solid arrowheads contain the 26 kDa CA protein from BIV-infected cells.

Transcriptional activity

Production of Gag, CA, or Env8 proteins was not detected from any of transfer vector or eukaryotic expression plasmids in any of the experiments outlined thus far. The block to protein production could be at a translational level, or at a transcriptional level. Total RNA was isolated from a number of cells with transfer vectors or pCR3.1 eukaryotic expression vectors, and several tests were performed to determine if the transfer vectors and the eukaryotic expression plasmids were being transcribed to RNA within the cells.

RNA isolation. RNA was isolated from PG13 cells, from PG13/LNc8 (these carry only the neo^R gene), and PG13--LNCX/CA. Total RNA was also taken from normal FBL cells, FBL cells infected with recombinant LNCX/CA retrovirus, and neo^R FBL--LNCX/CA cells. RNA was isolated from normal 293 cells and from 293 cells transiently transfected with pCR3.1/gag3, pCR3.1/gag, LXSN/gag3, or LXSN/gag. Total RNA from BIV-infected cells was used as a positive control for *gag*.

RT-PCR. Total RNA from normal PG13 cells, PG13/LNc8, PG13--LNCX/CA, unselected FBL--LNCX/CA, and neo^R FBL--LNCX/CA was analyzed by RT-PCR (Figure 13). The templates were amplified using primers for neo^R, CA , and a primer pair outside the multi-cloning site of LNCX, all of which give 0.70-0.76 kb products. No 0.7 kb bands were present in either normal PG13 (lanes 4-7) or normal FBL cells (lanes 8-11) or from reactions without reverse transcriptase (lanes 2, 4, 8, 12, 19, and 23), as expected. A 0.7 kb CA band amplified from the BIV RNA (lane 3). From PG13/LNc8, only the neo^R primers amplified a 0.7 kb band (lane 9). From PG13--LNCX/CA, FBL--LNCX/CA, and neo^R FBL--LNCX/CA, 0.7 kb bands amplified in the CA, neo^R, and LNCX reactions (lanes 13-16, 20-22, and 24-26). These results indicated that in both packaging cells and bovine cells with the

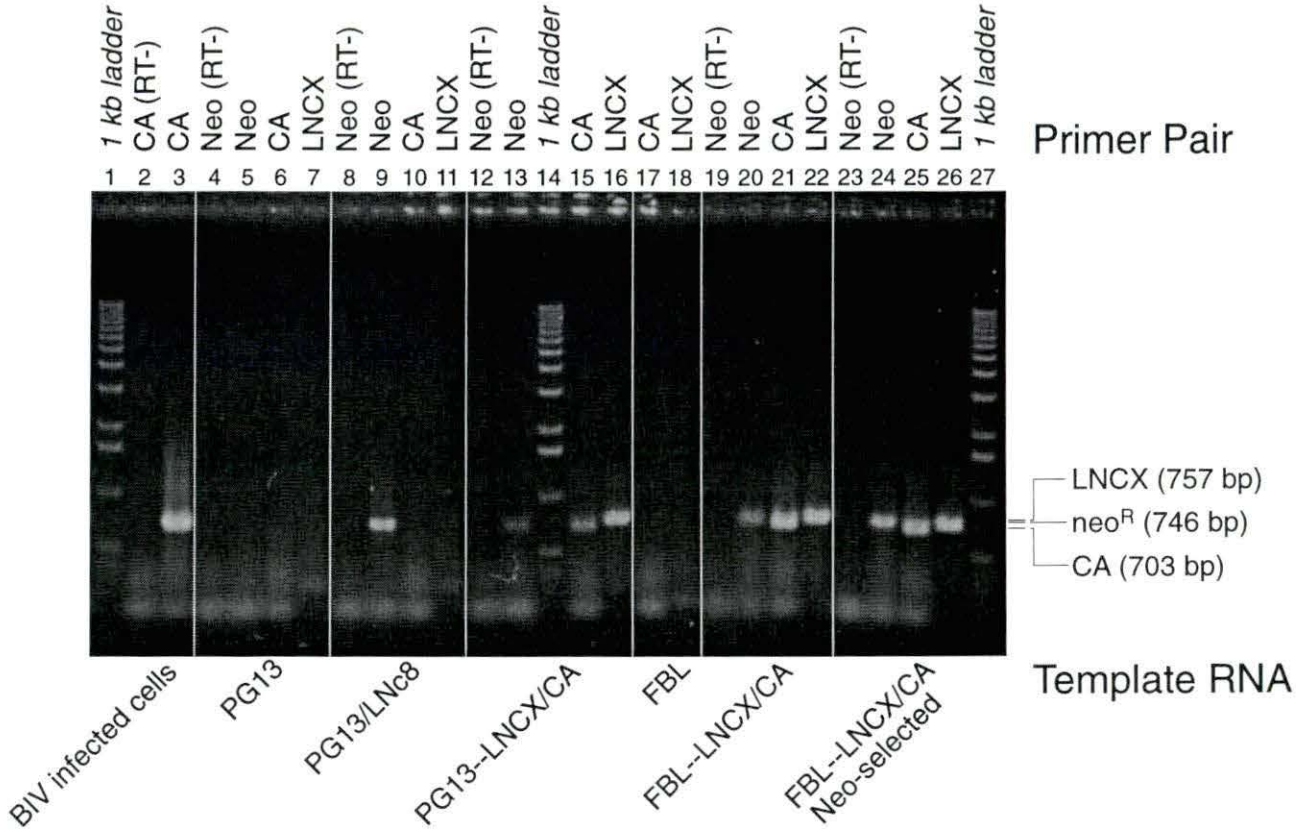

Figure 13. Analysis of gene expression by RT-PCR in stably transfected and infected cells. Total RNA from cells with the LNCX/CA transfer vector was amplified using primers specific for the CA and neo^R genes, or with primers outside the LNCX multi-cloning site. Primers are indicated above the gel and RNA templates are labeled below. Reactions labeled RT- were run without reverse transcriptase to test for DNA contamination. Positive controls included BIV-infected cells and PG13/LNc8 cells. Negative controls included normal PG13 cells and normal FBL cells. PG13--LNCX/CA cells were stably transfected with LNCX/CA plasmid. FBL--LNCX/CA were infected with LNCX/CA retroviral vector and used either unselected or selected for neo^R. The expected RT-PCR product sizes from the CA, neo^R, and LNCX primer pairs are indicated.

