MOLECULAR CHARACTERIZATION OF NATURAL RECOMBINANT ALVS AND THEIR USE AS VECTORS FOR GENE DELIVERY INTO STABLE CELL LINES

by

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(Under the Direction of Guillermo Zavala)

ABSTRACT

Avian Leukosis viruses (ALV) previously found as contaminant of Marek's Disease vaccines were molecularly characterized. All three isolates, named PDRC-1039, PDRC-3246 and PDRC-32-49, were found to be recombinant viruses. The mosaic viruses mostly consist of endogenous ALV sequences but, the surface protein (gp85) is of exogenous origin and very similar to ALV-A subgroup. Due to their high similarity to each other, the viruses were considered to be from a common source, despite being from different vaccine companies. One of the viruses, PDRC-1039, was used to construct a novel ALV based retroviral defective vector system for gene delivery, named pBZ system. Eight different vectors were constructed to allow versatility of vector utilization. All vectors are bicistronic with one drug resistant gene, which allow selection of transduced cells. All vectors were tested for transfection and transduction using Green Fluorescence Protein (GFP). The titers achieved with transduction were similar for all vectors. An avian fibroblastoid cell line (DF-1) was used for transduction and expression of GFP. Selected cells were positive for fluorescence during all passages tested, which varies from 5 to 25 depending on the vector design. One of the pBZ vectors, the pBZ3.0, was used to transduce the Infectious Laryngotracheitis Virus (ILTV) glycoprotein I (gI) into DF-1 cells. After

transduction and drug selection with G-418, single colonies were tested for ILTV gI expression by immunoflourescence and westernblot assays. Two clones of DF-1 ILTV gI positive cells were expanded and used as antigen in an inactivated vaccine. SPF chickens were subcutaneously injected with the DF-1 ILTV gI vaccine. Birds were challenged with ILTV USDA reference strain at 28 days of age. All vaccinated chickens mounted a humoral response against the ILTV gI protein. Serum samples from birds vaccinated twice (1 and 10 days of age) had higher antibody levels. There was no difference in protection and viral load in the trachea among vaccinated and control birds. The pBZ system can be used to stably transduce DF-1 cells to express exogenous proteins, and these recombinant proteins can be used to induce humoral responses in chickens.

INDEX WORDS: Avian Leukosis Virus, ALV, Retrovirus, Retroviral Vector, Gene delivery, Transduction, Stable expression, Recombinant protein expression, Avian cells, Vaccine

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DEDICATION

This dissertation is dedicated to:

- the memory of my father, Benedito Corrêa Barbosa, who always emphasized the importance of education and was my role-model for hard work, persistence and personal sacrifice, and who instilled in me the inspiration to set high goals and the confidence to achieve them, without ever forgetting that a man's principles and dignity are his most valuable patrimony. Thank you. Rest in peace father.
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- my daughter, Katherine, who is the most beautiful and precious thing that I have.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Disease prevention and control are fundamental for the success of the poultry industry. Sound biosecurity practices and vaccination are paramount tools for disease control. Effective conventional vaccines have been in use for decades in commercial poultry and consist mostly of naturally avirulent or laboratory-attenuated organisms. Such is the case for viral strains used to control diseases like Marek's Disease, Newcastle, infectious bronchitis and many more. Most vaccine viruses can be easily propagated in embryonated chicken eggs and/or avian primary or secondary cell cultures. However, some important pathogens are difficult to propagate *in vitro* and thus they may not be suitable for replication to high, immunizing virus titers or for economically feasible vaccine production. In addition, vaccine production for some viruses such as avian influenza virus may be highly regulated since a laboratory breach could lead to serious consequences. All such factors and more, combined with potential shortages of SPF embryonated eggs and their cost, justify exploring new vaccine production methodologies that are not dependent on non-adherent cell lines and/or SPF chick embryos and/or primary or secondary cell cultures. The main research goal of this work was to develop a system for subunit vaccine production that would be inexpensive and user-friendly. Such a system would be designed to obviate the use of embryonated eggs for vaccine production, and could potentially be used for immunizations against avian pathogens. To accomplish this goal we chose to explore

the proprieties of non-oncogenic retroviral vectors for gene delivery and expression into well established avian cell lines.

Because of their distinct advantages over other vectors, retroviral vectors have been used for specific gene delivery in several systems. One of the salient advantages of using retroviral vectors is permanent gene transfer for its continued expression. The most important advantage that retroviral vectors offer is their ability to transcribe their single stranded RNA genome into a double stranded DNA molecule that stably integrates into the target cell genome. Thus, retroviral vectors can be used to deliver genes of interest to be stably inserted into the host cell genome for steady gene expression. The genetic structure of retroviruses and the production of double stranded proviral DNA during the replication cycle make it relatively easy to manipulate them in the laboratory. Such characteristics also allow for the construction of replication-defective vectors for transferring desired genes into target cells without the production of parental viruses. After infection with a replication-defective vector, the resulting provirus lacks a complete set of viral genes and thus it cannot generate infectious viral particles.

Our first objective was to molecularly characterize three recently isolated Avian Leukosis Viruses (ALVs). These viruses were found as contaminants of commercially produced Marek's Disease vaccines. All three isolates were fully sequenced and previously characterized *in vitro*, and demonstrated to be non-oncogenic in SPF chickens up to 31 weeks of age. These unique viruses combine very interesting characteristics, including presence of an envelope gene from an exogenous virus (ALV-A), allowing it to infect a large variety of avian cells. The three viruses are recombinants (ALV-E/A) since they carry an exogenous ALV envelope gene (ALV-A) and also contain endogenous (ALV-E) regulatory sequences in their long terminal repeats (LTR),

which decreases considerably the potential for insertional activation of host cellular genes. In fact, all modern commercial meat-type and egg-type chickens and SPF chickens have several such endogenous retroviral sequences inserted into their genomes without any detectable detrimental effects.

Our second objective was to construct a replication-defective vector using these non-oncogenic natural recombinant viruses. To accomplish this objective we used one of the contaminant ALVs, an isolate named PDRC-1039, as donor of the sequences necessary for the construction of the vectors. Eight different retroviral vectors were constructed using the regulatory sequences of PDRC-1039. All vectors are bicistronic and encode one drug resistance gene, either neomycin or zeocin. The other cassette in the vectors allows the expression of any gene of interest. We tested all vectors to transcribe and express the Green Fluorescent Protein (GFP) and their ability to transduce avian fibroblastoid cells. Transduced cells could be clone selected for the drug resistant gene present in the respective vector. Finally, one of the newly designed vectors was used to express ILTV gI protein into an avian cell line. Clone selected cells that express the desired protein were used to vaccinate SPF chickens. Immune responses and protection against challenge were evaluated in vaccinated and control chickens.

This system has shown to have clear benefits, such as the ability to express foreign viral immunogenic proteins without the need for SPF embryonated eggs or primary cell cultures. It would also allow for large scale production of proteins from fastidious viruses, which propagate poorly in standard laboratory systems or highly contagious viruses, which need high biosecurity for vaccine production.

CHAPTER 2

LITERATURE REVIEW

Viruses from several different families have been modified to generate viral vectors for gene delivery. These viruses include retroviruses, adenoviruses, adeno-associated viruses, herpes simplex viruses, picornaviruses and alphaviruses (52, 57, 101). Retroviruses were the first viruses to be modified for gene delivery (69, 119), and retroviral vectors are used in the majority of all gene therapy trials (124). Several characteristics of simple retroviruses have made them useful as vectors for gene transfer/delivery with distinct advantages over other vectors or methods of transduction (86). First, the normal replication cycle of retroviruses includes the integration of the viral genome into the host's chromosomal DNA, and these viruses have evolved a very efficient mechanism of stable gene transfer for this purpose (19). Second, integration takes place in a predictable manner with the long terminal repeat (LTR) sequences flanking the viral genes (30). Third, retroviruses have a broad host range, which allows gene transfer into and expression of foreign genes in many cell types, including cells that are normally refractory to gene transfer by other methods (12). Fourth, non-transforming retroviruses lack cytopathic effect on infected cells, which is especially important for gene expression studies and the generation of stable cell lines (30). Moreover, all retroviral proteins required for the assembly of infectious virions can be supplied in *trans*, enabling the expression of exogenous genes up to

approximately 8,000 nucleotides (87). Finally, retroviral vectors can be conveniently manipulated in their plasmid form and are somewhat flexible in terms of vector design (16).

Retroviruses

Retroviruses comprise a large and diverse family of enveloped RNA viruses defined by common taxonomic denominators that include structure, composition and replicative properties (31). The virions are 80 – 100 nm in diameter, and their outer lipid envelope incorporates and displays the viral glycoproteins (30). The shape and location of the internal protein core are characteristic for various genera of the family. The virion has two copies of RNA which range from 7,000 to 12,000 nucleotides in size, and it is linear, single stranded, non-segmented, and of positive polarity (30). The main characteristic of the retroviridae family is its replicative strategy which includes as essential steps reverse transcription of the virion RNA intro linear double stranded DNA and the subsequent integration of this DNA into the genome of the cell (116). These steps are done by two unique enzymes present in the virions. First is the RNA-dependent DNA polymerase ordinarily known as *reverse transcriptase* and the following step is consummated by an enzyme called *integrase* (31, 121).

Based on viral genome organization retroviruses are divided into two main groups: simple and complex (122). Simple retroviruses usually carry only elementary information, whereas complex retroviruses code for additional regulatory non-virion proteins derived from multiple spliced messages (122). All retroviruses contain three major coding domains with information for virion proteins: *gag*, which directs the synthesis of internal virion proteins that form the matrix, the capsid, and the nucleoprotein structures; *pol*, which contains the information for the reverse transcriptase and integrase enzymes; and *env*, from which the surface (SU) and transmembrane (TM) components of the viral envelope protein are derived. An additional,

smaller, coding domain present in all retroviruses is *pro*, which encodes the viral protease (31, 122).

Replication cycle

Retroviruses are RNA viruses that replicate through an integrated DNA intermediate (117). Retroviral particles encapsidate two copies of the full-length viral RNA, each copy containing the complete genetic information needed for virus replication. Retroviruses possess a lipid envelope, and the virally-encoded envelope protein that is embedded in the membrane interacts with a cellular receptor to enter the host cell (67). Using the viral enzyme *reverse transcriptase*, which is present in the virion, viral RNA is reverse-transcribed into a double stranded DNA copy (115), which is integrated into the host genome by another viral enzyme known as *integrase.* The integrated viral DNA is referred to as provirus and becomes a permanent part of the host genome (117). The cellular transcriptional and translational machinery carry out the expression of viral genes (113). The host RNA polymerase II transcribes the provirus to generate RNA, and other cellular processes modify and transport the RNA out of the nucleus (31). Some viral RNAs are spliced to allow expression of some genes whereas other viral RNAs remain in their full-length form (113). The host translational machinery synthesizes and modifies the viral proteins. The newly synthesized viral proteins and the newly synthesized full-length viral RNAs are assembled together to form new viruses that bud out of the host cells (31).

Genome Structure of Retroviruses

Based on their genome structures, retroviruses can be classified into simple and complex retroviruses (122). Simple and complex retroviruses encode *gag* (group-specific antigen), *pro* (protease), *pol* (polymerase), and *env* (envelope) genes. In addition to these genes, complex retroviruses also encode several accessory genes (122).

The *Retroviridae* family is divided in three subfamilies: *Orthoretrovirinae*, *Spumaretrovirinae* and the unclassified *Retrovirinae* (20). The most important is the *Orthoretrovirinae*, which is divided into 6 genera: *Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, and *Lentivirus* (20). The avian leukosis viruses are classified within the A*lpharetrovirus* genus.

The viral DNA contains redundant sequences at the two ends of the genome designated long terminal repeats (LTRs) (122). LTRs can be further divided into U3 (unique 3'), R (repeat), and U5 (unique 5') regions. The viral promoters and transcriptional enhancers are located in the U3 region (122). The R region is essential for reverse transcription and replication of all retroviruses (31). The U5 region contains sequences that facilitate the initiation of reverse transcription (31). Immediately downstream of the 5' LTR is a primer binding site (PBS) that has sequence complementarity to a portion of a cellular tRNA (122) . Different specific tRNAs are used by different viruses as primers for the initiation of reverse transcription (122). The packaging signal (Ψ) or encapsidation signal (E) are sequences that interact with the viral proteins to accomplish specific packaging of the viral RNA (122) For the most part they are located closer to the 5'end of the viral genomic RNA, but for some virus this locations have not been completely identified (6, 42, 122). Retroviruses preferentially package full-length genomic RNA over spliced viral messages. For most retroviruses, like the Moloney murine leukemia virus (MLV), this preference is likely due to the absence of all or part of the packaging signal on subgenomic RNAs (36). In avian leukosis viruses (ALV), however, the minimal packaging signal, MΨ, is located upstream of the 5' splice site and therefore is present in both genomic and spliced RNAs (9). The packaging of MΨ alone is almost as efficient as the genomic Rous Sarcoma virus (RSV) RNA. This suggests that few, if any additional packaging sequences and/or

structures exist outside of MΨ for encapsidation (8). In contrast, *env* mRNA is not efficiently packaged, even with the presence of MΨ sequence (8).

The coding regions of all retroviruses contain at least three genes. The *gag* gene near the 5' end of the viral genome codes for Gag polyproteins that make up the viral capsid (122). After assembly of the virus particle, the Gag polyprotein is proteolytically cleaved into several proteins including matrix, capsid, and nucleocapsid (113). The *pol* gene encodes *reverse transcriptase* and *integrase* (19, 115). *Reverse transcriptase* transcribes the viral RNA to generate the viral DNA, whereas *integrase* integrates the viral DNA into the host chromosome to form a provirus (19, 115). In all retroviruses, a spliced mRNA is used to express the *env* gene (113). The *env* gene codes for the envelope polyprotein, which is cleaved into the transmembrane domain (TM) and the surface domain (SU) (113). The sequences that encode the viral protease (Pro) are always located between *gag* and *pol* and are most often expressed as either a part of the Gag polyprotein or as a part of the Gag-Pol polyprotein (122).

The region between *env* and the 3' LTR contains a purine-rich region known as polypurine tract (PPT) that is important for reverse transcription (31) The RNA sequence within the PPT is relatively resistant to RNase H degradation. This is important for the initiation of the syntheses of the second strain of DNA during reverse transcription. After serving as primer for DNA syntheses, the PPT RNA sequence is degraded allowing the completion of the reverse transcription. Although all strains of retroviruses generate a defined plus-strand primer from the PPT, some viruses generate additional plus-strand primers from the RNA genome (115).

The 3' LTR has the same sequence as the 5' LTR, and they both flank the entire proviral genome. The genesis of the identity of the LTR elements lies in the process of reverse transcription (RT). The U3 is derived from the sequence unique to the 3′end of the RNA, R is

derived from a sequence repeated at both ends of the RNA, and U5 is derived from the sequence unique to the 5′end of the RNA. So, during the RT step of replication the enzyme "jumps" from one end of the template to the other using the homology of the R sequences (115, 122). Short sequences at the two ends of the LTR are important for integration and are referred to as attachment sites (*att*), which interact with the integrase and are necessary for efficient integration of the viral DNA (19).

Retroviral Vectors

When a replication-competent retrovirus infects a natural host cell, it can form a provirus in the host genome, express viral genes, and release new infectious particles to infect other host cells or hosts (121). In most gene therapy applications, it is not desirable to deliver a replication-competent virus into a patient because the virus may spread beyond the targeted tissue and cause adverse pathogenic effects (127). Therefore, in most retroviral systems designed for gene delivery, the viral components are divided into a vector and a helper construct to limit the ability of the virus to replicate freely (86). The term vector generally refers to a modified virus that contains the gene (s) of interest and *cis*-acting elements needed for gene expression and replication (67). Most vectors contain a deletion (s) of some or all of the viral protein coding sequences so that they are not replication-competent (86). Helper constructs are designed to express viral genes lacking in the vectors and to support replication of the vectors (85). The helper function is often provided in a helper cell format although it can also be provided as a helper virus or as co-transfected plasmids (16). Helper cells are engineered culture cells expressing viral proteins needed to propagate retroviral vectors; this is generally achieved by transfecting plasmids expressing viral proteins into culture cells (85). Most helper cell lines are derived from cell clones to ensure uniformity in supporting retroviral vector replication (67).

Helper viruses are not used often because of the likelihood that a replication-competent virus could be generated through high frequency recombination. Helper functions can also be provided by transient transfection of helper constructs to achieve rapid propagation of the retroviral vectors (27). This transient expression system, normally achieve higher titers, than packaging cell lines (14, 27).

Most retroviral vectors are maintained as bacterial plasmids to facilitate the manipulation and propagation of the vector DNA (86). These DNA vectors can be introduced into helper cells by conventional methods such as DNA transfection, lipofection, or electroporation (86). The helper cell expresses all of the viral proteins (Gag, Gag-Pol, and Env) but lacks RNA containing the packaging signal (85). Viral RNA is necessary for the formation and release of infectious viral particles, but it is not necessary for the formation of "empty" noninfectious viral particles (67). When the vector DNA is introduced into the helper cells, vector RNA containing a packaging signal is transcribed and efficiently packaged into viral particles (2, 58). The viral particles contain viral proteins expressed from helper constructs and RNA transcribed from the vector (86). These viral particles can infect target cells, reverse transcribe the vector RNA to form a double-stranded DNA copy, and integrate the DNA copy into the host genome to form a provirus (86). This provirus encodes the gene (s) of interest and is expressed by the host cell machinery. However, because the vector does not express any viral proteins, it cannot generate infectious viral particles that can spread to other target cells (85).

Packaging Systems

Helper cells are designed to support the propagation of retroviral vectors (85, 125). In helper cell lines that were initially developed, all of the viral genes were expressed from one helper construct (85). The helper constructs for these cell lines were cloned proviral DNAs that

lacked packaging signals (111). These helper cells can support efficient propagation of retroviral vectors. However, a major problem with these helper cells is that replication-competent viruses can be frequently generated during the propagation of the viral vector (85). The helper construct contains most of the viral genome and thus shares significant sequence homology with the retroviral vector (67). The sequence homology can facilitate recombination between the helper construct and the retroviral vector to generate replication-competent viruses (94). Although the helper RNA lacks the packaging signal, it can still be packaged into a virion with a low efficiency (approximately 100- to 1,000-fold less than RNAs containing Ψ) (44). Retroviral recombination occurs frequently between the two co-packaged viral RNAs to generate a DNA copy that contains genetic information from both parents (85). If the helper RNA and the vector RNA are packaged into the same virion, the large regions of sequence homology between the two RNAs can facilitate homologous recombination during reverse transcription to generate a replication-competent virus (67). A similar recombination event can also occur between the helper RNA and RNA derived from an endogenous virus at a lower efficiency to generate replication-competent viruses (67).

For safety reasons, new packaging cell lines have been developed. Further modifications were employed to separate the viral genome, to express gag-pol and env (94). Also known as "split genomes" packaging cells (67). In these helper cells, the viral Gag/Gag-Pol polyproteins are expressed from one plasmid and the Env proteins are expressed from another plasmid. Also, the two helper constructs also contain deletions of viral *cis*-acting elements to reduce or eliminate sequence homology with the retroviral vector (33-34, 85). In these helper cells, genes encoding viral proteins are separated into two different constructs and the viral *cis*-acting elements are located in the vector (86). Therefore, several recombination events have to occur to reconstitute

the viral genome. In addition, reducing the regions of homology decreases the probability that these recombination events will occur. However when homology sequences are present in the packaging cell and the vector (34), recombination events can still result in production of contaminant defective viruses (56). Therefore, helper cells containing split-genome helper constructs that share the minimal necessary sequences with vectors, are considered safer than helper cells containing one-genome helper constructs (67, 85).

Helper cell lines can be designed to express the viral proteins in an inducible manner (67). This is especially useful when some viral proteins are cytotoxic and cannot be easily expressed at high levels. This approach has been used mainly for Human Immunodeficiency Virus (HIV) (4, 129), or for pseudotyping with different envelope proteins (92). Expression of the cytotoxic proteins is limited to the stage in which virus is propagated (73). High viral titers can be achieved by controlling the expression of the cytotoxic proteins, (106).

With the development of efficient transfection methods, transient transfection systems have also been developed for propagation of retroviral vectors (24). In these systems, helper functions are generally expressed from two different constructs, one expressing *gag-pol* and another expressing *env* (24, 67). These two constructs generally share little sequence homology, similar to the split genome packaging system (67). The retroviral vector and the helper constructs are transfected into cells, and viruses are harvested a few days after transfection, with high titers (24).

Other systems include the "Pseudotyping" envelopes, where the viral particles contain a viral genome from one virus and the viral envelope from a different virus (51, 95, 103). The most commonly used pseudo-envelope is the G protein of vesicular stomatitis virus (VSV) (21). VSV G protein has a very broad host range and can infect a variety of cells that cannot normally be

infected with retroviruses (21). Modifications of the surface (SU) portion of the Env protein have also been tested, aiming to modify the interaction of SU with cell receptors, giving or eliminating the ability of the virus to infect certain cells (67).

Avian Leukosis Virus-Based vectors

Vectors derived from all retroviruses genera have been developed (29, 80, 84, 86). The main difference between the oncovirus and lentivirus vectors is the ability to infect non-dividing cells (86). Oncovirus vectors can only be used to efficiently deliver genes into dividing cells. The requirement for cell proliferation can sometimes be used as an advantage to selectively target rapidly dividing cells (for example, cancer cells) (23, 29, 80).

Avian Leukosis Virus-based vectors can be divided into replication competent and replication defective vectors. RSV is the only known acute oncogenic retrovirus that is replication-competent (31). In addition to *gag-pol* and *env*, RSV also encodes the oncogene v-*src* between *env* and the 3' LTR (122). A splice acceptor site upstream of the v-*src* allows the gene to be expressed as a spliced mRNA (122). The ability of RSV to code for an additional gene has inspired the generation of RSV-based replication-competent retroviral vectors. Various modifications have been made to generate a replication-competent viral vector (59, 68-69). In such constructs, the v-*src* was replaced by a splice acceptor site and restriction enzyme sites. DNA fragments can be inserted in the restriction sites to generate a replication-competent vector that expresses the gene of interest. The vector behaves as a regular ALV, infecting cells, integrating its genome and replicating, to infect new cells (68).

ALV has also been used to generate vectors that require helper cells for their propagation (27, 34, 111). The basic structure of an ALV vector also contains the 5' and 3' LTRs, PBS, PPT, and a packaging signal Ψ. Initially, the packaging signal of ALV was believed to extend into the

gag open reading frame, and thus the relevant portions of *gag* were included in ALV-based vectors to achieve efficient packaging (27, 34). Today, it is well known that the packaging signal (Ψ), only extends into the 5' UTR, before the start codon of *gag* (2), allowing the deletion of these overlapping sequences present in the early ALV-vectors. Elimination of these sequences can potentially decrease the production of competent and defective contaminant viruses.

Design of Retroviral Vectors

Vector design involves the construction of a "provirus" that contains all of the signals needed in *cis* for vector packaging, reverse transcription, and integration, but which lacks the coding regions for most or all of the viral proteins, and a corresponding packaging cell line to produce the viral proteins required for viral assembly and transduction (67, 94). Retroviral vector designs have many different modifications that serve various purposes. These modifications may be introduced to permit the expression of more than one gene, regulate gene expression, activate or inactivate the viral vectors, and eliminate viral sequences to avoid generation of a replication-competent virus (86). New vector systems are constantly being developed to take advantage of particular properties of the parent retroviruses, such as host range, usage of alternative cell surface receptors, and levels of tissue-specific expression (86).

U3 Promoter-Driven Gene Expression

Full-length viral RNA is expressed from the retroviral promoter located in the U3 region of the 5' LTR (67). The viral RNA contains the R, U5, 5' untranslated region, a gene of interest, 3' untranslated region, U3, and R (67). The gene inserted between the 5' and 3' untranslated regions can be translated from the full-length RNA that is transcribed from the U3 promoter (86).

During the propagation of viral stocks, it is often desirable to express a selectable marker gene in the vector so that helper cells transfected or infected by the viral vectors can be selected

(67, 94). Therefore, it is often necessary to design retroviral vectors that express a selectable marker gene as well as a gene of interest (86). Drug resistance genes are frequently used as selectable markers, but other marker genes, such as the green fluorescent protein gene, can also be used to select for transfected or infected cells (1, 83, 86). The expression of two foreign genes in a retroviral vector can be achieved by expressing the 3' gene with the aid of an internal promoter, RNA splicing, or an internal ribosomal entry site (IRES) (67, 94).

Vectors That Use an Internal Promoter to Express Additional Genes

This type of vector is designed to express more than one gene (gene A and gene B). A full-length RNA and subgenomic RNA can be synthesized from this vector (86). The full-length RNA that is expressed from the viral U3 promoter is used to translate the gene A protein and the subgenomic RNA that is expressed from the internal promoter is used to translate the gene B protein (67, 86).

Several retroviral vectors containing internal promoters have been successfully utilized for expression of two genes (17, 50). However, potential interference between the U3 and the internal promoter can occur. For example, it has been shown that the SNV U3 promoter and the herpes simplex virus thymidine kinase promoter can interfere with each other in SNV-based retroviral vectors (45-47). Promoter interference can lead to reduced levels of transcription from either the U3 or the internal promoter (47).

Vectors That Use Splicing to Express Additional Genes

All retroviruses express *env* by regulated splicing (31, 113). The splice donor site that is used to express *env* is located in the 5' region of retroviruses (113, 122). During replication, some full-length viral RNAs are spliced to produce subgenomic viral RNAs that are used to express the Env proteins (113). Splicing vectors were developed by using the same principle to express two

different genes by using the viral splice donor and splice acceptor sites (34, 43). The splice acceptor site is located between genes A and B. The full-length RNA is used to translate the gene A protein, and the spliced RNA is used to translate the gene B protein (34, 43). Although two mRNAs are synthesized from this vector, only the full-length viral RNA contains the packaging signal and is efficiently encapsidated into viral particles (34, 56).

The advantage of splicing vectors is that only one promoter is necessary, and the potential for promoter interference is eliminated (67). However, the disadvantage of splicing vectors is that the efficiency of splicing can be significantly influenced by the vector RNA sequence (43). As a result, expression of two genes using splicing vectors is unpredictable, and splicing vectors are not used as commonly as other vectors (94).

Vectors That Use Translational Control Signals to Express Additional Genes

It was first demonstrated in picornaviruses that sequences in the mRNA can serve as signals that allow the ribosome to bind to the middle of an mRNA and translate a gene far from the 5' end of the mRNA (70, 96). These sequences (named internal ribosomal entry sire, or IRES), are now commonly used in retroviral vectors (1, 83, 123). The IRES sequence is inserted between gene A and gene B. Only one mRNA is synthesized from this vector. However, the mRNA is bicistronic because of the presence of IRES, and it is used to translate both gene A and gene B (67). Gene A is close to the 5' cap, and ribosomes that bind to the 5' end of the mRNA use normal mechanisms to synthesize the gene A protein efficiently (110, 112). Gene B is immediately 3' to the IRES, and ribosomes that bind to the IRES can synthesize the gene B protein in a cap-independent manner (112). This strategy has been used successfully to express many different genes (83, 110). The gene expressed with an IRES is generally translated less efficiently than the gene located near the 5' end of the mRNA (37). Therefore, the gene of interest

is often placed at the 5' end near the cap, whereas the selectable marker is placed immediately 3' to the IRES. Recently, newly designed vectors with wild type IRES sequences achieved high expression of the gene located 3' of IRES (83), when compared to IRES modified sequences, which are present in most of commercially available vectors.

Recombinant protein expression *in vitro*

Recombinant DNA technology allows placing a gene within a new genetic environment, away from the normal regulatory constraints of their natural environment and allows for their expression in a controlled manner (40). This technology is used to transcribe and translate prokaryotic or eukaryotic genes to achieve high-level expression of gene products (93). Proteins expressed using this technology are called "recombinant proteins". A number of functional studies can be performed using the recombinant purified protein, such as protein-protein interaction experiments, enzyme kinetics, functional studies of the protein, subunit vaccines, and structural studies including protein crystallization, protein structure studies and NMR (Nuclear Magnetic Resonance).

There are two main systems for the expression of recombinant protein: A prokaryotic (bacterial) or eukaryotic (insect, yeast, mammalian or avian cell) system.

Prokaryotic systems

Prokaryotic recombinant protein expression systems have several advantages, which include ease of culture and very rapid cell growth (93). Also, expression can be induced easily in bacterial protein expression systems using Isopropyl β-D-1-thiogalactopyranoside (IPTG). Purification is quite simple in prokaryotic expression systems and there is a plethora of commercial kits available for recombinant protein expression (93). However, if the recombinant

protein has eukaryotic origin and it is needed for functional or enzymatic studies, prokaryotic systems may be problematic, as most proteins become insoluble in inclusion bodies and are difficult to recover as functional proteins (72, 108). Furthermore, most if not all post-translational modifications are not added by bacteria and therefore such proteins of interest may not be functional (26).

The gram-negative bacterium *E. coli* was the first organism utilized for the production of recombinant proteins. It is still extensively used for industrial applications (40). A large amount of knowledge has been generated about its molecular biology, biochemistry, and physiology (5, 114). *E. coli* is easy to grow to high cell densities, and has simple nutritional requirements that can be satisfied with fully defined simple media (93). Despite its proven success, recombinant protein production in *E. coli* has several drawbacks. *E. coli* is usually not capable of efficiently producing very long or short proteins (41). Proteolytic cleavage and disulfide bond formation seldom occur, and post-translation modifications, including glycosylation, acylation, and isoprenylation, are not performed (93). If neither of such modifications is required for obtaining an adequate product, then bacteria may be the host of choice. Other concerns about the expression of recombinant protein in *E. coli* include variability in the level of expression, protein solubility, and protein purification (81).

Eukaryotic systems

Yeast and Fungi

Yeasts have been utilized by humans since the very early ages (93). Their various applications in the food industry and for single-cell protein production have taken yeast fermentations to the largest volumes ever performed (102), on the scale of thousands of liters per batch. The yeast *Saccharomyces cerevisiae* was the first yeast species to be manipulated for

recombinant protein expression (76). *S. cerevisiae* can, as other yeasts, secrete recombinant proteins to the culture medium and intracellular proteins are usually properly folded (76). As other eukaryotes, yeasts are also capable of performing most post-translational processing typical of mammalian cells (93).

However, extracellular proteases and differences in glycosylation in proteins expressed in yeast, compared to those of mammalian and avian cells, limit their use for some specific protein applications. Unmodified yeasts are proper for production of proteins that do not require mammalian-type glycosylation and are resistant to proteases. One example of this type of protein is insulin, which has been commercially produced in *S. cerevisiae* after enhancing its folding and secretion capacities through genetic engineering (76).

Facultative methylotrophic yeasts, such as *Pichia pastoris, Pichia methanolica, Candica boidinii, and Pichia angusta* are hosts with great potential and with various recombinant proteins within reach or already in the market (55). Very large fermentations of methylotrophic yeast were performed in the 1970's, but it was not economically attractive. *P. pastoris* was proposed as a host for recombinant protein production in the 1980's (26). Very high cell densities have being described, obtaining up to 1g/L of secreted recombinant protein (25). Examples of recombinant proteins produced in methylotrophic yeast that are available commercially include the hepatitis B vaccine, human serum albumin, phytase, insulin-like growth factor, and the avian infectious bursal disease Virus VP2 subunit vaccine (55, 126).

A potential disadvantage of *P. pastoris* is that transgenes are placed under the influence of a promoter of the alcohol oxidase I (AOX1) gene, which requires methanol to induce gene expression (15). This has some implications for the process; first, large tanks of flammable methanol are needed in the production facilities, and methanol, which is toxic to humans, must

be thoroughly removed from the final product. Methanol is also toxic to the cells, making it necessary to specifically design methanol-feeding strategies to implement and guarantee its continuous supply during the induction stage while avoiding its accumulation to inhibitory levels (66, 93).

Insect cells

The initial studies demonstrating the potential of recombinant baculovirus infection of insect cells for the production of heterologous proteins were published in the early 1980's (97, 109). Several improvements in vector design facilitating the differentiation and isolation of recombinant viruses from wild-type virus associated with the widespread availability of these reagents and the simplicity of insect cell culture have resulted in this system being one of the most widely used approaches for heterologous protein expression (3, 120). Numerous recombinant proteins have been produced in insect cells using this system. Examples include many classes of proteins, which are widely used in pharmaceutical research for target validation studies, assay development, antibody generation and structural analysis (35, 82, 109, 120).

Recently, the safety and immunogenicity of a baculovirus-expressed hemagglutinin influenza vaccine has been described (35, 91). In order to mimic the glycosylation patterns of membrane bound and secreted proteins produced in mammalian cells significant progress has been made in engineering both viral vectors and host cell lines (77).

Another valuable application of the system has been the production of virus-like particles (VLPs) and protein complexes (90). This approach has proven particularly valuable for the production of human papilloma virus (HPV) VLPs to develop vaccines for the prevention of infection with human HPV (62). Recent studies have also demonstrated the feasibility of this

system for the production of severe acute respiratory syndrome (SARS) coronavirus-like particles and Hepatitis C VLPs (71, 88).

In addition to its role as an insect cell protein expression system, baculovirus mediated gene delivery into mammalian cells has recently evolved into a particularly useful research tool (78). It has been shown that baculoviruses modified to contain mammalian cell-active promoters, such the human Cytomegalovirus (hCMV), could be used to efficiently transduce several cell types of mammalian origin (22, 77-78). Undoubtedly, the application of baculovirus for expression of recombinant proteins in insect cells will continue to serve as an important tool in biotechnology and pharmaceutical research programs.

Mammalian and Avian cells

Animal cells have been cultured *in vitro* for more than a hundred years, primarily for viral replication, which served to study the biology of these organisms. Cell culture also has been used for vaccine production, by means of replication of attenuated viruses (live vaccines) or wild type viruses (inactivated vaccines) (11, 13, 75). The complex biology of mammalian and avian cells has delayed their used for recombinant protein production (93), compared to previously described systems. However, the majority of recombinant protein products recently approved by the Food and Drug Administration (FDA) have been produced using animal cells (93). Such successful recombinant protein production in animal cells came after overcoming many troubles, including the cellular fragility and the complex nutritional requirements of these cells (32). For instance, animal cells require hormones and growth factors that are normally provided by bovine serum, but to avoid possible contamination in large-scale production systems, serum-free media have been developed (93).

One of the main reasons to use animal cells for recombinant protein expression is the requirement of post-translation modifications like glycosylation occurring in the "natural" environment of the protein (100). However due its complexity, post-translation modifications are in some cases species-specific and special care has to be taken to avoid unwanted immune responses to recombinant proteins expressed in heterologous cell species (100).

Gene transfer is a particularly relevant issue in cell culture. The development of mammalian cell culture methodologies included designing of a variety of vector systems and gene transfer methods (32). Basically, the most frequently used methods for gene transfer in mammalian and avian cells are: electroporation, liposomes, and viral based vectors.

Electroporation is an established technique that has been used in a variety of applications, including but certainly not limited to introducing exogenous nucleic acids into cells. The electroporation technique was first used to introduce DNA into murine fibroblasts (89). Later improvements adapted the technique to introduce DNA into a broad range of established cell lines (98-99). Electroporation has unique advantages over methods that involve endocytosis and/or phagocytosis since direct uptake of DNA bypasses the lysosomal compartment, limiting the possibility of enzymatic degradation (98). During the electroporation process, cells are exposed to several electric pulses of varying intensity and duration, creating a transmembrane potential across the poorly conducting cell membrane. Once this transmembrane potential reaches a certain threshold, which is proportional to the cell radius and the applied electric field strength, the molecular structure of the membrane will be rearranged to create hydrophilic pores that are permeable to DNA (98). These pores can be transient in nature and resealed to preserve the integrity of the cell. An excessive electric field can cause a transmembrane potential

exceeding a tolerable threshold resulting in pores that cannot be resealed leading to the destruction of the cell (98).

Liposomes are typically formed by mixing a cationic lipid with a neutral lipid (53). The positively charged cationic lipid interacts with the negatively charged nucleic acid to form a liposome/nucleic acid complex, which can be taken up by cells via endocytosis (74). After entering the cells, liposome/nucleic acid complexes appear within endosomes. The endosomes release their contents into cytoplasmic compartments and the nucleus (53). The release of nucleic acids from the endosome is believed to be facilitated by the neutral lipid in the liposome/nucleic acid complex. Some neutral lipids may also assist fusion of the complex with the outer cell membrane to enhance the lipofection efficiency (48-49). Recent refinements of the liposome technology have led to the development of uniform-sized micelles to deliver DNA with greater efficiency and reproducibility (74).