LNCX/CA transfer vector, the CA and neo^R RNA was produced. In addition, in the LNCX reactions in cells with the transfer vector, a single clear band was present, indicating that the CA RNA was not spliced out of RNA transcripts.

The presence of CA RNA was measured in total RNA from 293 cells transiently transfected with either pCR3.1/gag3, pCR3.1/gag, LXSN/gag3, or LXSN/gag (Figure 14A). The expected PCR product for the CA primers is 703 base pairs (bp), and a 0.7 kb product was detected in BIV-infected controls (panel A, lane 2), as well as 293--LXSN/gag3 (lane 6), 293--LXSN/gag (lane 10), and 293--pCR3.1/gag3 cells (lane 8). The 0.7 kb band was not detected in normal 293 cells (lane 4), or in cells to which no reverse transcriptase had been added (lanes 3, 5, 7, 9, 11, and 13), so the *gag* gene was not present in 293 cells before transfection, and no contaminating *gag* DNA was present in RT-PCR reactions. The expected RT-PCR product was not detected in 293--pCR3.1/gag cells (lane 12), which could either indicate that that transfection was not successful, or that something was wrong with the plasmid. LXSN/gag, the derivative of pCR3.1/gag, was transcribed, so the lack of *gag* RNA was not due to a lack of the correct *gag* DNA. A poor transfection seems the most likely reason. In any case, the *gag* gene of LXSN/gag3, LXSN/gag, and pCR3.1/gag3 was transcribed by 293 cells, and thus the lack of protein was not due to an absence of RNA caused by a problem such as promoter-cell incompatibility.

A further RT-PCR analysis was performed on a total RNA from 293 cells using several other primer pairs (Figure 14B). An attempt was made to amplify RNA from 293--pCR3.1/gag and 293--pCR3.1/gag3 cells using a 5' primer from inside the gag gene and a 3' primer from outside the pCR3.1 multi-cloning site (panel B, lanes 8 and 9). The expected sizes are 1547 bp for pCR3.1/gag and 937 bp for pCR3.1/gag3. Neither RT-PCR product was detected. This was not very surprising