The use of recombinant viruses to infect mammalian and avian cells has emerged as one of the preferred means to deliver exogenous genes to these cells. Adenoviruses, adeno-associated viruses, and retroviruses/lentiviruses have all proven to have utility for gene transfer. All these methods carry the conspicuous advantages of high efficiency gene transfer into mammalian cells, which can result in high expression of the gene product. Adenoviruses are virions that have a double-stranded DNA genome with sizes ranging from 26 to 45 kb. Adenoviruses can replicate in either quiescent or dividing cells after infection. A relatively high level of protein expression can be achieved following transduction, and estimates of as high as 35% of total cellular protein have been reported (54). Since transduced genes remain epichromosomal, activation or inactivation of host genes following integration is avoided. Postinfection viability of the host cells remains at nearly 100% for most mammalian cell types; thus, the process is well tolerated.

For these reasons recombinant adenovirus is the vector of choice for many protein over-expression studies, but not for stable expression (65, 130).

Over the past decades, retroviruses have been used as a means to deliver genes into mammalian and avian cells. Retroviruses exhibit several characteristics which render them excellent vehicles for the transfer and expression of exogenous genes (86) (also described previously in this review). First, the relatively small genome of retroviruses is easily manipulated to allow the insertion of foreign genes and the ability to complement defects in viral replication *in trans* allows the use of replication-defective retroviruses. Second, the viruses can easily be produced at high titers in culture. Moreover, the efficiency of infection of susceptible cells is extremely high, approaching 100% in some cases. Third, retroviruses carry powerful transcriptional enhancer elements ensuring high levels of expression in a wide range of cell types.

Simple retroviruses only integrate into the DNA of replicating cells (86). As a result of this chromosomal integration, the progeny of the infected cells inherit the retroviral DNA (31). When these retroviruses are used for gene delivery as defective vectors, they deliver exogenous genes into target cells, but cannot replicate on their own and infect unrelated cells (18). These attributes make replication-defective recombinant retroviruses ideal for use as tracers of cell lineage during development and stable insertion of exogenous genes in susceptible cells (17, 63, 79).

Examples of Avian Diseases in need of better vaccines

Infectious Laryngotracheitis virus

Infectious laryngotracheitis (ILT) is a highly contagious acute respiratory disease of chickens, of worldwide distribution. The disease causes great economic losses during severe

outbreaks due to a decrease in egg production and high mortality rates (60). Infectious laryngotracheitis virus (ILTV) is a member of the genus Iltovirus, within the family *Herpesviridae*, subfamily *Alphaherpesvirinae* (38). ILTV establishes a primary lytic infection in the trachea followed by periods of latency in the trigeminal ganglia with episodes of reactivation where viral replication resumes in the trachea (60).

Modified-live attenuated ILT vaccines have been the principal tool utilized to control the spread of the disease (60). Traditionally, two types of modified-live ILT vaccines have been used to control ILTV; vaccines attenuated by multiple passages in embryonated eggs, also known as CEO (chicken embryo origin) vaccines (107); and the Tissue Culture Origin (TCO) vaccine, which was generated by multiple passages in tissue culture (28). Several CEO vaccines and one TCO vaccine are commercially available. The CEO vaccines are labeled for administration by water and spray in addition to the eye drop method. The TCO vaccine is labeled for eye drop administration only. In addition to modified live attenuated ILT vaccines, there is also a fowl poxvirus-vectored (FPV) ILT recombinant vaccine (FP-LT); and a Herpesvirus of Turkeys-vectored (HVT) recombinant vaccine. Both recombinant vaccines are commercially available for subcutaneous or *in ovo* administration (39, 118).

Modified-live ILT vaccines have been associated with a variety of adverse effects including spread of vaccine virus to non-vaccinated birds (64, 104-105), production of latently infected carriers (7), and increased virulence as a result of consecutive back passages *in vivo* (61). Also, live attenuated ILT vaccine viruses have been implicated in field outbreaks of the disease (61).

ILTV inactivated vaccines lack problems such as residual virulence, reversion to virulence, or spread of infection and establishment of latent infection, which occur with modified

live vaccines. Inactivated ILT vaccines have been made from inactivated whole ILTV (10) and from affinity-purified glycoproteins (128). Inactivated vaccines are capable of stimulating immune responses in chickens, resulting in some levels of protection against ILTV challenge (10, 128). Such methodologies have not been cost effective, but new technologies for recombinant protein expression may allow the utilization of such type of vaccines in the future.

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CHAPTER 3

MOLECULAR CHARACTERIZATION OF THREE RECOMBINANT ISOLATES OF AVIAN LEUKOSIS VIRUS OBTAINED FROM CONTAMINATED MAREK'S DISEASE VACCINES[1]

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Summary

Three natural recombinant avian leukosis viruses (ALV) (PDRC-1039, PDRC-3246 and PDRC-3249) expressing a subgroup A gp85 envelope protein and containing long terminal repeats (LTR) of endogenous ALV-E viruses were isolated from contaminated commercial Marek's Disease vaccines, cloned and completely sequenced. Their full genomes were analyzed and compared with representative strains of ALV. The proviral DNA of all three isolates displayed 99.3% identity to each other, suggesting a possible common ancestor, even though the contaminating viruses were obtained from three separate vaccine serials produced by two different vaccine manufacturing companies. The contaminating viruses have a genetic organization typical of replication-competent *Alpharetroviruses*. The proviral genomes of PDRC-1039 and PDRC-3246 are 7497bp long and the PDRC-3249 is three base pairs shorter, due to a deletion of a Threonine residue within the gp85 coding region. The LTR, *gag*, *pol* and the transmembrane region (TM, gp37) of the *env* gene of all three viruses are highly similar to endogenous counterpart sequences (>98%). Only the SU (gp85) region of the *env* gene is highly similar to exogenous ALV-A (98.7%). Locus-specific PCR analysis for ALV endogenous sequences (*ev* loci) in the chicken embryo fibroblasts used to produce the original vaccine vials identified the presence of *ev*-1, *ev*-2, *ev*-3, *ev*-4 and *ev*-6 in all three vaccines. Homologous recombination most likely took place to involve the SU region of the *env* gene, since the recombinant viruses only differ in this particular region from the consensus ALV-E. These results suggest that the contaminating ALV isolates probably emerged by recombination of ALV-A with endogenous virus sequences prior to vaccine preparation.

Key words: avian leukosis virus recombinant, endogenous virus, exogenous virus, vaccine

Abbreviations: ALSV = avian leukosis sarcoma virus; ALV = avian leukosis virus; ALV-A = avian leukosis virus subgroup A; ALV-B = avian leukosis virus subgroup B; ALV-C = avian leukosis virus subgroup C; ALV-J = avian leukosis virus subgroup J; C/EBP = CCAAT/enhancer binding protein; CEF = chicken embryo fibroblasts; env = envelope gene; *ev* = endogenous virus; gag = GAG gene; gp37 = glycoprotein 37, or transmembrane protein (TM) in the envelope gene; gp85 = glycoprotein 85, or surface envelope protein (SU) in the envelope gene; LTR = long terminal repeat; MDV = Marek's disease virus; PAS = polyadenylation site; PCR = polymerase chain reaction; ORF = open reading frame; pol = polymerase gene (POL); PRE = pentanucleotide repeat element; R = repeat region of the LTR; REV = reticuloendotheliosis virus; SPF = specific pathogen free; U3 = unique region 3 in the LTR; U5 = unique region 5 in the LTR.

Introduction

The avian leukosis sarcoma viruses (ALSV) induce a variety of neoplasms such as B-cell lymphomas, erythroblastosis, myelocytomas and other types of neoplasia (25, 27). Avian leukosis viruses (ALVs) isolated from chickens are classified based on their host range, cross-neutralization and viral interference into 6 groups (A, B, C, D, E and J) (9). Further classification as endogenous (ALV-E) and exogenous (ALV-A, B, C, D and J) viruses is based on their mode of transmission. Exogenous viruses can be transmitted

horizontally or congenitally via the egg, while endogenous viruses are transmitted primarily through the chicken germ line (genetic transmission), but can also be transmitted horizontally and congenitally (5, 8, 24). ALV-E is expressed from *ev* loci, which are inheritable proviral elements. At least 22 *ev* genes have been described in White Leghorn chickens (4). Most of these *ev* genes are structurally incomplete (defective) and therefore do not encode all sequences necessary for production of infectious virus particles. However, some of the *ev* loci, such as *ev*-2, -14, -18, -19, -20 and -21, have the ability to produce infectious virus particles, albeit with low or no oncogenicity. Other *ev* genes such as *ev*-7, -10, -11, -12, can also produce infectious virus particles in the presence of a nearly intact provirus like *ev*-1 (7, 36). Recombination is believed to occur in these cases due to the large amount of ALV RNA produced in cells that carry certain *ev* genes (4). Recent studies describing the sequences of *ev*-1, -3 and -6 proved that the DNA sequences in those loci (except for some deletions) are very similar (19), and it has been proposed that all *ev* genes may have derived from one another (3).

Recombination in retroviruses is thought to be facilitated by the diploid nature of their genomes (14). Various types of mutations including deletions may arise during reverse transcription from inter-strand homologous recombination between viruses of the same or similar species or from non-homologous recombination with host genome sequences (14). The emergence of the novel subgroup ALV-J in the late 1980's is now credited to a possible recombination of an exogenous virus backbone with envelope gene sequences of an ancient endogenous retrovirus family (EAV-HP, or EV/J) (29, 32). A natural recombinant ALV containing an ALV-B envelope and the LTR of ALV-J has

been isolated from commercial egg-type chickens (22). Furthermore, a virus with an ALV-A envelope and an LTR from ALV-J was identified after propagation of ALV-J in cells that continuously transcribe and express the ALV-A envelope gene (21).

The long terminal repeat (LTR) of endogenous viruses differs from the LTR of exogenous viruses in that it contains less functional transcription regulatory elements (2, 26), partially explaining why endogenous viruses are less oncogenic, replicate at a slower rate or may be transcriptionally silent.

Alpharetroviruses and *Gammaretroviruses* have been described as contaminants in vaccines intended for use in commercial poultry. Accidental contamination of commercial vaccines with reticuloendotheliosis virus (REV) has been documented (11, 14, 20, 23, 31, 34, 35). Vaccine contamination with ALV is not considered a common occurrence, but it has been reported recently (10, 38). REV and ALV may be introduced accidentally in vaccine preparation, potentially by contamination of flocks used to produce specific pathogen-free (SPF) embryos for vaccine production or by accidental contamination of master or working seeds at vaccine manufacturing plants. We describe herein the molecular characterization of the full genome of three previously described (38) ALV isolates obtained from contaminated Marek's disease vaccines.

Materials And Methods

Cells and viruses. The viruses examined in the present study were all previously isolated from commercially produced Marek's disease vaccines (38). After isolation and purification as previously described (38), the viruses were propagated in DF-1 cells (15)

for three consecutive passages and the virus stocks were kept at -80C until used. DF-1 cells constitute a fibroblastoid cell line derived from line 0 chicken embryos and thus display a C/E phenotype; i.e., they are permissive to exogenous ALV, but not to endogenous viruses (30). The PDRC-1039 isolate was obtained from a vaccine lot produced by one vaccine company herein designated as "A"; isolates PDRC-3246 and PDRC-3249 were obtained from two different vaccine lots produced by vaccine company "B" as previously described (38, 39).

DNA isolation, cloning and sequencing. Infected DF-1 cells or original vaccine fibroblasts were used for DNA extraction and purification using a commercially available DNA extraction kit (High Pure Template Preparation kit, Roche Diagnostics GmbH, Mannheim, Germany). For full provirus genome cloning, total genomic DNA from infected DF-1 cells was used as a template for PCR amplification using four different sets of overlapping primers (Table 1). The PCR reactions were carried using a proofreading polymerase enzyme (Platinum® *Pfx* DNA polymerase, Invitrogen, Carlsbad, CA), as per the manufacturer's recommendations. The PCR products were visualized in agarose gels using a "Dark Reader" (Clare Chemical Research, Dolores, CO), which does not use UV light in order to avoid cDNA damage. Specific products were gel-purified using a commercial gel extraction kit (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA). The purified products were cloned into pCR2.1 or pCR XL plasmids depending on their sizes following the manufacturer's recommendations (Topo cloning kit, Invitrogen, Carlsbad, CA). After screening and purification, the plasmids were used as templates for sequencing using the dideoxynucleotide method with the BigDye v3.1 terminator kit (Applied Biosystems, Foster City, CA). Sequencing readings were performed in an ABI

PRISM™ model 310 or ABI PRISM™ 377 sequencer (Perkin Elmer Corp., USA). At least 2 clones for each segment from single PCR amplifications were sequenced. The overlapping primers used for cloning and sequencing are listed in Table 1.

Endogenous virus sequence identification. Endogenous *ev* loci present within the genomic DNA of the CEFs in the original vaccine vials were identified based on methods previously described by Benkel (1). Briefly, genomic DNA from vaccine vials was extracted and purified as described above. The PCR primers used to detect *ev* loci were directed against the *ev* LTRs and sequences flanking known chromosome integration sites. Amplification of the targeted sites produced fragments of expected lengths indicating the presence or absence of specific *ev* loci. Eleven different loci were tested, including *ev*-1, -2, -3, -4, -6, -7, -9, -12, -15, -16 and, -21. All of such loci have been described in egg-type chicken lines. The annealing temperatures described for a touchdown PCR (1) were modified to 2 cycles of 63 C, 2 cycles of 60 C, 3 cycles of 57 C and 25 cycles of 55 C.

Sequence analysis. The overlapping proviral sequences cloned were aligned for preparation of contiguous sequences using the Seqman 6.1 function in the DNASTAR sequence analysis software (DNASTAR, Inc., Madison, WI). The sequences resolved were aligned with sequences available in GenBank using the Clustal W alignment with weighted residues method in the MegAlign 6.1 sequence analysis function in DNASTAR (DNASTAR, Inc., Madison, WI, Madison, WI).

Results

The complete genomes of the ALV isolates PDRC-1039, PDRC-3246, and PDRC-3249 were each amplified in four different overlapping regions (Table 1). PCR size products based on the isolate PDRC-1039 were the following; LTR-F and ALV-BR primers amplified a 1109 bp product (nucleotide position 1 to 1109), ALV-BF and ALV-HR primers amplified a 3583 bp product (nucleotide position 489 to 4072), ALV-HF and CapA primers amplified a 2430 bp product (nucleotide position 3472 to 5902) and H5 and LTR-R primers amplified a 2289 bp product (nucleotide position 5208 to 7497). The PCR products were successfully cloned and sequenced using either the primers described in Table 1 or the plasmid primers M13 Forward and M13 Reverse. The complete proviral genomes of all three isolates were determined after producing contiguous sequences for each one of the viruses. The PDRC-1039 and PDRC-3246 isolates (vaccine manufacturing companies A and B, respectively), contained 7,497bp long, whereas PDRC-3249 (vaccine manufacturing company B) contained 7,494bp. The complete proviral sequences of PDRC-1039, PDRC-3246 and PDRC-3249 were deposited in GenBank with the reference numbers EU070900, EU070901, and EU070902 respectively. All three isolates display a genetic organization typical of replication-competent *Alpharetroviruses*. The *env* gene is expressed from a spliced mRNA with a 5' leader sequence (first 18 bp of the *gag*/*pol* ORF). The *gag*/*pol* gene is situated in the same continuous ORF. Overall the three virus isolates share at least 99.3% identity, suggesting the possibility of all three viruses sharing a common ancestor. The difference in length between the isolates is an insertion of three bases (ACA) encoding a threonine

residue at position 114 of the surface envelope protein gp85 (SU), also seen in the ALV-C reference isolate (Figure 1) and in the *ev*-6 locus (data not shown).

The sequence identities between the three isolates and representative strains of ALV-A (GenBank NC 001408), ALV-B [RCASBP(B) vector] (18), ALV-C (GenBank AF033808), ALV-J (HPRS103 strain, GenBank Z46390), *ev*-1 (GenBank AY013303), *ev*-3 (GenBank AY013304) and *ev*-6 (GenBank AY013305) are shown in Table 2. The *gag/pol* genes are the most conserved genes of members of the ALSV, sharing at least 95.5% nucleotide identity. All three contaminating isolates exhibited very similar sequences to the *ev*-1 locus with at least 99.0% identity for the *gag* gene and up to 99.6% for the *pol* gene.

Sequence analysis of the proviral LTRs revealed that all three contaminating isolates contain 274 bp in their LTR region (Figure 2), consistent with endogenous LTRs, but not with exogenous virus LTRs. The proviral LTRs showed low identity to exogenous ALVs, represented by less than 59% compared to ALV-A, ALV-B and ALV-J; and 71.5% nucleotide identity to ALV-C. However, the LTRs of all three contaminating ALVs were highly similar to endogenous loci, represented by at least 98.2% identity of PDRC-1039 to *ev*-1 and -6, and as high as 99.6% identity of PDRC-3246 to *ev*-3 (Table 2). Within the LTR, the R region of the contaminating viruses showed little divergence compared to *ev* loci, with the PDRC-3246 isolate being 100% similar to *ev* loci, while isolates PDRC-1039 and PDRC-3249 displayed a single point mutation on nucleotide 14 (Figure 2), leading to a lower nucleotide sequence identity in this short but critical region (95.7%). U5 in all three isolates was 100% similar to the *ev*

loci analyzed and showed only 82.9% to 93.4% identity to the U5 of exogenous viruses (Table 2).

The U3 region, which contains important transcription regulatory elements (26, 37) showed the least identity between the nucleotide sequences of the contaminating viruses and equivalent sequences of exogenous reference viruses. The U3 sequences in the contaminating isolates exhibited less than 40% identity to U3 in ALV-A, ALV-B and ALV-J. The same region displayed only 46% identity to U3 in ALV-C. In contrast, the U3 sequences in the contaminating ALVs were most similar (97.7%) to the U3 sequences of endogenous loci. None of the differences noted in the U3 sequences of the contaminating viruses involved any of the known regulatory elements present in the U3 region of *ev* loci (Figure 2). The CCAAT enhancer box present within the first 20 bp of U3 in exogenous viruses is not present in the contaminating viruses or in the *ev*- loci (26). However, the three contaminants and all *ev* loci examined share the same consensus sequence (TT/GNNGC/TAAT/G) (28) at position 104 to 113, which constitutes a functional enhancer box (Figure 2). The CCAAT-like box is only found in exogenous viruses ALV-C and ALV-J (Figure 2). Only the first of two CArG boxes ($CC(A/T)_6GG$) known to be present in exogenous viruses is present in the contaminant ALVs, a feature that is characteristic of endogenous loci LTRs (Figure 2) (37). The Y Boxes (with sequence ATTGG, also known as inverted CCAAT boxes) are conserved in exogenous and endogenous viruses (37), but the PRE-Boxes (with sequence GGTGG) are absent in the vaccine contaminants and the *ev* loci. The TATA box (TATT/ATAA consensus) and the polyadenylation signal (AATAAA) are well conserved in all exogenous, endogenous and recombinant contaminating viruses (Figure 2).

The gp85 (SU) and gp37 (TM) coding regions in the envelope gene of the contaminants most probably have two different origins. The gp85 amino acid sequence in the contaminants is highly similar (>98%) to gp85 in ALV-A; less than 88% similar to endogenous virus sequences; and only 51% similar to ALV-J (Table 2). Both hypervariable regions (hr) within gp85 of the contaminants are highly similar to ALV-A (Figure 1). The variable regions (vr) 1 and 2 showed nucleotide residue substitutions similar to those in ALV-C and endogenous virus sequences, but vr3 is identical to ALV-A. Relative to gp85 in PDRC-3246 (vaccine company B) and PDRC-1039 (vaccine company A) have an insertion of a threonine residue in position 114, which is only seen in ALV-C. Two residue substitutions (glutamic acid on residue 9 and proline on residue 160; the former within variable region 2) are seen in both the contaminants and endogenous viruses. The contaminant viruses PDRC-1039 and PDRC-3246 have an amino acid substitution at residue 73, where the predicted glycosylation site 73NRT75 is change to 73DRT75. This substitution is caused by a point mutation (A-G) at nucleotide 217 of the *env* gene, changing the codon from an asparagine to an aspartic acid residue. Lost of this N-linked glycosylation site may cause antigenicity changes in this isolates, but it remains to be tested.

The gp37 amino acid sequences of the contaminant viruses are almost identical to equivalent sequences in endogenous viruses (≥99% of identity). The same region is only 95.4% similar to ALV-A, and less than 60% similar to ALV-J (Table 2). Thus, within the envelope gene of the contaminant viruses gp85 was most similar to equivalent ALV-A sequences, whereas gp37 was most similar to endogenous virus sequences.

To identify the profile of *ev* loci in the original MDV vaccine preparations we used *ev*-specific loci PCR analysis for *ev*-1, -2, -3, -4, -6, -7, -9, -12, -15, -16 and, -21. All three vaccine stocks (PDRC-1039, PDRC-3246 and PDRC-3249) produced exactly the same results, since the same *ev* loci (*ev*-1, -2, -3, -4, and -6) were detected in the genomic DNA of the chicken embryo fibroblasts of all three vaccines. For this analysis, the DF-1 cell stock used for isolation of contaminant viruses was utilized as a control and this stock was negative for all *ev* loci tested (Table 3).

Discussion

The complete proviral genomes of three recombinant replicating avian leukosis viruses isolated from contaminated Marek's Diseases vaccine were fully sequenced. All three viruses were shown to be extremely similar to each other at the nucleotide level (>99.3%), demonstrating that most likely they share the same ancestor. The use of proof reading DNA polymerase for the PCR reactions supports the results that the three isolates are closely related viruses. However these minor changes can still be mistakes added by the DNA polymerase, as its error rate is low but not null. Nucleotide and amino acid sequence analyses and comparison against previously known endogenous and exogenous ALV sequences revealed that the LTRs, *gag*, *pol* and TM (gp37) of PDRC-1039, PDRC-3246 and PDRC-3249 are highly similar to corresponding sequences in endogenous viruses. Thus most of the contaminant ALV genomes have an endogenous origin, with the gp85 coding region of the envelope gene being the sole exception, as this region in all three contaminant viruses was highly similar to the gp85 region of exogenous ALV-A

(Table 2). Hypervariable regions 1 and 2 and vr3 on gp85 in the contaminating viruses are identical to each other and to gp85 of ALV-A, except for an arginine residue at position 213 in isolate PDRC-3246 (Figure 1). vr1 in PDRC-1039 and PDRC-3246 (vaccine companies A and B, respectively) is identical to corresponding ALV-A sequences, but PDRC-3249 (vaccine company B) is identical to endogenous sequences. vr2 of gp85 in all the contaminants is identical to corresponding sequences in *ev*-1, *ev*-3 and *ev*-6, and ALV-C. The LTR sequences of the contaminating ALVs are most similar to LTR sequences of endogenous viruses. All the putative regulatory elements present in the *ev* loci are also seen in the contaminant ALVs (Figure 2). Absence of one CCAAT like box, one CArG like box, one Y box (inverted CCAAT box) and one PRE box in the contaminant ALVs (Figure 2) may explain the relatively slow replication of these viruses (results not shown) and their inability to induce tumors in White Leghorn type chickens, as seen in previous *in vivo* studies (39).

These results agree with our previous hypothesis (38) that these viruses may be the product of recombination between an exogenous virus (ALV-A) and endogenous ALV sequences. A variety of relatively frequent recombinatorial and mutational events are known to occur in retroviruses including avian retroviruses, mainly because of the diploid nature of their genomes and the lack of a proof reading mechanism during reverse transcription (21, 22, 29). Retrovirus recombination may occur as a result of simultaneous cell infection with heterozygous virions (16). The presence of two RNA genomes in the same virus particle may facilitate the exchange of genetic information, and thus heterozygote virions can occur (17). Although two RNA genomes are packaged in each retroviral virion, it is believed that only one provirus is produced from each

infection event (17). Our results show that almost the entire genomes of the vaccine contaminant viruses herein described are closely related ALV-E (*ev* loci) and only part of the SU protein of the envelope gene is closely related to an exogenous virus (ALV-A). Molecularly engineered ALV- E/*env*A recombinant viruses produced by Brown, *et al*. (2) could be described as very similar to all the PDRC isolates where the backbone of an ALV-E virus carries part of the envelope gene of ALV-A. That particular study demonstrated that a recombinant virus (ALV-E/*env*A) was not able to cause tumor development and not all the birds seroconverted after one month post-inoculation (2), resembling our previous findings from *in vivo* experiments using the vaccine contaminant viruses as challenge viruses (39).

All *ev* sequences described for the chicken genome are highly similar, supporting the hypothesis that they all originated from the same ancestor (1, 4). It is believed that they were incorporated into the chicken genome after speciation and before domestication (12) since they are present in the Red Jungle Fowl (*Gallus gallus,* the modern chicken ancestor), but not in any other Jungle Fowl, such as Grey (*G. sonnerati*), Green (*G. various*), and Ceylonese (*G. lafayettei*) (12). Our search for *ev* loci in the total genomic DNA from the original contaminated vaccine vials revealed 5 *ev* loci in each vaccine. The embryos used for vaccine production may have been carriers of a variety of *ev* loci while the phenotypes of endogenous virus particles expressed in the vaccine substrates may have varied with the embryo pools used. However, all three vaccines tested in the present study showed an identical pattern for *ev* loci. Most of the *ev* loci are incomplete and do not code for complete viral genomes, but some of them can code for at least one viral protein. For instance, the *ev*-3 locus codes for Gag and Env proteins. *ev*-6

only transcribes Env proteins and *ev*-4 does not transcribe any ALV protein (4). The *ev*-1 *pol* sequence has an adenosine insertion at position 5026 (19). This additional residue produces a frameshift in the ORF, resulting in a premature termination codon located 41 bp downstream of the mutation and 298 bp upstream of the Pol polyprotein in the wild-type virus stop codon. These results suggest that despite high proviral DNA sequence identity between *ev*-l and the vaccine contaminants, *ev*-l is not likely the donor of the sequences present in the vaccine contaminants. However, the replication-capable *ev*-2 locus (4) was identified in the original vaccine preparations, presenting the possibility of this endogenous virus being the origin or one of the possible endogenous sequence donors for the vaccine contaminating isolates. We did not investigate the possible presence of *ev*-18 and *ev*-19 in the vaccines. Such loci may contribute to the production of ALV-E particles and potentially could have contributed to the emergence of these contaminant viruses.

Despite the presence of replication-competent endogenous virus sequences, the only possible mechanism for the vaccine contaminants to acquire the exogenous ALV-A SU sequences would be by coinfection between ALV-E and ALV-A. In one experimental study, one isolate of ALV-J acquired the ALV-A SU region of the *env* gene from a CEF cell line resistant to ALV-A (21) due to the presence of a defective recombinant endogenous virus (RAV 0-A_1) that expresses ALV-A envelope proteins (6). The authors believed that homologous recombination must have been responsible for the emergence of an ALV-J/A recombinant virus. We also hypothesize that homologous recombination between sequences from endogenous ALV and exogenous ALV-A may have taken place. Our results support this possibility, since only part of the SU coding

sequence is similar to ALV-A and the rest of the viruses still resemble mostly ALV endogenous virus sequences.

Presence of recombinant viruses such as the ones herein described in contaminated vaccines presents several difficulties in vaccine testing. At the time of production of the contaminated vaccines the complement fixation for avian leukosis (COFAL) was routinely used by the vaccine industry for detection of ALV contamination. This assay was clearly insufficient for detection of ALV in the contaminated vaccines. Cytopathogenesis induced by Marek's disease vaccine virus may have compromised the viability of the CEFs used as substrate for ALV propagation. In addition, the contaminating ALVs propagate slowly in cell culture (G. Zavala, pers. observ.), thus limiting the possibility of detection. On the other hand, it is known that proteins encoded by endogenous viruses may be detected by antigen-capture enzyme-linked immunosorbent assays for detection of p27 antigen (26). The vaccine contaminants herein described contain LTRs similar to endogenous viruses possibly leading to low ALV antigen expression levels that are very similar to endogenous sequence expression. Thus, it is possible that one could misread test results based on previous endogenous virus detections and fail to detect replicating recombinant viruses present in commercial vaccines. Replication-competent *ev* loci such as *ev*-12, *ev*-10 and *ev*-19 have been linked to detrimental effects on egg production, also *ev*-12 has been associated with egg weight and egg shell thickness in Leghorn type chickens (13). Heavy breed White Leghorn lines selected for egg production traits also showed low frequency of genes know to code for complete virus except for *ev*-21 that was presumably present in slow-feathering lines (4). These detrimental effects were not seen in chickens expressing only defective *ev* loci

such as *ev*-1, *ev*-3 and *ev*-6 (4). It would be cost-prohibitive and impractical to attempt to eliminate all the *ev* loci from SPF chicken stocks used for vaccine preparation but it would be worth exploring the feasibility of elimination of nondefective *ev* loci from SPF chicken breeding stock, thus reducing the opportunity for emergence of novel replication-competent ALVs.

(33, 37)

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Table 3.1. Primers used for cloning and sequencing of genomic proviral DNA.

PCR Oligonucleotide Name	Sequence 5'-3'[A]	Purpose[B]
ALV-E-LTR-1	TGTAGTCAAATAGAGCCAGAGGC[1]	Cloning
ALV-E-LTR-1R	TGAAGCCTTCTGCTTCATTCAG[1]	Cloning
ALV-BF	GCAAGGAAGCCCGACGACT[2]	Cloning
ALV-BR	CCGCCAGGGAAGGATACAAACC[2]	Cloning
ALV-C2F	CATCCTGCTATCATTGC[2]	Sequencing
ALV-DF	AAGGGTTTAGCTGATGGGATGGT[2]	Sequencing
ALV-DR	GCACTGCGCCTGATAATGT[2]	Sequencing
ALV-EF	TTACGGATCAAGGCATAGC[2]	Sequencing
ALV-ER	GCGGGGAAGAGGAGCAG[2]	Sequencing
ALV-FR	CCGCAAGCGGAATAGAAAAA[2]	Sequencing
ALV-GF	GCTGGCTGGCCCCTGATG[2]	Sequencing
ALV-HF	TTAGGAATCCCGCCACGACT[2]	Cloning
ALV-HR	GGCCCTCCCTCCAGACTACC[2]	Cloning
ALV-IF	GACGCCTCCTCAAGCACCCATAAG[2]	Sequencing
ALV-IR	ATGCTCAAGCGTAAAGTCTGTCTG[2]	Sequencing
ALV-JF	GCAGCAGGCTAGGGAGGTTGTTCA[2]	Sequencing
ALV-JR	TCCCCAGACTAGCACGTTCCATCC[2]	Sequencing
H5	GGATGAGGTGACTAAGAAAG[3]	Cloning
CapA	AGAGAAAGAGGGGTGTCTAAGGAGA[4]	Cloning
BS-up	GATGAGGTGACTAAGAAAGATGAG[5]	Sequencing
U5-R ENDO	TGCTTCATTCAGGTGTTCG[6]	Sequencing

[A] References: [1]This present publication; [2]G. Zavala, M. W. Jackwood, D. Hilt, J.R. Glisson, and S. Hafner, manuscript in preparation; [3]Smith, *et al.* (31); [4]K. Venugopal, pers. comm.; [5]Spencer, *et al.* (33); [6]M. Garcia, pers. comm. [B] PCR size products based on the isolate PDRC-1039 were the following: LTR-F and ALV-BR primers amplified a 1109 bp product (nucleotide position 1 to 1109), ALV-BF and ALV-HR primers amplified a 3583 bp product (nucleotide position 489 to 4072), ALV-HF and CapA primers amplified a 2430 bp product (nucleotide position 3472 to 5902) and H5 and LTR-R primers amplified a 2289 bp product (nucleotide position 5208 to 7497).

Table 3.2. Nucleotide and amino acid percent identity between three contaminating ALV recombinant isolates and reference ALV sequences.

	PDRC-1039 (GenBank EU 070900)							PDRC-3246 (GenBank EU 070901)							PDRC-3249 (GenBank EU 070902)						
	Gag[2]	Pol	Env		LTR	U3	R	U5	Gag	Pol	Env		LTR	U3	R	U5	Gag	Pol	Env		LTR	U3	R	U5
			gp85	gp37							gp85	gp37							gp85	gp37				
ALV-A[1]	97.6 (97.7)	99.2 (99.4)	98.7 (98.5)	95.4 (94.1)	58.4	35.7	95.7	87.0	97.7 (97.6)	99.1 (99.4)	98.7 (98.0)	95.3 (93.6)	57.5	35.1	91.3	87.0	97.8 (98.3)	99.3 (99.4)	98.0 (98.2)	95.8 (94.6)	57.8	35.2	95.7	87.0
ALV-B[1]	95.8 (96.4)	98.2	84.2 (78.5)	94.1 (93.1)	58.6	35.4	95.7	88.2	96.1 (96.7)	98.2	84.2 (78.2)	94.0 (92.6)	57.7	34.9	91.3	88.2	95.7 (96.3)	98.1	84.7 (79.0)	94.4 (93.6)	58.0	34.9	95.7	88.2
ALV-C[1]	96.4 (96.3)	98.7 (99.3)	86.5 (84.0)	94.2 (94.4)	71.5	46.9	100	93.4	96.7 (96.7)	98.4 (98.3)	86.7 (84.5)	94.1 (93.9)	70.4	46.3	95.7	93.4	96.3 (96.0)	98.6 (99.0)	87.1 (84.5)	94.6 (94.9)	70.8	46.3	100	93.4
ALV-J[1]	95.6 (96.3)	97.8 (99.0)	51.0 (36.8)	56.7 (54.4)	55.7	36.4	87.0	82.9	96.0 (97.0)	97.6 (98.7)	51.2 (36.9)	56.7 (54.9)	55.4	36.0	91.3	82.9	95.5 (96.3)	97.7 (98.7)	51.0 (36.8)	56.5 (54.4)	55.1	36.0	87.0	82.9
ev-1[1]	99.0 (98.6)	99.6 (98.0)	86.5 (82.6)	99.2 (98.0)	98.2	97.7	95.7	100	99.1 (98.4)	99.6 (98.0)	86.8 (83.3)	99.0 (97.5)	99.3	98.9	100	100	99.2 (99.1)	99.6 (98.0)	87.8 (83.3)	99.5 (98.5)	98.9	98.9	95.7	100
ev-3[1]	NA	NA	86.7 (83.0)	99.2 (98.0)	98.5	98.3	95.7	100	NA	NA	87.0 (83.7)	99.0 (97.5)	99.6	99.4	100	100	NA	NA	88.0 (83.7)	99.5 (95.5)	99.3	99.4	95.7	100
ev-6[1]	NA	NA	86.9 (82.8)	99.0 (98.0)	98.2	97.7	95.7	100	NA	NA	87.3 (83.6)	98.9 (97.5)	99.3	98.9	100	100	NA	NA	87.7 (83.1)	99.3 (98.5)	98.9	98.9	95.7	100

[1]ALV-A (GenBank NC 001408); ALV-B [RCASBP(B)] vector (16); ALV-C (GenBank AF033808); ALV-J (HPRS-103 strain, GenBank Z46390); *ev*-1 (GenBank AY013303); *ev*-3 (GenBank AY013304); *ev*-6 (GenBank AY013305).

[2] Nucleotide similarities are shown in the top line and amino acid similarities are shown in parentheses.

NA = Sequences not available

Table 3.3. Identification of *ev* loci in the original MDV vaccine preparations by *ev*-specific PCR analysis.

	Vaccine Chicken Embryo Fibroblasts			
Loci[1]	PDRC-1039	PDRC-3246	PDRC-3249	DF-1 cells
ev-1	+/+	+/+	+/+	-/-
ev-2	+	+	+	-
ev-3	+/-	+/-	+/-	-/-
ev-4	+/-	+/-	+/-	-/-
ev-6	+	+	+	-
ev-7	-/-	-/-	-/-	-/-
ev-9	-/-	-/-	-/-	-/-
ev-12	-/-	-/-	-/-	-/-
ev-15	-/-	-/-	-/-	-/-
ev-16	-/-	-/-	-/-	-/-
ev-21	-/-	-/-	-/-	-/-

[1] Tests are able to determine the presence or absence of *ev* loci in both alleles; i.e. a single sample can be +/+ (presence in both allele), +/- (presence in only one allele) or -/- (absence in both allele). *ev*-2 and *ev*-6 only discriminate positive and negative samples, as described by Benkel, 1998 (1).