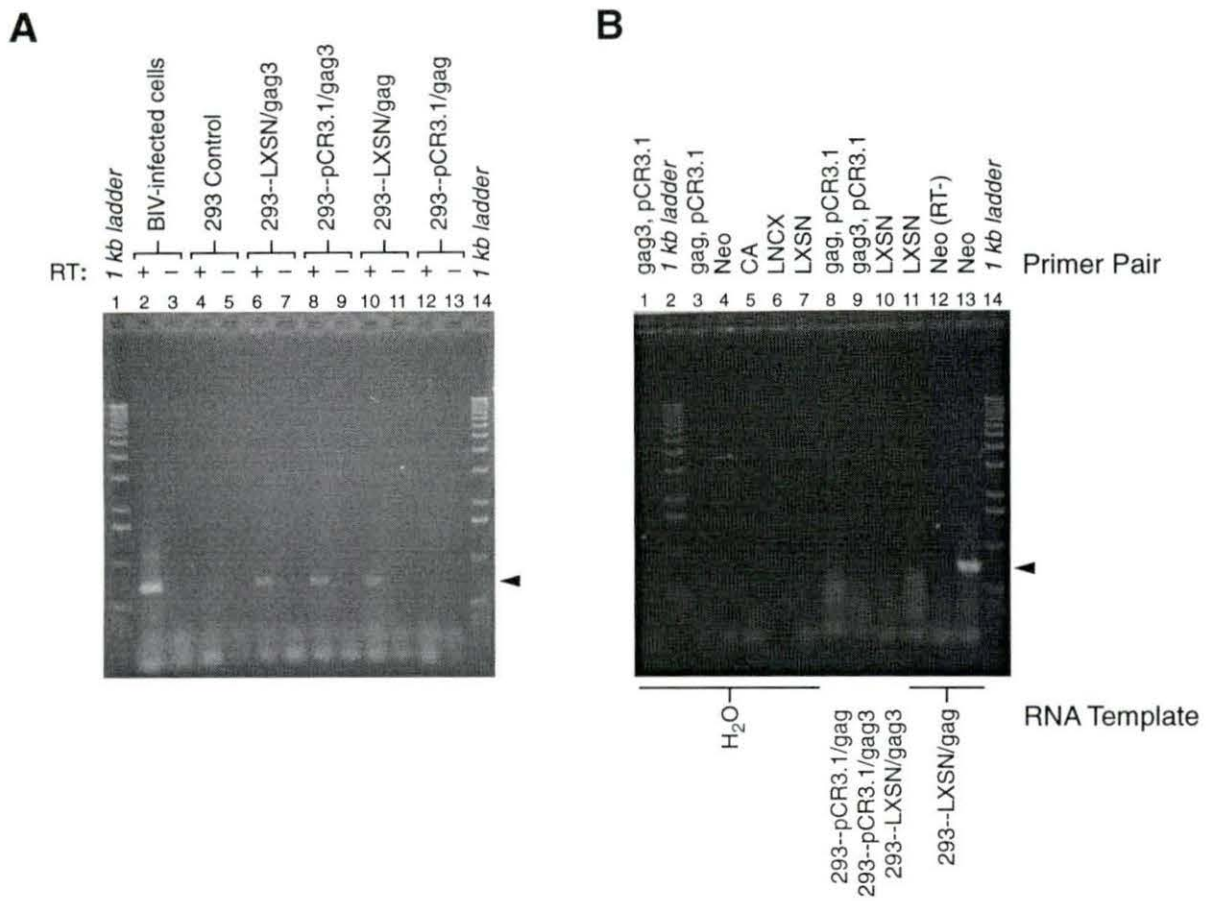

Figure 14. Analysis of gene expression by RT-PCR in transiently transfected cells. Total RNA was isolated from 293 cells transiently transfected with the indicated transfer vector. In panel A, RT-PCR was performed with and without reverse transcriptase for all six total RNA samples using primers specific for BIV CA. The arrowhead indicates the expected position of the 703 bp CA RT-PCR product. In panel B, RT-PCR was performed with templates indicated by the lower labels using primers indicated by the upper labels. The solid arrowhead in panel B indicates the expected position of the 746 bp neo^R RT-PCR product.

for the 293--pCR3.1/gag RNA since no CA RNA had been detected, but for 293--pCR3.1/gag3 it may be a result of a poor primer pair since no positive control was used. A similar amplification of 293--LXSN/gag and 293--LXSN/gag3 was performed using primers outside the multi-cloning site of LXSN (panel B, lanes 10 and 11). The expected product for LXSN/gag is 1501 bp, and for LXSN/gag3 an 891 bp product is expected. Neither product was detected, but again no positive control was used. It is possible that only the interior of the gene was transcribed, but this seems highly unlikely. On the other hand, when RT-PCR was performed on 293--LXSN/gag RNA using primers for neo^R RNA, the expected 746 bp product was found (panel B, lane 13). This reinforced the conclusion that the LXSN transfer vectors were capable of being transcribed in 293 cells.

Northern blotting. RT-PCR can detect an extremely small amount of RNA, and a positive reaction gives no indication of the amount of RNA present, so some of the RNA samples were electrophoresed in a denaturing agarose gel and analyzed by Northern blot hybridization (Figure 15). A BIV gag probe gave a strong signal from total RNA from BIV-infected cells (lane 2). In 293--LXSN/gag cells (lane 7), the BIV gag probe detected a 4.3-4.5 kb band, which is the expected size of RNA transcribed off the 5' LTR and terminating at the 3' LTR polyA site. A neo^R probe detected the 4.3-4.5 kb band and a 1.6-1.7 kb band, the expected size of RNA transcribed from the SV40 promoter to the 3' LTR polyA (lane 7). The two very high molecular weight bands visible in 293--LXSN/gag RNA with both gag and neo^R probes also hybridized with a pUC19 probe. Since the pUC19 probe is homologous with the plasmid backbone of pLXSN outside the LTRs, this indicated that the large band is the transfected LXSN/gag plasmid DNA. It appeared to have a very high molecular weight because DNA migrates more slowly than RNA of the same size on denaturing agarose gels. Detectable levels of gag and neo^R