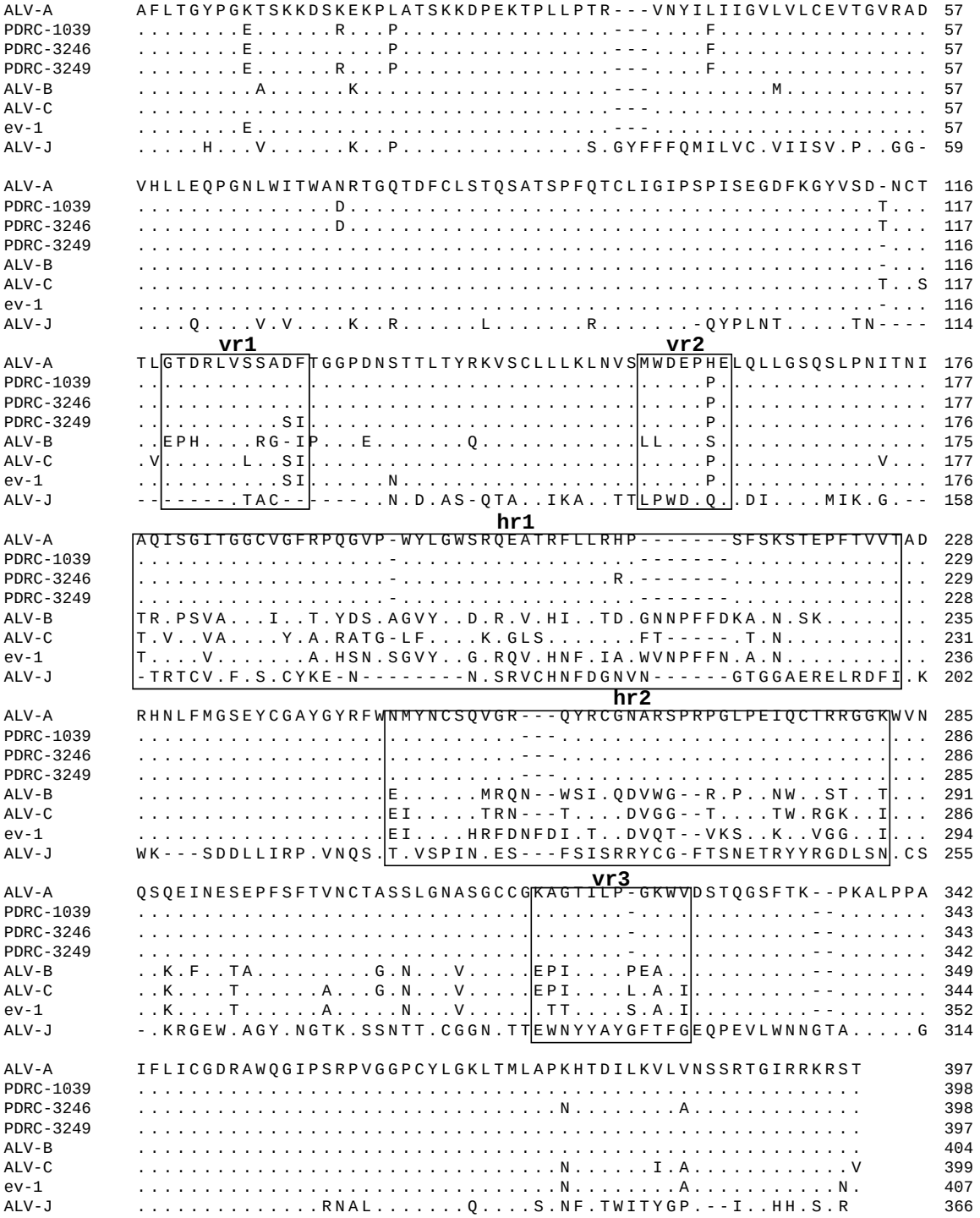

Figure 3.1. Alignment of deduced amino acid sequences of gp85 (surface envelope protein) of recombinant isolates (PDRC-1039, PDRC-3246 and PDRC-3249) and representative ALVs. ALV-A (GenBank NC 001408); ALV-B [RCASBP(B) vector,

(16)]; ALV-C (GenBank AF033808); ALV-J (HPRS-103 prototype, GenBank Z46390); *ev*-1 (GenBank AY013303). Boxes represent the variable (vr) and hypervariable (hr) regions. Positions of identity are indicated by dots while nucleotide differences are appropriately marked. Dashes (-) represent gaps produced in the alignment.

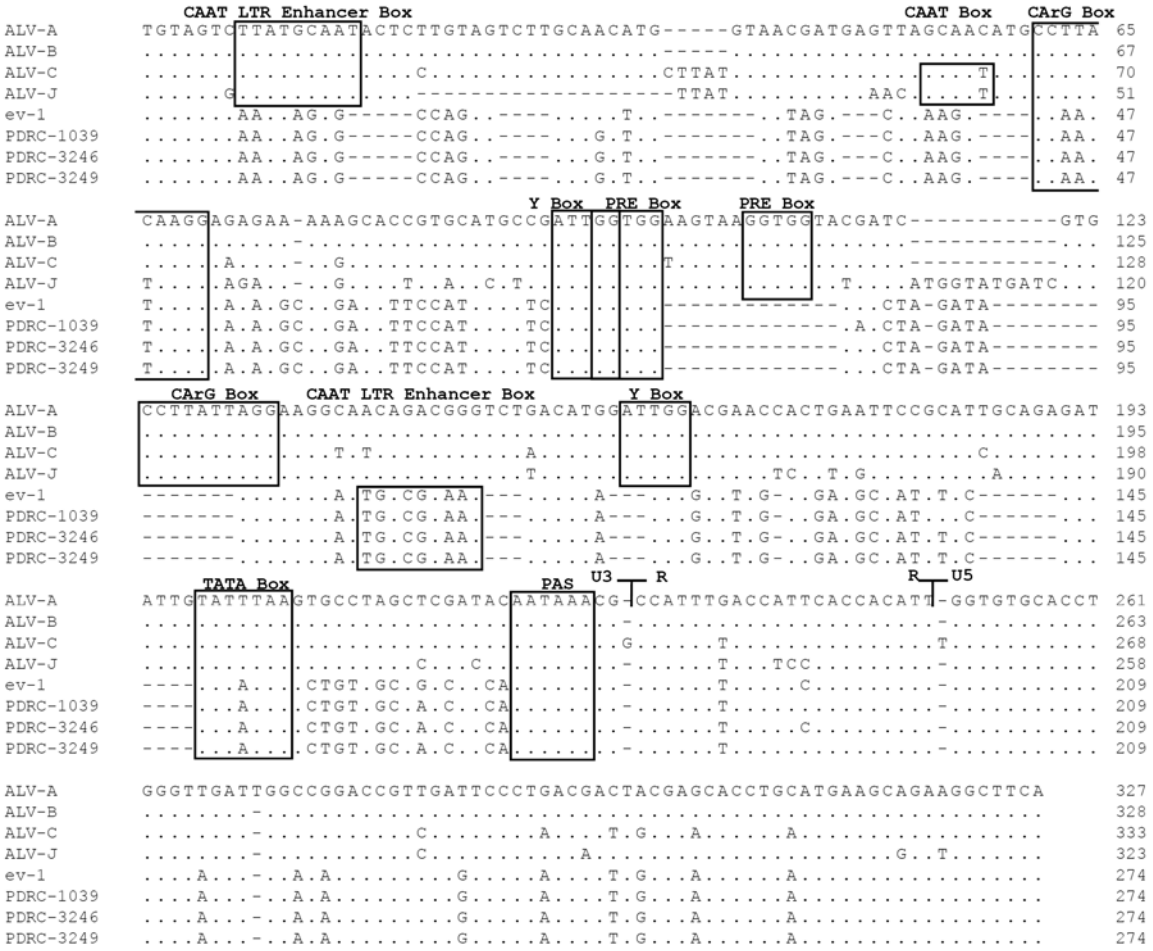

Figure 3.2. DNA sequence comparison of the ALV proviral LTR region. ALV-A (GenBank NC 001408); ALV-B [(RCASBP(B) vector (16)]; ALV-C (GenBank AF033808); ALV-J (HPRS-103 strain, GenBank Z46390); *ev*-1 (GenBank AY013303). Positions of identity are indicated by dots while nucleotide differences are appropriately marked. Dashes (-) represent gaps produced in the alignment. The locations of U3, R and U5 are indicated. Location of putative transcription regulatory elements are indicated in boxes and labeled. PAS = Polyadenylation Site.

CHAPTER 4

CONSTRUCTION OF A NOVEL AVIAN LEUKOSIS VIRUS BASED GENE DELIVERY VECTOR[1]

[1] Barbosa, T, Zavala, G and Cheng, S., (2010) To be submitted to *Avian Diseases.*

Summary

Gene delivery systems have been used for transient and permanent expression of exogenous genes in several cell types, either *in vivo* or *in vitro*. Retroviral vectors have been largely used for this purpose including avian leukosis virus vectors, which have been use to some extent. Most retroviral vectors have been constructed without convenient restriction sites for cloning or different drug selection markers. We have developed a new avian leukosis virus based gene delivery system, using the recently isolated natural recombinant virus ALV PDRC-1039, an ALV-E/A recombinant virus, which has been proven to be non-pathogenic to chickens up to 31 weeks of age. The pBZ system herein decribed contains a large multicloning site (MCS) that allows for flexibility for insertion of a vast number of genes. In addition, eight additional new vectors herein described differ in their construction and drug selection markers, allowing expression of recombinant proteins. We have inserted the Green Fluorescent Protein (GFP) gene in all of the pBZ vectors. Fluorescence was detected after transduction and selection of avian cells. GFP was also detected after several passages of these clonally selected cells. These newly constructed vectors may be used in the future to express exogenous genes in avian cells for *in vitro* and *in vivo* applications.

Introduction

Retroviral vectors have been used for specific gene delivery in several systems (12, 18, 23). Such vectors can transduce genes into the chromosomes of a wide variety of mammalian and avian cell types. They have several distinct advantages over other non-viral and viral vectors, and thus they are often used as preferred gene transfer vectors. Advantages of retroviral vectors include their high efficiency of transduction into a variety of cell types and their ability to

integrate into the host cell chromosome allowing for a stable expression of the incorporated genes (1, 13, 15). Replication-competent retroviral vectors can be obtained just by adding sequences to the 3'end of existing viruses (18). However, a much more common design of retroviral vectors involves the replacement of retroviral coding sequences to create replication-defective vectors (6, 26). In these vectors, the amount of foreign DNA that can be accommodated is much larger than can be inserted in replication-competent vectors. Replication-defective vector systems normally contain three plasmids. The coding sequences are divided into two different expression plasmids, one having the *gag-pol* genes and the second containing the *env* gene (9, 22). This strategy aims to minimize the reconstitution of full-length provirus by recombination and to increase the efficiency of translation of viral proteins. A third vector contains the *cis* elements necessary for encapsidation, integration and the promoter region (LTR). The *gag-pol* plasmid provides all of the necessary enzymes and structural proteins in *trans*; the *env* plasmid provides the Env protein for the desired pseudotype for interaction and invasion of target cells (9, 22).

The traditional approach of constructing a clonal cell line that stably expresses a gene of interest relies on recombination of the transfected DNA with the host genome. In mammalian and avian cells this process is inherently inefficient because the recombination frequency is quite low and the site of integration strongly affects the level of expression (19). Stable expression of cloned DNA promoted by the viral LTR occurs after integration of a retroviral vector into the host genome (7). Maximal levels of protein expression are typically present 2 days after infection (10). An integration site study found that MLV (Murine Leukemia Virus) vectors have a strong bias in favor of integration near transcriptional start sites, while ALV vectors have only a weak preference for active genes and no preference for transcriptional start regions (21). These

important findings support the potential benefit of ALV-based vectors as gene delivery systems, without disrupting active regions of the cellular chromosome.

The 5′ LTR facilitates expression of retroviral proteins in most of the naturally occurring infection of oncogenic retroviruses and the expression of multiple viral coding regions is achieved by alternative splicing (5). However, most retroviral vector designs are not limited to the use of the single retroviral promoter with alternative splicing for expression of genes of interest. Other strategies have been explored by researchers, including the use of multiple promoters, insertion of genes in reverse orientation, the use of an internal ribosome entry sites (IRES), fusion proteins, and cleavage factors (9, 14). Each system has a different expression level that can be achieved and it depends on several factors including cell type, promoter sequence, and stability of the expressed product (20). Co-expression of the gene of interest with a drug selectable marker is desirable as it has shown increased expression levels of the target gene (9).

We have previously isolated and characterized three Avian Leukosis Viruses (ALV) found as contaminants in Marek's Disease vaccines (28). All three isolates were fully sequenced and characterized *in vitro*, and demonstrated to be non-oncogenic in SPF chickens up to 31 weeks of age, or 34 weeks post-infection (29). These unique viruses combine very interesting characteristics, including presence of an envelope gene from an exogenous virus (ALV-A), allowing it to infect a large variety of avian cells. The three viruses are recombinants (ALV-E/A) since they carry an exogenous ALV envelope gene (ALV-A) and also contain endogenous (ALV-E) regulatory sequences in their LTR, which decreases considerably the potential for insertional activation of host cellular genes (2). In fact, modern commercial meat-type and egg-

type chickens and specific pathogen free (SPF) chickens carry several of such endogenous retroviral sequences inserted into their genomes without detectable detrimental effects (3).

Most defective retroviral vectors based on ALV contain residual *gag, pol* and/or *env* coding sequences in their construction. The presence of such sequences may lead to recombination during viral stock production, potentially resulting in rescue of replication-competent viruses. Therefore, retroviral vectors containing such coding sequences should be avoided whenever possible. Aiming to develop a safer ALV-based vector, we describe the construction of the pBZ system based on the natural ALV recombinant ALV PDRC-1039. The pBZ vectors lack all the ALV protein coding regions, but remain effective in expressing exogenous proteins in avian cells.

Materials and Methods

Viruses and cell lines

The ALV isolate PDRC-1039, a naturally occurring recombinant avian leukosis virus isolated from contaminated Marek's Diseases vaccines (2), was used as template for production of plasmids necessary for construction of a replication-defective ALV-based vector. Transfections were performed in human embryonic kidney cells 293T (HEK 293T) and the ALV packaging cell line ISOLDE (11). Transduction was done in DF-1 cells, a spontaneously immortalized fibroblastoid cell line derived from line 0 chickens (C/E phenotype for ALV) (16). HEK 293T and DF-1 cells were cultured in growth medium, consisting of DMEM medium containing 10% fetal bovine serum (FBS) under 5% CO_2 at 37ºC. For long incubation periods (more than 4 days) maintenance medium was used (DMEM medium plus 2% FBS).

Nucleic acid extraction

DNA and RNA were extracted from infected cells using a High Pure PCR Template Preparation Kit and High Pure RNA Isolation Kit, respectively (Roche, Mannheim, Germany), following the manufacturer's recommendations.

Cloning strategy

All constructs were made using classical molecular biology techniques, as described in Sambrook, et al. (24). All restriction endonucleases and ligases were purchased from New England BioLabs (Ipswich, MA) and used following the recommendations from the manufacturer. The strategy for construction of the replication-defective ALV based gene delivery system included three plasmids. Vector plasmids were constructed using the PDRC-1039 ALV-A/E isolate as backbone, and the Green Fluorescent Protein (GFP) was used as a reporter gene to study the efficiency of transfection and transduction of different constructs. Table 1 lists the primers and sequences used for construction of vectors. The pBZ1.0 plasmid contains the regulatory sequences from the isolate PDRC-1039. Additional plasmids were conctructed to contain either the *gag-pol* genes (p1039_gagpol) or the *env* gene (p1039_env). pBZ1.0 has both LTRs, the encapsidation signal and the PPT (polypurine tract) region, which is necessary for reverse transcription postinfection. To construct the pBZ1.0 delivery plasmid, we designed specific primers based on the PDRC-1039 isolate (GenBank EU070900 – Table 1). The 5'end LTR plus the encapsidation signal were amplified by PCR with a forward primer containing a *Spe*I site. The reverse primer included part of the MCS (Multi Cloning Site) containing the *Not*I, *Cla*I, *Eco*RI, *Xba*I, *Eco*RV restriction sites. A second set of primers was used to amplify the Encephalomyocarditis virus (EMCV) IRES (Internal Ribosomal Entry Site) from pIRES (Clontech Laboratories, Mountain View, CA) kindly provided by Dr. Mark Jackwood (Poultry

Diagnostic and Research Center, University of Georgia). The EMCV IRES forward primer also contains part of a MCS with the *Eco*RV, *Bsi*WI, *Fse*I, *Xma*I, *Sma*I, *Asc*I, *Age*I, *Bsp*EI, *Stu*I and *Bsr*GI restriction sites and the EMCV IRES-reverse primer includes a *Bam*HI restriction site. Another set of primers was used to amplify the Neomycin gene from pcDNA 3.1 (Invitrogen, Carlsbad, CA) and the forward and reverse primers included the *Bam*HI and *Hind*III restriction sites, respectively. The last set of primers was designed to amplify the 3'end of the PDRC-1039, with the forward primer including a *Hind*III restriction site and the reverse primer with an *Apa*I restriction site. All PCR products were cloned into pcR2.1 (Invitrogen, Carlsbad, CA) using the TOPO TA cloning kit. The plasmids were named pcR-5'LTR+Ψ, pcR-IRES, pcR-Neomycin and pcR-3'UTR respectively. Selected clones were sequenced and compared to original sequences. Those that did not show any mutations were chosen for future applications.

pBZ1.0 construction: A diagram summarizing the construction strategy is shown of Figure 1. The expression plasmid pcDNA3.1 was used as a backbone plasmid for pBZ construction. The neomycin gene was deleted by digestion with *Bst*Z171 and *Stu*I, followed by re-ligation with T4 DNA ligase. The resulting pcDNA3.1ΔNeo was digested with *Bam*HI and *Apa*I and ligated by three-way ligation with the *Bam*HI and *Hind*III fragment of pcR-Neomycin and the *Hind*III and *Apa*I fragment of pcR-3'UTR. After confirmation of insertion the resulting plasmid was digested with *Spe*I and *Bam*HI and followed by a three-way ligation with the *Spe*I and *Eco*RV fragment of p5'LTR+Ψ and the *Eco*RV and *Bam*HI fragment of pcR-IRES. The pBZ1.0 plasmid was sequenced to confirm the orientation of the fragments and absence of mutations.

p1039_gagpol construction: Primers were designed based on the genome sequence of PDRC-1039 isolate (GenBank EU070900) to amplify the *gag* and *pol* regions. Forward and

reverse primers included the *Pme*I and *Not*I restriction sites, respectively. The PCR 4.5Kb product was digested with *Pme*I and *Not*I and cloned into the same sites of pcDNA3.1 plasmid. The p1039_gagpol plasmid was sequenced to confirm absence of mutations.

p1039_env construction: Primers were designed to amplify the *env* gene of the PDRC-1039 isolate, including the Leader sequence and both the Surface (SU, gp85) and Transmembrane (TM, gp37) proteins. Forward and reverse primers included the *Bam*HI and *Not*I restriction sites, respectively. The RT-PCR 1.8Kb product was digested with *Bam*HI and *Not*I and cloned into the correspondent sites of pcDNA3.1. The insert was sequenced to confirm absence of mutations.

pBZ vectors: In order to have a variety of delivery vectors with different selection agents and different secondary promoters, we used the pBZ1.0 as backbone for the construction of several other pBZ vectors as seen in Figure 2. Details of their construction strategy are available upon request.

pBZ-GFP constructs: The Green Fluorescent Protein (GFP) gene was amplified by PCR from the pEGFP plasmid (Clontech Laboratories, Mountain View, CA). The *Not*I and *Eco*RV restriction sites were added to the forward and reserve primers, respectively. The 800bp PCR product was digested with *Not*I and *Eco*RV and cloned into the same sites of all pBZ constructs.

Virus production and transduction

HEK 239T cells were seeded and grown to 50-60% confluence in 6-well plates and transfected (Lipofectamine LTX, Invitrogen, Carlsbad, CA) when they reached 70 – 80% confluence with 1.6 µg of the delivery vector pBZ and with 0.8 µg of p1039_gagpol and 0.8µg of p1039_env plasmids. Supernatants were collected 48 and 72h post-transfection, passed through 0.45 µm filters, and pooled. Exponentially growing DF-1 cells were used for

transduction and viral titration. A schematic representation of the virus production is shown on Figure 3. Alternatively, ISOLDE ALV packaging cells were transfected with the delivery vector pBZ. Virus rescue was done as described above.

Selection of transduced DF-1 cells

DF-1 cells were tested for susceptibly to G-418 (Geneticin) and Zeocin (Invitrogen, Carlsbad, CA). DF-1 cells were plated and once they reached 50% confluence the medium was replaced with medium plus Geneticin or Zeocin in different concentrations (range from 50 to 1000 μg/mL). Cells were kept at 37ºC for 7 days, when cell growth was evaluated by visualizing the cells under light microscope. The lowest concentration to inhibit DF-1 cell growth was used to select DF-1 cells transduced with recombinant pBZ vectors.

Selection of DF-1 GFP positive clones

DF-1 cells were transduced with different amounts of pBZ recombinant viruses, and selected for G-418 or Zeocin resistance two days after. The cell culture medium was changed every 3-4 days until the formation of isolated cell clones. Individual cell clones were trypsinized and transferred to 48- or 24-well plates. GFP expression was confirmed by direct observation. Viral titrations were done in DF-1 cells plated in 6-well plates. Serial dilutions of filtered supernatant were inoculated 6 to 12 hours after seeding DF-1 cells. Fluorescence was observed 3-4 days post inoculation. Colony formation was observed 2-3 weeks after cultivation in selection-medium.

Results

As a first step to design the novel pBZ vectors we previously sequenced in full the ALV isolate PDRC-1039 (2). Based on its sequence we designed primers to amplify different

fragments of the genome (Table 1). These fragments were not overlapped and they were assembled to construct the pBZ system. The pBZ1.0 was the first assembly vector and it contains all the regulatory sequences from PDRC-1039, a multicloning site (MCS), the EMCV IRES sequence, and the Neomycin resistance gene (*neo*).

We designed a MCS that allows for a wide range of insertion of different fragments. The design of pBZ MCS also tolerates bidirectional cloning since compatible ends are located in both directions (Table 2). Altogether, the pBZ MCS has 13 unique restriction sites, including eight sites for bidirectional cloning. The MCS for pbZ4.0 and pBZ5.0 is shortened (*Not*I, *Eco*RI, *Xba*I, *Eco*RV, *Bsr*Gi), but allows fusion protein expression. The ATG site of Neomycin and Zeocin has been removed from pBZ4.0 and pBZ5.0, respectively. For expression of fusion proteins, the gene of interest is inserted in frame with the drug resistance gene and without a stop codon.

We used the pBZ1.0 as backbone to construct seven different pBZ derivative vectors, all with a different setup either for the expression of the gene of interest or the selection gene (Figure 2). The numbering of the pBZ vectors follows the order of construction and it does not reflect levels of expression of the gene (s) of interest. The design for all of the eight vectors is shown on Figure 2. As illustrated in Figure 2, the vectors were constructed in separate sections, which allows for easy modification in their structure. Restriction sites are strategically located to flank each element. Plasmid vectors pBZ4.0 and pBZ5.0 contain a shorter version of the MCS, but allow the expression of fusion proteins with neomycin and zeocin, respectively.

The EMCV IRES sequence was amplified from the commercial available pIRES which has a mutation in the 11th ATG site inside the EMCV IRES sequence. This site was converted from AATATG (wild type EMCV IRES) to AAGCTT (*Hind*III restriction site). It has been previously proven that such modification decreases drastically the expression of a protein

downstream the EMCV IRES sequence (20). We designed primers to mutate back the *Hind*III site into its original ATG site. To facilitate cloning of any cassette after the EMCV IRES sequence into the pBZ vectors, we included the *Bam*HI site after the 11th EMCV IRES ATG and kept the neomycin or the zeocin genes *in frame* with it (Figure 1 and 2).

We co-transfected HEK-293T cells with the pBZ vector plus the packaging vectors (P1039_gagpol and p1039_env) to produce retroviral particles (Figure 3). The viral supernatant was then used to transduce exponentially growing DF-1 cells, and fluorescence was observed two days post transduction (Figure 4). In parallel we also transfected the ALV packaging ISOLDE cells and viral supernatants were also collected and used to transduce DF-1 cells. Fluorescence was readily detected in all pBZ vectors after transduction in DF-1 cells. Vector titers measured by fluorescence detected 2 days after transduction are shown on Table 3.

In order to select individual clones of the transduced cells we titrated the susceptibility of DF-1 cells to G-418 (Geneticin, Invitrogen) and Zeocin. Serial dilutions of each (Geneticin and Zeocin) were done in DF-1 growth medium (DMEM + 10%FBS). Concentrations varied from 50µg/mL to 1000µg/mL. The lowest concentrations to inhibit DF-1 cell growth were selected to clone purified transduced cells. DF-1 cells were selected with 200µg/mL of G-418 or 150µg/mL of Zeocin. Selection started 48h post transduction of DF-1 cells and continued for at least two weeks. Isolated colonies were identified using light microscopy, trypsinized and transferred to 24- or 48-well plates. Isolated colonies, for all eight constructs, were split every 3-5 days after the first passage and kept for at least five passages (Table 3). Fluorescence was detected in all vectors up to the latest passage tested (5 to 25 passages – Table 3). pBZ 4.0 and pBZ5.0 which express fusion protein, had the lowest intensity of fluorescence amongst all vectors, and their GFP expression decreased with the serial passages. After the expansion of single cell clones, all

colonies were tested with commercially available ALV specific anti-p27 ELISA (IDEXX Laboratories, Westbrook, ME). All clones transduced with the pBZ vectors were negative for p27 expression, indicating the absence of replicating ALV in the cell cultures (results not shown).

Discussion

We have constructed an ALV vector system based on the PDRC-1039 isolate. This system allows the efficient transduction of exogenous genes into chicken cells. No detectable production of infectious virions was observed, thus the system could be useful for the introduction of exogenous genes into avian cells in which the continuous production of virus is unacceptable. In contrast with most ALV-based vector systems previously described (4, 12, 25), the pBZ system does not contain any region of homology between the delivery vector and the helping vectors, which provide the necessary proteins for virus assembly in *trans.* This absence of homology among the pBZ system plasmids reduces greatly the possibility of recombination events during virus assembly and reverse transcription (27). In addition, to the best of our knowledge the pBZ system is the first ALV-based vector that contains such large number of restriction sites (13 unique sites), allowing substantial flexibility for construction of delivery vectors.

The 5′ LTR drives expression of retroviral proteins in the naturally occurring infection by oncogenic retroviruses and the expression of multiple viral coding regions is achieved by alternative splicing (5). The previously described ALV-based vectors rely mostly in the use of the single retroviral promoter (LTR) with alternative splicing for expression of the second gene of interest (4, 8, 12). However, other strategies have been explored for construction of

mammalian retroviral vectors, including the use of multiple promoters; insertion of genes in the reverse orientation; use of internal ribosome entry sites (IRESs); fusion proteins; and cleavage factors (9). Each system has a different expression level that can be achieved depending on several factors including cell type, promoter sequence, and stability of the expressed product (20). The unpredictability of vector performance has been described (17), suggesting that vectors need to be constructed and tested in desired cell types to determine their optimal activity. Our novel ALV-based pBZ system, contain eight different vector designs, which will facilitate this task in future studies.

Expression of a gene of interest in the pBZ vector system is always associated with the expression of a drug selectable marker. This co-expression is desirable and it has been shown previously that it increases expression levels of the target gene (9). One of the most used strategies for dual expression is the use of IRES sequences in between the genes aimed to express. The commercially available EMCV IRES sequence within the pIRES has been shown to be mutated at the 11^{th} ATG site (20). This single mutation was responsible for up to 10-fold decrease of the expression of the second cassette (20). We mutated back the EMCV IRES sequence to its wild type sequence, restoring the 11^{th} ATG site and we were able to readily select DF-1 cells clones with selection markers, which were expressed located downstream of the EMCV IRES sequences in the pBZ vectors. The activity of EMCV IRES sequences in the pBZ vectors was as good as when a secondary promoter (SV40) was used to drive the transcription of the resistance gene, as seen on the number of resistant colonies isolated after drug selection (Table 3).

Our main objective was to develop a new retroviral vector, based on the recently isolated ALV PDRC-1039. This isolate has an ALV-A related envelope gene and the rest of the genome

is very similar to endogenous ALV sequences. Part of the rationale behind using the PDRC-1039 isolate to construct the pBZ system was based on the use of an endogenous-like LTR. The endogenous-like LTR is present in mosty all avian cells, and likely would not represent any detrimental effect on cell growth. In addition, the avoidance of use of an exogenous-like LTR to transduced cells, as present in previous described ALV-based vectors, is desirable, once exogenous ALV represent a threat to commercial poultry production. Furthermore, the PDRC-1039 has an ALV-A related envelope, which increases the range of cells that can be transduced compared to ALV-E envelope.

We have successfully transduced DF-1 cells with all eight ALV vectors presented herein. DF-1 cells were clonally selected and these clones expressed the gene of interest (GFP) for several (5-25) passages. The pBZ system can now be tested to produce stably transduced cells lines to express different genes.

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Table 4.1. Oligonucleotides used for construction of the PBZ system, origin and location of amplicons.

Plasmid Name	Insert	Origin[1]	Location[2]	Primers used for amplification 5' to 3' sequence [3]	Restriction Sites on 5' end of primer[4]
P1039_gagpol	Gag and Pol	PDRC-1039 Proviral DNA	554-5066	F – ATGGAAGCCGTCATAAAGGTG R – TTAACCGGGGGTCCTTCT	*Pme*I *Not*I
p1039_env	Envelope	PDRC-1039 Proviral DNA	554-571, 5252-7057	F – ATGGAAGCCGTCATAAAGGCA R – TTAACCGGGGGTCCTTCT	*Bam*HI *Not*I
pcR-5'LTR+Ψ	5'LTR+Ψ	PDRC-1039 Proviral DNA	1-553	F – TGTAGTCAAATAGAGCCAGAGGC R – GCTTGATCCACCGGGCGAC	*Spe*I *Not*I, *Cla*I, *Eco*RI, *Xba*I, *Eco*RV
pcR-3'UTR	3'UTR	PDRC-1039 Proviral DNA	7058-7497	F – TGAAGCCTTCTGCTTCATTCAG R – TGAAGCCTTCTGCTTCATTCAG	*Hind*III *Apa*I
pcR-IRES,	EMCV-IRES	pIRES	1130-1710	F – AATTCCGCCCCTCTCCCT R – CATATTATCATCGTGTTTTTCAAAGG	*Eco*RV, *Bsi*WI, *Xma*I, *Sma*I, *Asc*I, *Age*I, *Bsp*EI, *Stu*I and *Bsr*GI *Bam*HI
pcR-Neo	Neomycin	pcDNA3.1+	2136-2930	F – ACCATGATTGAACAAGATGGATTGC R – ATTATCATCGTGTTTTTCAAA	*Bam*HI *Hind*III

[1] Original template used for the amplification reaction: PDRC-1039 (GenBank EU070900), pIRES (Clontech Laboratories, Mountain View, CA), pcDNA3.1+ (Invitrogen, Carlsbad, CA).

[2] Position of nucleotides included into the amplified region, based on the original sequences described in [1]

[3] F – Forward and R – Reverse primers.

[4] Restriction site sequences added to the 5' end of the oligonucleotides used for the amplification reactions.

Table 4.2. Restriction sites present on the Multicloning site of pBZ vectors.

Site[1]	**Compatible end**[2]
*Not*I	*Eae*I/*Eag*I/*Psp*OMI
*Cla*I	*Bsp*DI / *Acl*I/ *Bst*BI / *Nar*I
*Eco*RI	*Mfe*I
*Xba*I	*Avr*II / *Nhe*I / *Spe*I
*Eco*RV	Any Blunt End
*Bsi*WI	*Bsr*GI /*Acc*65I
*Xma*I	*Age*I / *Bsp*EI / *Bsr*FI / *Ngo*MIV / *Tsp*MI
*Sma*I	Any Blunt End
*Asc*I	*Bss*HII / *Mlu*I
*Age*I	*Bsp*EI / *Bsr*FI / *Ngo*MIV / *Tsp*MI / *Xma*I
*Bsp*EI	*Age*I / *Bsr*FI / *Ngo*MIV / *Tsp*MI / *Xma*I
*Stu*I	Any Blunt End
*Bsr*GI	*Bsi*WI / *Acc*65I

[1] Sites present in the multicloning site (MCS), 5' to 3' direction.

[2] Restriction enzyme which produces a compatible end for cloning into the original site present in the pBZ vector.

Table 4.3. Transient vector-derived virus titer from HEK-293T cells

	GFP positive cells[a]	Selected colonies[b]	Number of passages tested
pBZ1.0-GFP	$3.2x10^3$	$1.9 x10^3$	20
pBZ2.0-GFP	$3.1 x10^3$	$2.0 x10^3$	10
pBZ3.0-GFP	$5.9 x10^3$	$3.2 x10^3$	25
pBZ4.0-GFP	$2.1 x10^3$	$5.3 x10^2$	5
pBZ5.0-GFP	$2.0 x10^3$	$6.0 x10^2$	5
pBZ6.0-GFP	$3.3 x10^3$	$1.7 x10^3$	5
pBZ7.0-GFP	$5.4 x10^3$	$3.0 x10^3$	5
pBZ8.0-GFP	$3.2 x10^3$	$1.8 x10^3$	5

[a] Titer of vector-derived virus measured by fluorescence microscopy 2-3 days after transduction. Titer given by milliliter of rescue supernatant.

[b] Titer of vector-derived virus measured by counting selected colonies after 2-3 weeks. Selection with G-418 or Zeocin depending on the design of the vector. Titer given by milliliter of rescue supernatant.

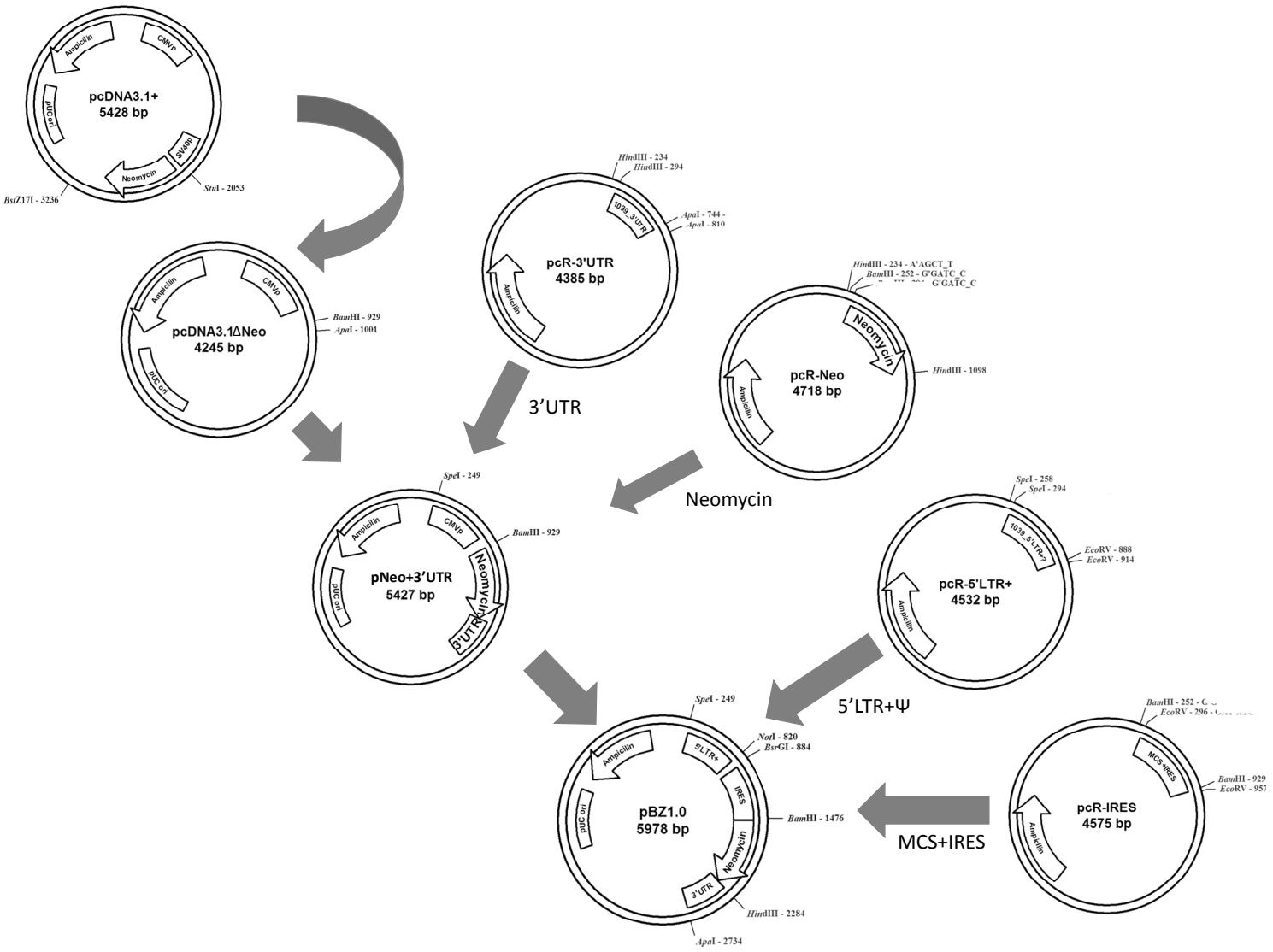

Figure 4.1. Schematic representation of pBZ1.0 Construction. Restriction sites used for construction are indicated in each respective plasmid. Restriction sites on pBZ1.0 are shown as also indicated in Figure 2, for comparison with other pBZs. The

multicloning site is indicated by the *Not*I and *Bsr*GI restriction sites, sites in between are not shown due to space limitation. The list of all sites on MCS are on Table 1.