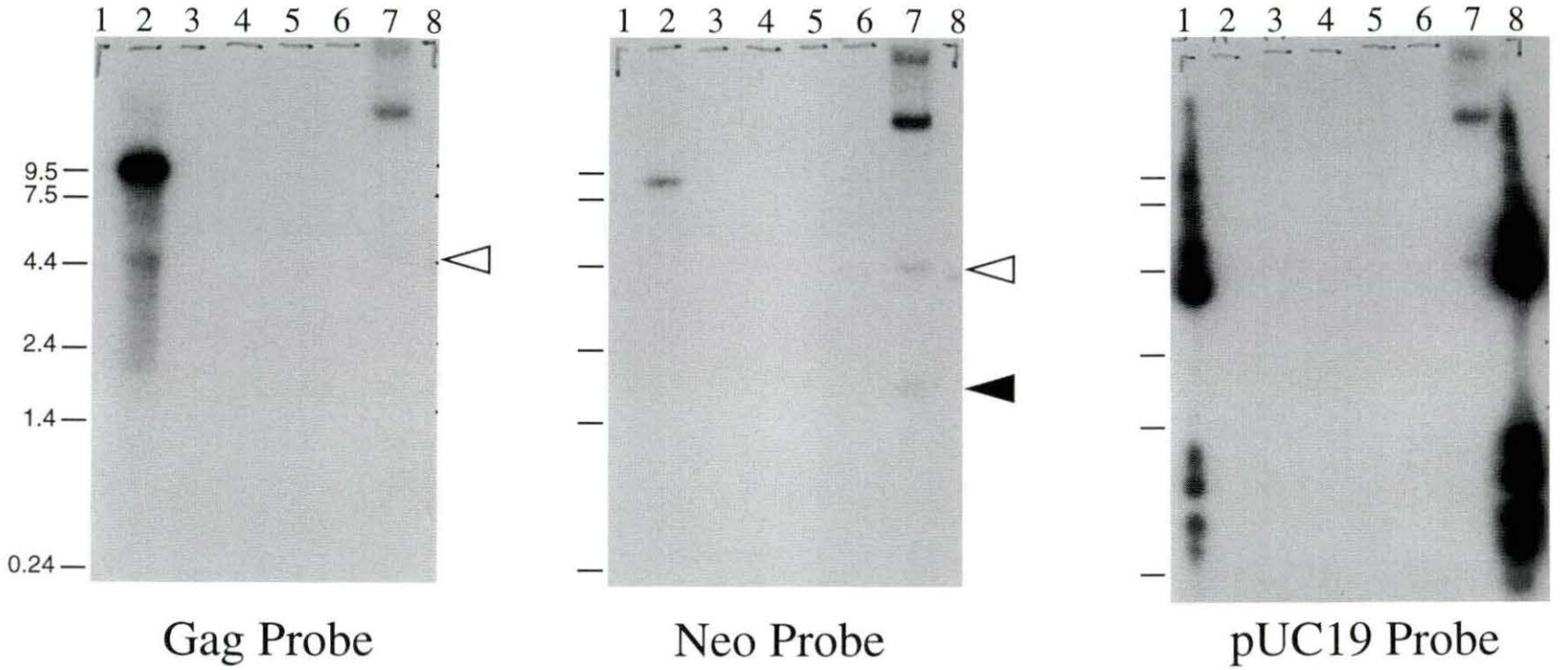

Figure 15. Northern blot of RNA from cells containing transfer vectors. Total RNA was electrophoresed through a formaldehyde/agarose gel and blotted to a membrane. Lanes 1 and 8 contain 0.24 - 9.5 kb RNA ladders. Lane 2 contains RNA from BIV-infected cells. Lane 3 contains PG13 RNA, lane 4 has PG13/LNc8 RNA, and lanes 5 and 6 have PG13--LNCX/CA RNA. Lane 7 has RNA from transiently transfected 293--LXSN/gag cells. All lanes contain 4 μg of total RNA, except lane 6 which has 7 μg. The open arrowhead indicates the 4.3-4.5 kb band in lane 7, and the closed arrowhead indicates the 1.6-1.7 kb band in lane 7. The membrane was hybridized with ^{32}P-radiolabeled BIV gag DNA, neo^{R} DNA, and pUC19 DNA. The hybridized DNA was stripped from the membrane between different probes. The very strong gag-hybridizing band in lane 2 was not completely stripped before probing with neo^{R} DNA, but was stripped off before probing with pUC19.

transcription did take place in the 293--LXSN/gag cells (open and closed arrowheads), but the RNA bands are very faint, indicating that the level of transcription was quite low. A low level of transcription in the 293 cells indicates that some protein may have been produced, but it probably was not in sufficient amount to detect by ICC or Western blotting

No signal was detected in any of the lanes with PG13 RNA, PG13/LNc8 RNA, or PG13--LNCX/CA RNA with either a gag or neo^R probe. If the expected transcription was taking place, the neo^R probe should have detected RNA in the PG13/LNc8 lane and PG13--LNCX/CA lanes, and the gag probe should have detected RNA bands in the PG13--LNCX/CA lanes. The PG13/LNc8 and PG13--LNCX/CA cells did possess neomycin-resistance, and so they must have produced the neo^R protein and neo^R RNA. Apparently, if RNA was being produced, the level was too low to detect by Northern blot hybridization.

Dot blotting. A method which is more sensitive at detecting RNA than Northern blot hybridization is dot blot hybridization. Because all the RNA in a sample is concentrated in one spot on the membrane, different sizes of RNA cannot be distinguished, but smaller quantities can be detected. PG13, PG13/LNc8, and PG13--LNCX/CA were analyzed by dot blot hybridization at ten-fold serial dilutions (Figure 16). Serial dilutions of pLNCX/CA plasmid DNA were used as a positive control. Duplicate blots were made and probed with either BIV gag (Figure 16A) or neo^R DNA (Figure 16B). The gag probe detected the plasmid DNA, but did not detect PG13--LNCX/CA RNA. On the other hand, the neo^R probe detected the plasmid DNA, as well as PG13/LNc8 RNA and PG13--LNCX/CA RNA, though the signal in PG13--LNCX/CA cells was much weaker. This indicated that neo^R transcription off the LNCX/CA transfer vector was taking place at a somewhat low level in PG13--LNCX/CA cells, but transcription of the CA RNA had been reduced