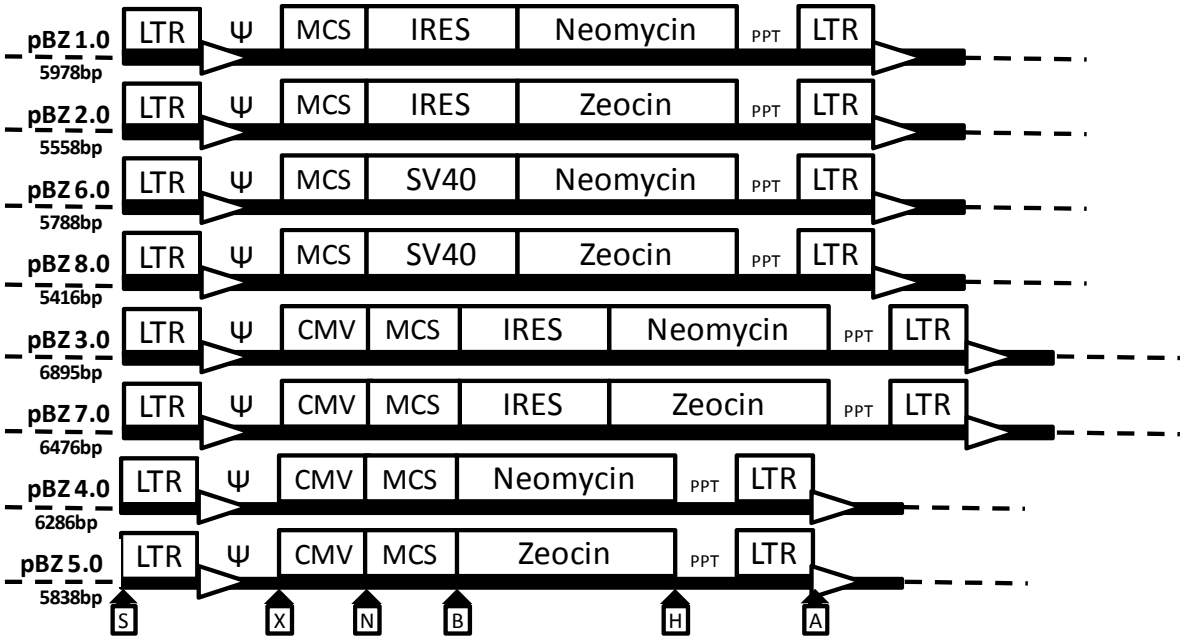

Figure 4.2. Schematic representation of the pBZ retroviral vectors. Vectors are numbered in order of construction. The diagrams represent the linear form of the plasmid vectors, which contain the regulatory sequences. Plasmid sizes are indicated for each vector. The psi letter Ψ is the ALV encapsidation sequence. LTR corresponds to the ALV PDRC-1039 Long Terminal Repeat. MCS (Multicloning site) is detailed in Table 1. The MCS for pbZ4.0 and pBZ5.0 is shortened (*Not*I, *Eco*RI, *Xba*I, *Eco*RV, *Bsr*Gi) and allows fusion protein expression. The CMV promoter is derived from commercial vector pcDNA3.1. The Internal Ribosomal Entry Site (IRES) is from pIRES, with modifications (see text for details). Drug resistance genes are indicated. The Polypurine Track (PPT) is from ALV-PDRC 1039. Letters at the bottom represent restriction sites, which have been used to construct the pBZ vectors, as follow: S (*Spe*I), X(*Xho*I), N(*Not*I), B(*Bsr*GI), H(*Hind*III), A(*Apa*I). Arrows indicate the direction of transcription. Not drawn to scale.

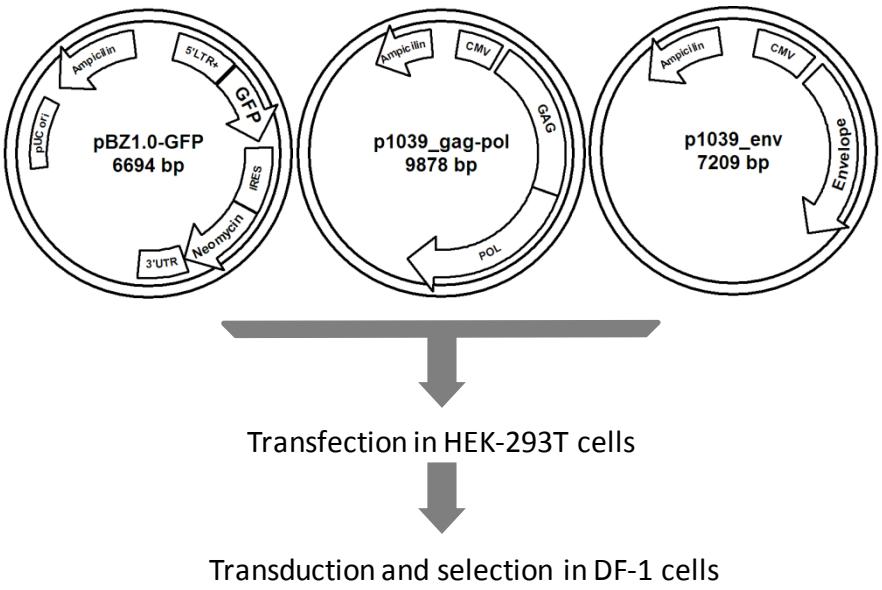

Figure 4.3. Schematic representation of protocol for recombinant viral production. Vector plasmid (pBZ) and packaging plasmids (p1039_gag-pol and p1039_env) are co-transfected into HEK-293 cells. Supernatant is collected 48h later and filtered. DF-1 cells are then used to transduction and selection. Refer to Material and Methods for details.

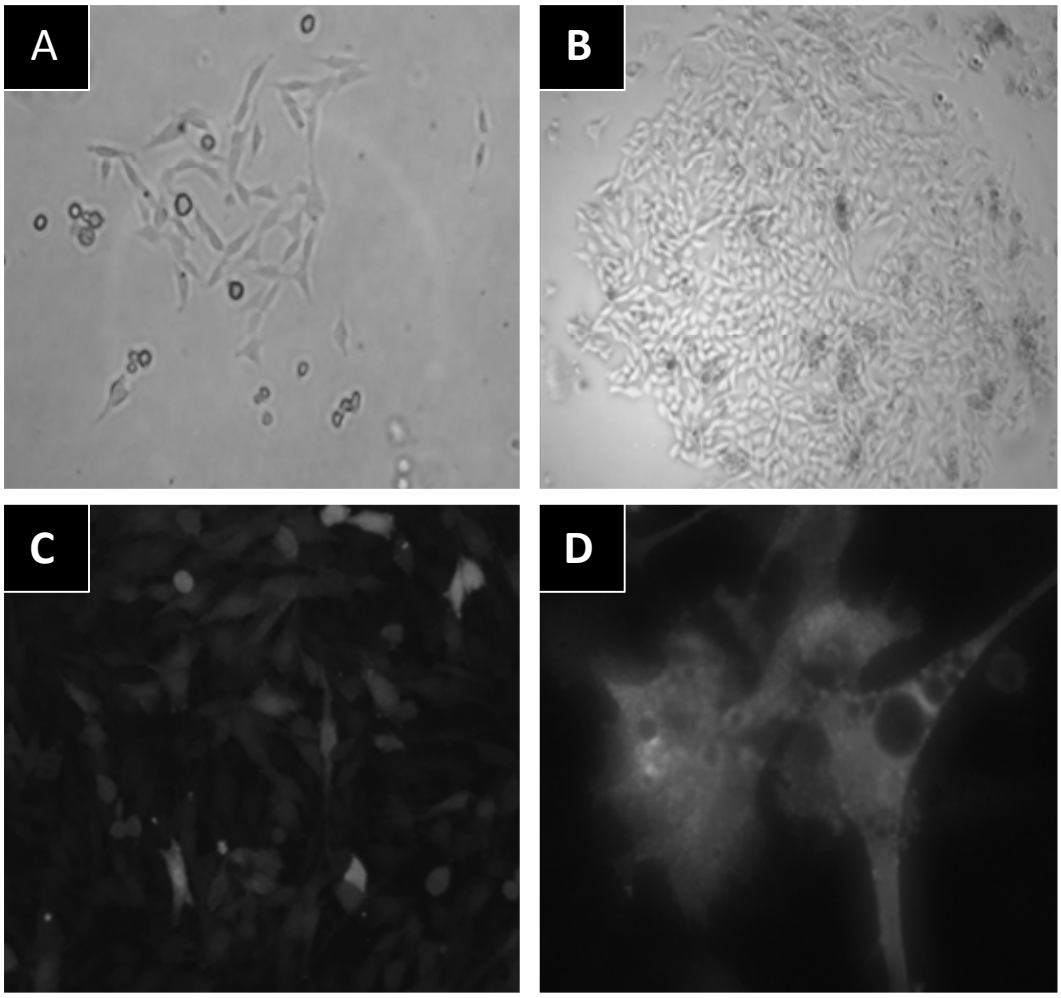

Figure 4.4. Selection and GFP expression of pBZ-1.0(GFP) vector in DF-1 cells. HEK-293T cells were co-transfected with pBZ1.0(GFP) and helper constructs. Viral supernatant was used to transduce DF-1 Cells. Cells were photographed under bright light on A and B, or fluorescent light on C and D. A. Single clone of DF-1 cells after 7 days of drug selection with Geneticin (200µg/mL). B. Same clone of DF-1 cells after 3 weeks on Geneticin selection. C. Expression of GFP under fluorescent light of DF-1 cell clone shown on B. D. Close-up of DF-1 cell expressing GFP.

CHAPTER 5

STABLE EXPRESSION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS GLYCOPROTEIN I (GI) IN AVIAN CELLS AND ITS USE FOR VACCINATION AND SEROCONVERSION IN CHICKENS[1]

[1] Barbosa, T, Zavala, G and Cheng, S., (2010) To be submitted to *Avian Diseases*

Summary

Stable expression of exogenous genes in cell culture may be achieved by using retroviral vectors. Infectious laryngotracheitis virus (ILTV) is a herpesvirus that causes a highly contagious acute respiratory disease in chickens. Several envelope glycoproteins are present in the surface of the virion. The function of most of these proteins is not well understood in ILTV. We have generated a recombinant cell line that continuously expresses the ILTV gI protein, using the ALV-based pBZ retroviral system. These cells were used to produce an inactivated vaccine. Vaccinated chickens were subsequently challenged with the standard ILTV USDA strain. Protection, viral load and serological responses were evaluated. All vaccinated chickens mounted a humoral response against the ILTV gI protein. An anamnestic immune response was observed in chickens that received the vaccine twice. A priming effect was also observed as an ILTV gI antibody titer increase after challenge only in the vaccinated groups. There was no difference in protection and viral load in the trachea among DF-1 ILTV gI vaccinated and DF-1 vaccianted control birds. The pBZ system can be used to stably transduce DF-1 cells to express exogenous proteins, and these recombinant proteins can be used to induce humoral responses in chickens.

Introduction

Retroviral vectors have been used for specific gene delivery in several systems (9, 22, 31). Retroviral vectors can transduce genes into the chromosomes of a wide variety of mammalian and avian cells types. Most retroviral vectors are replication-defective, with replacement of retroviral coding sequences by gene(s) of interest for expression (6, 41). In these vectors, the amount of foreign DNA that can be accommodated is much larger than can be accommodated in replication-competent vectors. Depending on the specificity of the envelope

gene, different cell types can be transduced for continuous expression of the gene of interest present in the retroviral vector.

Infectious laryngotracheitis (ILT) is a highly contagious acute respiratory disease of chickens. The disease is worldwide in distribution and causes great economic losses during severe outbreaks due to a decrease in egg production and high mortality rates (15). Infectious laryngotracheitis virus (ILTV) is a member of the genus *Iltovirus,* within the family *Herpesviridae*, subfamily *Alphaherpesvirinae* (10). Traditionally, two types of modified-live ILT vaccines have been used to control ILTV; namely, vaccines attenuated by multiple passages in embryonated eggs, also known as CEO (chicken embryo origin) vaccines (35); and the Tissue Culture Origin (TCO) vaccine, which was generated by multiple passages in tissue culture (8). In addition to modified live attenuated ILT vaccines, there is also a fowl poxvirus-vectored (FPV) ILT recombinant vaccine (FP-LT); and a herpesvirus of turkeys-vectored (HVT) recombinant vaccine (11, 37). Modified-live ILT vaccines have been associated with a variety of adverse effects including spread of vaccine virus to non-vaccinated birds (18, 33-34), production of latently infected carriers (2), and increased virulence as a result of consecutive back passages *in vivo* (16). Also, live attenuated ILT vaccine viruses have been implicated in field outbreaks of the disease (16).

ILTV inactivated vaccines lack problems such as residual virulence, reversion to virulence, or spread of infection and establishment of latent infection, which occur with modified live vaccines. Inactivated ILT vaccines have been made from inactivated whole ILTV (5) and from affinity-purified glycoproteins (43). They are capable of stimulating immune responses in chickens resulting in some levels of protection against ILTV challenge (5, 43). Inactivated ILT

vaccines have not been cost effective, but new technologies for recombinant protein expression may allow the utilization of those types of vaccines in the future.

Permanently transformed cell cultures have been used for production of exogenous proteins. In recent years, the demand for recombinant therapeutic protein production in cell culture has increase dramatically (17, 42). Shortage and high cost of embryonated SPF eggs have also been a problem for vaccine production. We have recently developed a novel ALV based gene delivery vector system (3) that has been used to stably transduce a reporter gene into an avian fibroblastoid cell line. Herein we describe the use of one of the pBZ vectors to stably transform DF-1 cells expressing the ILTV gI protein and its use as immunogens in vaccine preparation in chickens, as a proof of concept for exogenous protein expression into stable avian lines and its antigenic capabilities for vaccine preparation.

Materials and Methods

Cell lines

Transfections were performed in human embryonic kidney cells 293T cells (HEK 293T). Transduction was done in DF-1 cells, a spontaneously immortalized fibroblastoid cell line derived from line 0 chickens (C/E phenotype for ALV) (20). HEK 293T and DF-1 cells were cultured in DMEM medium containing 2 – 10% fetal bovine serum under 5 % CO_2 at 37ºC. SF-9 cells were cultured in HyClone SFX-Insect serum free medium (Hyclone, Logan, UT) at 28ºC. Chicken kidney (CK) cells were prepared from 3- to 4-week-old Specific Pathogen Free (SPF) chickens as previously described by (33). CK were seeded in 96-well plates and used for ILTV titration and virus neutralization after 24 hours.

Vector construction and virus rescue

The previously described pBZ3.0 vector (3) was used to insert the ILTV glycoprotein I (gI) gene. The ILTV gI ORF was PCR-amplified from a field isolate obtained from a recent outbreak in the USA. Specific primers were designed to amplify the entire ORF of ILTV-gI, based on the ILTV USDA isolate sequence (GenBank Accession U28832). The following primers were used for PCR amplification: ILTV gI Forward 5' GCGGCCGCATGGCATCGCTACTTGGAAC 3' and ILTV gI Reverse 5' GATATCTCActtatcatcatccttgtaatcCATTTTTATTGAGTCGGGC 3. The underlined nucleotides represent the *Not*I and *Eco*RV restriction sites respectively, which were used for cloning into the pbZ3.0 vector. The reverse primer also included the highlighted (lower-case) FLAG™ epitope sequence (Sigma-Aldrich, St. Louis, MO) in frame with the gI ORF. The cloned ILTV gI ORF was sequenced to confirm the site of insertion and absence of mutations in comparison to the ILTV USDA isolate.

Viral rescue was performed as previous described (3). Briefly, HEK 239T cells were seeded at 50-60% confluence in 6-well plates and transfected (Lipofectamine LTX, Invitrogen, Carlsbad, CA) when the cells reached 70 – 80% confluence with 1.6 μg of the delivery vector pBZ3.0-gI and with 0.8 μg of p1039_gagpol and 0.8μg of p1039_env plasmids. Supernatants were collected 48 h and 72 h post-transfection, passed through 0.45 μm filters, and pooled.

Selection of gI positive cell lines

Exponentially growing DF-1 cells were used for transduction. Two days after transduction DF-1 cells were selected with medium in the presence of 200 μg/mL of G-418 (Geneticin, Invitrogen, Carlsbad, CA). Isolated clones were transferred to 24-well plates and

assessed by IFA for expression of recombinant gI protein (see below). Two cell clones were selected for further expansion. DF-1 ILTV gI positive cell clones were kept in culture for at least 25 passages and frozen at -80ºC every five passages. Expression of ILTV gI was confirmed every three passages by IFA or Western blot.

Vaccine preparation

Transduced DF-1 cells were clonally selected, expanded and harvested four days after passage for immunization of chickens. The cells were counted and used for vaccine preparation. Cells were emulsified as 35% of final volume, as 9.1 mL containing $6.6x10^8$ DF-1 ILTV gI cells were mixed thoroughly to create an emulsion with 16.9 mL of Drakeol 6VR:Arlacel-80 mix (90:10). Each dose was calculated to have 10^7 cells in 0.4 mL total volume. DF-1 control cells that were emulsified utilizing an identical procedure were used for inoculations in negative control chickens.