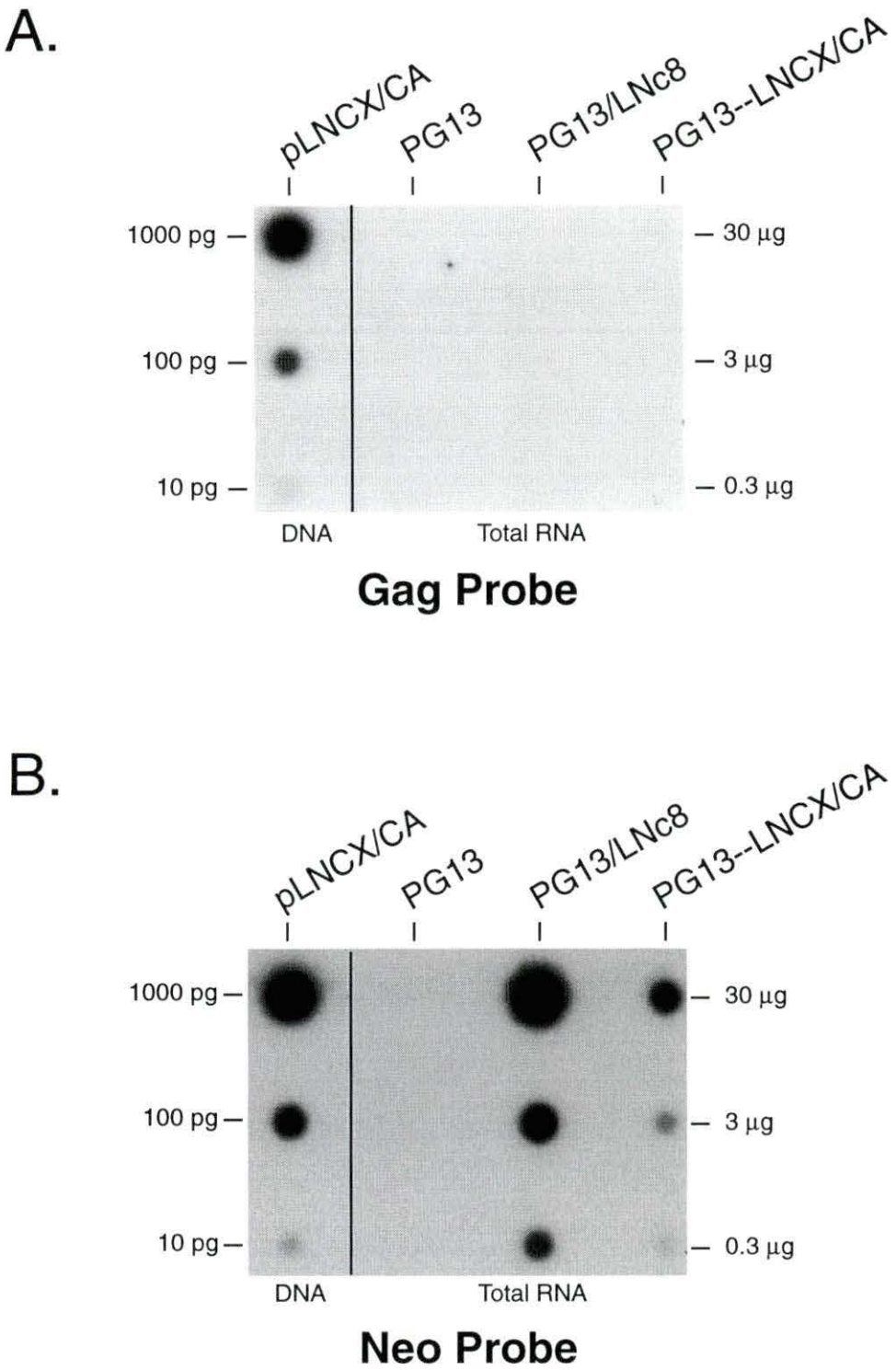

Figure 16. Dot blot of total RNA from packaging cells. A duplicate blot of packaging cell RNA and pLNCX/CA plasmid DNA was made. The blot shown in panel A was hybridized with ^{32}P-labeled BIV gag DNA, and the blot in panel B was hybridized with ^{32}P-labeled neo^{R} DNA.

to a very low level detectable only by RT-PCR. This result was somewhat surprising since the neo^R gene should have been transcribed in RNA species which contained the CA gene, but by recombination or some other method, this aspect of the LNCX/CA transfer vector had been eliminated. The very low level of CA transcription in the PG13--LNCX/CA cells detectable only by RT-PCR accounted for the absence of detectable CA protein.

Low levels of protein

In order get a better understanding of the levels of protein production that can be expected using retroviral gene delivery and in order to verify that the technique could be used to express genes in the bovine cells other than resistance markers, an LNCX transfer vector carrying green fluorescent protein (GFP) was introduced into PG13 packaging cells and into PBAC 79B cells.

LNCX/GFP. The GPE86--LNCX/GFP packaging cell line was kindly provided by John Levy at the Human Gene Therapy Research Institute (Des Moines, Iowa). This cell line both produces high levels of GFP protein and of recombinant LNCX/GFP retroviral vectors. The GPE86 retrovirus was used to infect PG13 cells, which were selected for neomycin resistance at serial dilutions. After three weeks, colonies were examined by fluorescent microscopy for fluorescence. Control PG13 cells had no visible fluorescence. The number of fluorescent and non-fluorescent neo^R PG13 colonies was counted. Approximately 3% of colonies showed any fluorescence, and only 1% fluoresced brightly. In some fluorescent colonies, a portion of the cells did not fluoresce. This suggested that as the cells underwent division, occasionally expression of the GFP protein was eliminated without loss of neomycin resistance, as was observed in the PG13--LNCX/CA cells. In a pool of neo^R cells, fewer than 1% of cells fluoresced after three weeks. Since fewer

fluorescent cells were visible when cells were grown in the more competitive conditions of a cell pool than in the non-competitive conditions of isolated colonies, the cells which did not express the GFP protein were presumably able to outgrow the GFP-expressing cells.

Supernatant from the PG13--LNCX/GFP cells was used to infect PBAC 79B cells, which were then selected for neomycin resistance with G418. Fluorescence was visible in more than ten percent of neo^R PBAC after three weeks and in one hundred percent of cells after six weeks. At three weeks, colonies with fluorescent cells did not contain non-fluorescing cells (data not shown). These results indicated that PBAC had a higher and more prolonged level of expression of transfer vector genes than did packaging cells.

Gene delivery of LNCX/CA to PBAC 79B cells. If LNCX/CA that was delivered to PBAC 79B was expressed in a manner similar to LNCX/GFP, very low levels of protein would be present in some clonal colonies. Supernatant was again collected from a pool of PG13--LNCX/CA cells, and the supernatant was used to infect PBAC 79B. The PBAC were selected for neomycin resistance for three weeks, and then stained by ICC using methanol fixation and a Gag-specific Rabbit polyclonal antibody. PBAC 79B infected with LNCX/GFP were used as a negative control, and their background level of staining was much lower than in normal PG13 cells or FBL cells. Compared with the very low background staining, some of the neo^R PBAC 79B--LNCX/CA colonies showed a very slight positive reaction which was higher than anything seen in the negative control cells (data not shown). Therefore, a low level of CA protein was produced in infected PBAC 79B cells, and this result indicated that protein expression probably had taken place in some of the other cells with transfer vectors, but a combination of a low number of

expressing cells and a low level of protein production had prevented detection of the BIV protein.

Summary

Several transfer vectors carrying BIV *gag* or env8 genes were constructed and used to transfect packaging cells. Retroviral vectors from a transfected packaging cell line were able to deliver BIV DNA to bovine cells, as detected by the presence of neo^R colonies. However, no BIV protein was detected in packaging cells, in the majority of infected bovine cells, or in transfected 293 cells. In packaging cells with the LNCX/CA transfer vector, the neomycin resistance gene (neo^R) was transcribed, but transcription of the BIV *gag* gene took place only at an extremely low level, as detected by dot blot hybridization and RT-PCR. Both neo^R and BIV *gag* RNA was detected by Northern blot in 293 cells transfected with the LXSN/gag transfer vector.

An LNCX transfer vector containing the green fluorescent protein (GFP) was successfully expressed in PG13 packaging cells, and recombinant LNCX/GFP retroviral vector was used to deliver and successfully express low levels of GFP in PBAC 79B cells. Bearing in mind the results with the GFP gene delivery, recombinant LNCX/CA retroviral vector was used to infect PBAC 79B cells, and a very low level of BIV CA protein was detected in some of the PBAC cells.

In high endothelial cells, detectable BIV CA protein and GFP protein were found. In 293 human kidney cells, transcription driven by both transfer vector promoters was detectable by Northern blot, but BIV protein was not detectable. In PG13 and FBL cells, both fibroblast cell types, BIV RNA was detected only by RT-PCR. GFP was weakly detectable in only a fraction of PG13 vector producer cells. These results indicated that high endothelial cells were suitable for expression of

genes delivered by retroviral vectors, and suggested that cultures of fibroblasts may be unsuitable for retroviral gene delivery because of a transcription level too low for detectable amounts of protein to be produced.

These results indicated that retroviral gene delivery may be a suitable method to deliver viral genes to bovine target cells for detection of virus specific CTL.

DISCUSSION

Cytotoxic T lymphocytes (CTL) are an important part of the immune system and are critical in the control of some intracellular pathogens, including viruses. The ability to detect cytotoxic T lymphocytes (CTL) which lyse virus-infected cells is an important tool in disease research, from the perspective of natural immunity and in vaccine development. Although a variety of different target cells and a number of gene delivery methods have been used for CTL research in mice and humans, technical difficulties have made detection of virus-specific CTL in cattle impossible for many pathogens.

One obstacle to development of a bovine CTL assay is the lack of a suitable target cell. Bovine target cells and CTL must be genetically matched at class I MHC loci, and since cattle are outbred, genetically matched target cells and CTL can be obtained only by isolating both groups of cells from the same animal. Because target cell cultures must be established before CTL can be taken from an animal, isolation of the target cells cannot kill the animal. Also, target cells must express a relatively high level of class I MHC to ensure that endogenous antigens are efficiently presented to T cells.

The virus can also pose problems in measuring CTL activity. Many viruses are only able to infect a limited range of host cells *in vitro*, and some viruses do not grow *in vitro* at all. Also, many of the viruses which can infect cultured cells lyse the cells they infect, creating a background of lysis above which CTL lysis of target cells cannot be detected.

One way that the problems of limited host cell range and viral lysis have been overcome in other animals is by using vectored gene delivery to introduce individual viral genes into target cells. This method is doubly beneficial in a CTL

assay because it allows mapping of CTL responses within the viral genome. Retroviral vectors are a promising gene delivery method for use in CTL assays because they can deliver genes to cells at high efficiency, the genes are stably expressed, and no viral lysis occurs. By using vectored delivery of viral genes, limited viral host range is a much smaller consideration, and the choice of target cells can be based more on a non-intrusive isolation method and a high level of class I MHC expression.

In the present study, a variety of primary cell cultures were evaluated for their potential as target cells in a CTL assay, and the usefulness of retroviral gene delivery as a vehicle for introducing viral genes into target cells was assessed. Among the different cell cultures examined, a peripheral blood adherent cell (PBAC) culture showed the most promise as a potential target cell. Retroviral vectors could deliver viral and non-viral genes to PBAC, and low levels of each gene's protein product were detected.

Nine primary cell cultures were examined for their relative levels of class I MHC expression and their susceptibility to retroviral gene delivery. These are both important characteristics for a target cell in a CTL assay using retroviral gene delivery. The cell culture with the best combination of these two characteristics was the PBAC 79B culture. PBAC are attractive as target cells because blood cell isolations cause little trauma to cattle and are easily performed. The three PBAC cultures we examined had different levels of class I MHC expression and susceptibility to gene delivery and were further characterized by cell marker analyses and functional assays to better determine their cell type and to differentiate between them. None of the cultures displayed leukocyte, epithelial, or vascular endothelial markers. PBAC 79B cultures were found to be high endothelial cells based on a cobblestone morphology typical of endothelial cells

and a level of *in vitro* lymphocyte binding and DiI-Ac-LDL uptake similar to that reported for high endothelial cells (45,71). PBAC 79A had a low susceptibility to gene delivery and PBAC 342 had a low class I MHC expression, making these other PBAC cultures much less suitable as target cells than PBAC 79B. The PBAC 79A cells grew much more slowly than 79B cells, had a lower susceptibility to retroviral gene delivery, and took up a very low level of DiI-Ac-LDL. Although PBAC 342 initially grew in a cobblestone pattern, after two passages the cells grew in a more bipolar fibroblastic morphology. The PBAC 342 had a low *in vitro* lymphocyte binding level.

PBAC 79A and PBAC 79B were isolated from the same blood sample, but exhibited different characteristics. The growth rate of the PBAC 79A cells dropped as they were cultured, and the low growth rate explained their lower susceptibility to gene delivery, since retroviruses only infect dividing cells. Uptake of DiI-Ac-LDL has also been found to sometimes drop as cells are cultured *in vitro*. The PBAC 79A cells' high lymphocyte binding and occasional cobblestone morphology indicate that they probably contain high endothelial cells and were originally identical to PBAC 79B. The 79A cells experienced a drop in growth rate and DiI-Ac-LDL uptake during culture, and consequently lost their suitability as potential target cells. The PBAC 342 culture, on the other hand, had a class I MHC expression and susceptibility to retroviral gene delivery in the range of the fibroblastic primary cell isolates. Also, they did not bind lymphocytes *in vitro*, and they grew in bipolar arrays. These characteristics all indicate that the PBAC 342 culture was composed primarily of fibroblasts, not high endothelial cells. Their initial morphology was characteristic of high endothelial cells, but the culture probably was overgrown by fibroblasts after several passages. All the fibroblasts that we tested were not suitable as potential CTL target cells.

In order for PBAC cultures to be routinely used as CTL target cells, it would be necessary to distinguish good potential target cells from bad ones. A doubling time of at most five days is necessary for good susceptibility to gene delivery. A level of lymphocyte binding higher than 0.4 lymphocytes per cell would indicate that the cultures are high endothelial cells, and not fibroblasts. PBAC cultures with a short doubling time and high lymphocyte binding correlate with a high potential as CTL target cells.

Retroviral vectors were used to deliver bovine immunodeficiency virus (BIV) genes to bovine cells to test the usefulness of this gene delivery method in a bovine CTL assay. Several retroviral transfer vectors were constructed with different portions of the BIV *gag* gene or the BIV *env* gene and using either the LNCX or LXSN transfer vector backbone. The BIV transfer vectors were transfected into packaging cells and into 293 human kidney cells, and in all experiments no BIV protein was detectable. In transiently transfected cells, the BIV genes were transcribed at a low level, and a small but undetectable level of translation may have also taken place. In stably transfected packaging cells, the transfer vector's antibiotic resistance gene was readily transcribed, but the BIV gene was transcribed at an extremely low level. In the packaging cells, very little BIV protein, if any, could have been produced. When recombinant retroviral vectors from packaging cells were used to deliver BIV genes to PBAC 79B cells, a barely detectable amount of BIV protein was produced in some antibiotic-resistant colonies. Thus, bovine viral genes could be delivered and expressed, albeit at a low level, to potential bovine CTL target cells.

A recombinant retroviral vector containing green fluorescent protein (GFP) was also used to infect bovine cells. Detection of GFP is much more sensitive than detection of the BIV genes. PBAC took up the GFP gene and showed green

fluorescence. The level of fluorescence was low, but it was more easily visible than the reaction with BIV protein had been. The expression of GFP in the PBAC confirmed the ability of retroviral gene delivery to introduce genes into potential bovine CTL target cells, and it confirmed that those genes were expressed.

A low level of expression of retroviral vector-delivered genes was observed, though the level varied between clonally derived populations of cells. When a retroviral vector infects a cell, the genes it carries are integrated at a random site in the host cell DNA. If the vector integrates into a chromosome region that is transcriptionally silent, the retroviral genes will likely also be silenced. Even if the retroviral vector integrates into a transcriptionally active region, the LTR or CMV promoters of the vector can become hypermethylated after integration causing a change in chromatin conformation which can also down-regulate transcription of the inserted genes (38). The problem of down-regulation of the retrovirus-delivered genes can be avoided by testing the expression levels of a large number of clonally derived populations of infected cells and selecting the highest expressors (56). Other researchers have used retroviral gene delivery of HIV and FIV genes to CTL target cells to measure the specificity of CTL lysis to viral genes in naturally infected animals (69,78). In the measurement of FIV-specific CTL, a low level of FIV CA gene expression was also found, but protein levels were sufficient to stimulate CTL to lyse cells. In the research using retroviral vectors carrying HIV *nef*, a number of different cell clones were screened and one with high expression levels was used. The field of retroviral gene delivery continues to develop, and as it does retroviral vectors which elicit higher and more consistent levels of expression will become available.

The genes delivered to bovine cells in this study using retroviral gene delivery were transcribed and translated at a low level, but a low level of expression may be

sufficient for use in a CTL assay. Unlike the humoral immune system, CTL can react to cells which present only 100 molecules of antigen on their surface (21,82), thus a low level of expression can be sufficient to elicit lysis of infected target cells. The most sensitive test of protein expression is CTL lysis of target cells, and that test has not yet been performed.

In summary, adherent high endothelial cells cultured from peripheral blood were found to have a high potential as target cells for a CTL assay. These cells could prove widely useful in measuring bovine CTL activity to viruses. Retroviral gene delivery was successfully used to deliver and express viral and non-viral genes to high endothelial cells. The use of retroviral vectors for construction of a bovine CTL assay system which is proposed in this research has a real potential to allow elucidation of bovine cellular immune responses to viruses which until now have been impossible to measure. The next step in this line of research is to use the CTL assay outlined here to try to detect cellular immune responses in virus-infected cattle.

APPENDIX A. SOLUTION RECIPES

Agarose/formaldehyde gel	1.2% agarose, 1X MESA buffer†, 6.7% (2.2 *M*) formaldehyde, and 0.1 μg/ml ethidium bromide
Alkaline Transfer Buffer	1.5 *M* NaCl, 0.25 *M* NaOH
Ambion's Gel Loading Buffer II	95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5 m*M* EDTA, 0.025% SDS
Denhardt's reagent	10 g/l Ficoll, 10 g/l polyvinylpyrrolidone, and 10 g/l BSA
DEPC H_2O	distilled water treated for at least 16 hours with 0.1-0.2% DEPC to inactivate RNases and autoclaved to remove the DEPC
DMEM	Dulbecco's modified Eagle's medium (Sigma), 4.5 mg/ml glucose, 1.2 mg/ml L-glutamine, 3.7 mg/ml sodium bicarbonate, 100 U/ml Penicillin, 100 μg/ml streptomycin, final pH 7.4
FACS buffer	PBS with 0.1% sodium azide and 1% BSA
2X HBS	280 m*M* NaCl, 10 m*M* KCl, 1.5 m*M* Na_2HPO_4, 12 m*M* dextrose, 50 m*M* Hepes, final pH 7.05
HBSS	Hanks' balanced salt solution (Sigma), 0.35 mg/ml sodium bicarbonate, final pH 7.4
IH Buffer 1	50 m*M* glucose, 10 m*M* EDTA, 25 m*M* Tris•HCl pH 8.0
1X MESA Buffer	40 m*M* MOPS pH 7.0, 10 m*M* sodium acetate, 1 m*M* EDTA
NZY broth	16 g/l NZ broth powder (Gibco), 5 g/l yeast extract, final pH 7.2
PBS	120 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* sodium phosphate buffer, final pH 7.4
Qiagen Wash Buffer	1.0 *M* NaCl, 50 m*M* MOPS pH 7.0, 15% ethanol
Qiagen Elution Buffer	1.25 *M* Nacl, 50 m*M* Tris-HCl pH 8.5, 15% ethanol
RBC Lysis buffer	6 m*M* Na_2HPO_4, 4-6 m*M* KH_2PO_4, final pH 7.4
Restoring buffer	460 m*M* NaCl, 6 m*M* Na_2HPO_4, 1.5-2.5 m*M* KH_2PO_4, final pH 7.4

SOB	20 g/l of bacto-tryptone, 5 g/l of yeast extract, 10 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* $MgSO_4$, 10 m*M* $MgCl_2$, final pH 7.5
SOC	SOB with 20 m*M* glucose
Sodium phosphate buffer	mixture of equimolar solutions of NaH_2PO_4 and Na_2HPO_4, to a desired pH
Southern I Buffer	1.5 *M* NaCl, 0.5 *M* NaOH
1X SSC	150 m*M* NaCl, 15 m*M* sodium citrate, final pH 7.0
1X SSPE	180 m*M* NaCl, 10 m*M* NaH_2PO_4, 1 m*M* EDTA, final pH 7.4
STV (saline-trypsin-versene)	8 g/l NaCl, 0.4 g/l KCl, 1 g/l glucose, 0.58 g/l sodium bicarbonate, 0.5 g/l trypsin, 0.2 g/l EDTA, $4.5x10^{-4}$% phenol red)
Super Broth	12 g/l peptone 140, 24 g/l yeast extract, 0.4% glycerol, 17 m*M* KH_2PO_4, 72 m*M* K_2HPO_4
1X TE	10 m*M* Tris pH 8.0, 1 m*M* EDTA
TAE buffer	40 m*M* Tris, 83 m*M* sodium acetate, 1 m*M* EDTA, final pH 7.9
TFB (transformation buffer)	10 mM MES pH 6.3, 45 m*M* $MnCl_2 \cdot 4H_2O$, 10 m*M* $CaCl_2 \cdot 2H_2O$, 10 m*M* RuCl, 3 m*M* hexamine cobalt (III) chloride
TN	10 m*M* Tris, 150 m*M* NaCl, final pH 7.4
TNFBS	TN† with 1% FBS
TBS	0.2 *M* NaCl, 50 m*M* Tris
TTBS	TBS† with 0.1% Tween-20 (Sigma)
5X Western running buffer	15 g/l Tris, 72 g/l glycine, 5 g/l SDS, final pH 8.3
2X Western sample buffer	4% SDS, 20 m*M* sodium phosphate buffer†, pH 7.0, 20% glycerol, 10% ß-mercaptoethanol, 0.2 *M* dithiothreitol, 0.02% bromophenol blue
Western transfer buffer	20% methanol, 25 m*M* Tris, 192 m*M* glycine, final pH 8.3
YT-carbenicillin plates	1.5 % agar, 8 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 5 g/l NaCl, 250 µg/ml carbenicillin

APPENDIX B. ABBREVIATIONS

ACD	acid, citrate, dextrose
APS	ammonium persulfate
BSA	bovine serum albumin (Fraction V, Sigma)
DEPC	diethylpyrocarbonate
DiI-Ac-LDL	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI)-labelled acetylated low density lipoprotein
DMF	N,N-dimethylformamide
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescent antibody cell sorting
FBS	fetal bovine serum
FITC	fluoroscein isothiocyanate
HBSS	Hank's buffered salt solution (se Solution Recipes)
IPTG	isopropyl ß-D-thiogalactopyranoside
MES	2-(N-morpholino)ethanesulfonic acid
MESA	MOPS, EDTA, sodium acetate (see Solution Recipes)
MOPS	3-(N-morpholino)propanesulfonic acid
PBAC	peripheral blood adherent cels
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline (see Solution Recipes)
PCR	polymerase chain reaction
PEG_{8000}	polyethylene glycol, 8000 average molecular weight
PVDF	polyvinylidene difluoride
RBC	red blood cell
RT-PCR	reverse transcription - PCR
SDS	sodium dodecyl sulfate
SSC	standard sodium citrate (see Solution Recipes)
SSPE	standard sodium phosphate-EDTA (see Solution Recipes)
STV	saline-trypsin-versene (see Solution Recipes)
TEMED	N,N,N',N'-tetramethylethylenediamine
TFB	transformation buffer (see Solution Recipes)

TN	Tris-NaCl (see Solution Recipes)
TTBS	Tween-20, Tris-buffered saline (see Solution Recipes)
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside in DMF

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