Experimental design

One-day-old SPF chickens were used to examine the immunogenic properties of DF-1 ILTV gI-positive cells. Chicks were divided into eight groups as summarized in Table 1. Birds were vaccinated subcutaneously in the crural area of the leg with the total volume of 0.4 mL. Groups 1 and 2 were vaccinated with the DF-1 ILTV gI vaccine once at one day of age. Groups 3 and 4 were vaccinated at one day of age and re-vaccinated at 10 days of age. Each vaccinated chick received 10^7 cells per application. Groups 5 and 6 were vaccinated with 10^7 DF-1 control cells at 1 and 10 days of age. Group 7 served as a non-vaccinated non-challenged negative control and Group 8 served as non-vaccinated challenged control. At 28 days of age, the chickens were challenged with the ILTV USDA reference strain (ATCC #N-71851). Chickens in

groups 1, 3 and 5 received 10^2 $TCID_{50}$ each and the chickens in groups 2, 4, 6 and 8 received 10^3 $TCID_{50}$ each. All intratracheal inoculations were done using a total volume of 100 µL. Clinical signs were scored individually as previously described (28). The clinical sign scores were recorded at 4, 5, 6, and 7 days post-challenge. Tracheal swabs for DNA extraction and ILTV detection were collected on days 5 and 7 post-challenge. Serum samples were collected pre-challenge (28 days of age) and 7 days post-challenge for antibody detection. Birds were kept in filtered air negative pressure isolators throughout the experiment with water and feed provided *ad libitum.* The birds were humanely euthanized 7 days post-challenge following methods approved by the American Association of Avian Pathologists (AAAP), The University of Georgia (UGA) and the American Association for Laboratory Animal Care (AALAC).

Western blot

Western blot analysis was carried out to assess expression of ILTV gI protein, similar as previously described (38). Briefly, protein extracts of transduced cell were loaded onto a 10% polyacrylamide gel and separated. Proteins were semi-dry transferred (TRANS-BLOT SD Semi-Dry Transfer Cell, Biorad, Hercules, CA) to nitrocellulose membranes and blocked with 5% skim milk in TBS. The membrane was briefly washed with TBS-Tween (0.05%) and incubated overnight with chicken polyclonal anti-ILTV antibody or anti-Flag monoclonal antibody (Sigma-Aldrich, St. Louis, MO). The membrane was then washed with TBS-Tween and incubated with anti-chicken or anti-mouse horseradish peroxidase conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) for 90 minutes at room temperature. The membrane was put through a final wash step, incubated with horseradish peroxidase (HRP) chemiluminescent substrate (Millipore, Billerica, MA) for one minute at room temperature, and analyzed.

Indirect Immunofluorescence

The ALV-based constructs' transduction efficiency was analyzed by indirect immunofluorescence analysis (IFA). Briefly, after transduction and clone selection, DF-1 cells were seeded in 24 well plates. An aliquot was transferred to 96-well plates and fixed 24 h later with a cold acetone–alcohol solution (60:40 v/v). The IFA test was performed following a modified pre-established procedure (4). Blocking was done with skim milk (5% in PBS), followed by incubation with chicken polyclonal anti-ILTV or anti-Flag monoclonal antibodies. After washing with PBS, cells were incubated with fluorescein isothiocyanate (FITC)–conjugated rabbit anti-chicken IgG or goat anti-mouse IgG (Jackson ImmunoResearch laboratories, West Grove, PA). Evan's blue (0.02%) was used for counterstaining. After washing, cells were examined using fluorescence microscopy. Seroconversion of vaccinated chickens was tested by IFA in Sf-9 insect cells infected with a Baculovirus expressing ILTV gI. Sf-9 cells were infected at an MOI of 0.1 and fixed 3 days later. Chicken sera obtained pre- and post-challenge were incubated for 1 h at 37ºC, followed by incubation with FITC-labeled rabbit anti-chicken IgG.

Virus neutralization.

Virus neutralization assay was performed as previously described (1), with some modifications. Briefly, two-fold dilutions of chicken sera (pre- and post-challenge) were prepared in 96-well plates and incubated with 10^2 $TCID_{50}$ of ILTV USDA strain for one hour at room temperature. After incubation, the mixtures were added to previously prepared monolayers of chicken kidney cells. The medium was replaced after 24 hours and the plates were incubated for a total of 6 days at 39ºC in a 5% CO_2 incubator. The cells were observed daily for detection

of cytopathic effects (CPE) and the CPE was recorded. Dilutions of serum that neutralized the formation of CPE were considered positive for anti-ILTV antibodies.

Detection of ILTV by Real-Time PCR

The presence ILTV DNA was determined by real-time PCR in a duplex assay normalized to an internal control (host DNA), combining previously reported assays (7, 23). ILTV specific primers and probe target the UL-44 gene of the ILTV genome. The chicken α2 (VI) collagen gene was used as an internal control. The RealTime-PCR reaction was performed using the iQ^{TM} Multiplex Powermix Kit (BioRad, Hercules, CA) as per manufacturer's recommendations. Briefly, each reaction contained 0.3µM of each primer and 0.2µM of the corresponding probe, 12.5µl iQ Multiplex Powermix, 5µl of DNA template in a total reaction volume of 25µl. The cycling parameters were: 50°C for 2 minutes, then 95°C for 2,5 minutes followed by 45 cycles consisting of denaturation at 94°C for 15 seconds and annealing/extension at 60°C for 60 seconds. Amplification and data acquisition were carried out using the termocycler Chromo4 System for Real-Time PCR Detection (BioRad, Hercules, CA).

The viral DNA amount was normalized to the amount of chicken genome to eliminate the sampling error. The viral load of each sample was calculated from the amount of the viral DNA (ILTV), normalized to the amount of the host gene (collagen), which serves as the endogenous internal control (27). The amount of both, the target and the internal control, were measured as the Ct value at the same time in a duplex reaction. The quantitation of the ILTV DNA present in each sample was calculated relative to the value obtained in the samples from the negative control group at the same age. Thus, the relative amount of the viral DNA was calculated as the $\log_{10}$ of the $2^{-\Delta\Delta Ct}$, as previous described by Livak *et al*. (25); where, $\Delta\Delta Ct = \Delta Ct_{challenge} - \Delta Ct_{control}$. The $\Delta Ct_{challenge}$ is the amount of ILTV genome normalized against the amount of the

collagen gene in the same sample for that bird. In addition, $\Delta Ct_{control}$ is the average amount of the ILTV genome normalized against the amount of the host gene of the negative control birds at the respective age.

Statistical analysis

The statistical significance of differences in $\Delta\Delta Ct$ and $Log_{10}\ 2^{-\Delta\Delta CT}$ values were evaluated by ANOVA Tukey test ($P<0.05$). As both tests showed the same results, only the $Log_{10}\ 2^{-\Delta\Delta CT}$ results are reported.

Results

The gI ORF of ILTV was inserted into the pBZ3.0 ALV-based retroviral vector. In this vector, the Cytomegalovirus (CMV) promoter initiates the transcription of the gI gene and the neomycin resistance gene. ILTV gI glycoprotein expression is achieved by regular cap mRNA processing of the gI gene; and the neomycin resistance is translated in a cap-independent manner through the IRES (internal ribosomal entry site) present in the vector. Recombinant virus production was accomplished by transfection of HEK-293T cells as previously described (3). Supernatant was harvested and used to transduce DF-1 cells. The transduced DF-1 cells were selected with G-418 until isolated colonies were observed. Isolated colonies were tested for ILTV gI expression by IFA (Figure 1). Twenty-eight different cell clones were tested by IFA and were tested positive for ILTV-gI expression. Two cell clones were selected for further expansion. Both clones were strongly positive for IFA using anti-Flag monoclonal and anti-ILTV polyclonal antibodies. Clones were subcultivated every 3-5 days and part of the cells were counted, concentrated by low-speed centrifugation and stored at -80ºC. Total protein cell extracts

were used for Western blot every five passages (Figure 1). Again, DF-1 ILTV gI clones were positive for both antibodies. Control cells did not react with either antibody. DF-1 ILTV gI cells were also tested for ALV replication by commercially available antigen capture ELISA (ALV - p27), where both clones were negative, certifying the absence of a replicating ALV virus, as expected (results not shown).

Frozen stocks of DF-1 ILTV gI cells were used for an oil-emulsion preparation. Each dose was calculated to contain 10^7 cells. The experimental vaccine was injected subcutaneously in SPF chickens in a manner similar to vaccinations performed in chickens with oil-emulsified vaccines. The vaccination schedule is detailed in Table 1. At 28 days of age all groups, except the non-vaccinated non-challenge group (group 7), were challenged with the ILTV USDA reference strain using two different doses ($TCID_{50}$ 10^2 and $TCID_{50}$ 10^3) as described on Table 1. Numerical differences on the clinical signs scores were noted among the challenge groups but no significant differences were found (Table 2). The non-vaccinated non-challenged group did not show any clinical signs related to ILTV infection. Two birds in group 2 and one bird in group 4 died after challenge. In addition, these groups had the highest numerical average for clinical signs.

Seroconversion against ILTV gI was tested on serum collected prior to and seven days post-challenge. For this purpose, Sf-9 cells infected with recombinant BacV-gI were used in indirect immunofluorescence assays (Figure 1). Results are summarized on Table 3. All chickens that were vaccinated had seroconverted by 28 days of age (dilution 1:50). None of the DF-1 control cell-vaccinated chickens or the non-vaccinated chickens were positive to ILTV gI antibodies by IFA. At seven days post-challenge the non-vaccinated and the DF-1 control groups still did not have antibodies against ILTV gI, most likely due to the short period after challenge.

The antibody titers of the DF-1 ILTV gI vaccinated groups correlated with the vaccination schedule. Birds in groups 3 and 4 were vaccinated twice and showed a higher percentage of positive birds (80-90%) at a 1:100 serum dilution, in comparison with groups 1 and 2 (50%), which were vaccinated only once. The antibody titers against ILTV gI increased seven days after challenge when compared to pre-challenge levels. Birds in groups 3 and 4 had 30-55% positive samples at a 1:500 dilution; and the groups in groups 1 and 2 did not have any positive samples at this dilution.

Virus neutralization was done in 96-well plates with two-fold dilution of serum pre and post-ILTVchallenge. ILTV-gI specific serum samples, as indicated by the IFA results, were tested for their ability to neutralize 10^2 $TCID_{50}$ particles of ILTV per well. Results are summarized in Table 4. Serum pre-ILTV challenge partially neutralized the ILTV replication in chicken kidney cells. However, serum from the birds in groups 2 and 4 (vaccinated with DF-1 ILTV-gI and challenge with ILTV), neutralized the USDA strain at significantly higher titers ($P<0.05$) when compared with serum collected from all the other groups. ILTV convalescence sera collected 21 days after ILTV USDA strain challenge, used as a positive control, had a titer above the dilution tested (≥ 256).

The viral load in the trachea after challenge was indirectly assessed by real-time PCR measuring the ILTV genomic copy number and normalizing it against the α2 (VI) collagen gene. Tracheal swabs were collected at five and seven days post-challenge and the results of viral DNA detection are shown on Table 2. The number of ILTV genome copies was higher at 5 days post-challenge compared to 7 days post-challenge. The birds that received the lowest challenge dose had the lowest level of ILTV genome copies. There was a difference in birds with differing

challenge doses, but the groups of birds that received the same amount of challenge virus did not differ in the levels of ILTV genome copies.

Discussion

Embryonated eggs are the major system for vaccine production for poultry worldwide. The expense involved in maintaining SPF chicken flocks accounts for a large part of the cost of vaccine manufacturing. Inactivated vaccines are used in poultry to increase antibody levels in commercial egg layers and broiler breeders. The production of inactivated vaccines also relies on embryonated eggs. A potential shortage of embryonated SPF eggs could represent a critical challenge for the future of vaccine production. Thus, alternative ways of vaccine production need to be explored. Recombinant protein expression in various systems has been accomplished and tested for vaccine applications. Fungi (30, 39-40), insect (26, 29, 36) and bacterial (14) systems have been used successfully. However, these heterologous systems, in most cases, depend on the purification of the proteins for mass applications. We set out to explore the use of avian cell lines for recombinant protein expression, which would have some advantages such as direct use without protein purification; a "natural" environment for protein folding and post-translational modifications; and easy and inexpensive maintenance of cells. This would be true especially for viruses that are difficult to grow in *in vitro* systems or that have regulatory restrictions regarding use in the laboratory.

Our previous work demonstrated that the pBZ retroviral vector system can be used to stably transform DF-1 cells with the purpose of expressing exogenous proteins (3). To validate this system using an avian viral protein, we chose the ILTV gI protein for continuous expression in DF-1 cells. ILTV gI expression was successfully achieved during several passages in DF-1

cells. The protein was identified by IFA and western blot. DF-1 cells are fibroblastoid cells of chicken origin that are easy to manipulate in laboratorial conditions (20). DF-1 cells can be grown in simple medium and split every 3 days with a high rate of replication. The ILTV gI protein was steadily expressed in both clones of ILTV gI DF-1 cells that were selected for this study. Relative levels of expression accessed by IFA and Western Blot were kept very similar throughout the passages tested (data not shown).

In order to test the antigenicity of the recombinant ILTV gI protein expressed in DF-1 cells for vaccine preparation, we injected SPF chickens subcutaneously with a preparation containing ILTV gI-expressing DF-1 cells as a vaccine. All birds that were injected with the vaccine seroconverted to ILTV gI. A boost effect was observed in chickens vaccinated twice since the second injection at 10 days of age resulted in higher antibody levels than in groups of chickens that received ony one vaccination. Also, ILTV gI-specific antibody levels increased in vaccinated birds only seven days post-challenge, demonstrating the priming effect of the vaccinations.

The ability of anti-ILTV IFA positive antibodies to neutralize ILTV-replication in cell culture, was tested by the virus neutralization assays. The pre-challenge serum, despite showing levels of anti-ILTV gI antibodies by IFA, did not neutralized the USDA strain *in vitro*. Serum samples from birds groups 2 and 4 when tested 7-days post-ILTV challenge neutralize the virus (Table 3). Geometric mean titers for birds in these two groups were significantly higher than for the other tested groups. It has been previously demonstrated that humoral response may have a very small role, if any, in protecting birds against ILTV challenge (32). However, it also has been shown that anti-ILTV serum can neutralized ILTV from replicating in cell culture (1, 19). Clearly, the antibody levels measured by IFA and VN did not correlate with protection, as

challenge groups of birds did not show differences with DF-1 vaccinated controls birds. Nevertheless, the ability of serum from birds previously vaccinated with ILTV-gI, to neutralize ILTV replication, is interesting. These VN titers also correlate with high IFA titers and they may be directly related, but this observation has not been studied.

ILTV gI is thought to be directly related to cell-to-cell spread of ILTV. Previous studies with glycoprotein I and glycoprotein E deletion mutants of ILTV (gI/gE-ve ILTV) have demonstrated that most likely the heterodimer gI/gE is directly involved in the cell-to-cell spread of ILTV (12). The authors reported that the gI/E negative ILTV was readily propagated in cell culture in the presence of parental ILTV. However, it did not grow in the absence of parental ILTV. The gI/gE negative ILTV failed to produce plaques but single infected cells could be identified by fluorescence microscopy (12). It has been suggested that the gI/gE heterodimer plays a major role in the cell-to-cell spread of ILTV *in vitro,* probably being more important in ILTV than in other herpesviruses (12). In other members of the *Herpesviridae*, the gI/gE heterodimer complex is most likely involved in the cell-to-cell spread as well, but is not as essential as it is for ILTV replication (13, 21, 24).

Our results demonstrate that the antibody response to ILTV gI did not protect against ILTV challenge at the antibody titers achieved in this experiment. The ILTV-related clinical signs observed and the amount of ILTV genome copies detected in the trachea were not different in chickens that mounted an immune response against ILTV-gI compared to sham-vaccinated birds. Despite numerical differences found in the ILTV genome copies, such differences were not statistically significant. Mortality was only observed in groups that did seroconvert against ILTV gI. This result is intriguing and deserves further investigation. The hypothesis that anti-ILTV gI antibodies would facilitate the replication of ILTV in the trachea was not proven but

cannot be discarded. However, ILTV gI positive birds died after challenge. Levels of anti-ILTV gI antibodies seems to be less important than the level of challenge virus, as only vaccinated groups with higher doses died as a result of ILTV challenge.

Our main goal was to demonstrate that proteins of viral origin could be stably expressed in cell cultures using the novel ALV-based pBZ retroviral vector system and that proteins thus expressed can induce an immune response in chickens. We have clearly shown that viral proteins can be expressed in sufficient levels to potentially be used as antigens in vaccine preparation. Further studies should reveal the potential of this system to express additional avian viral proteins in DF-1 cells and potential use for vaccinations. Such system could be an alternative method for the vaccine industry worldwide to produce inactivated vaccines, especially for viruses that are difficult or expensive to propagate in the laboratory.

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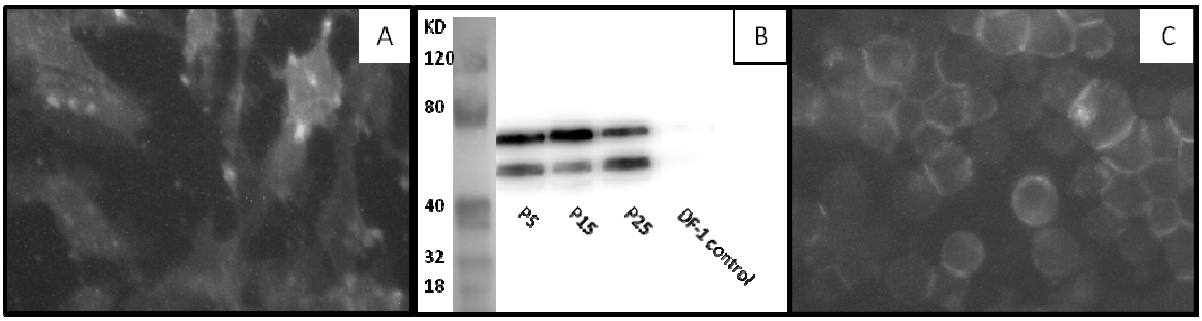

Figure 5.1. Expression and detection of ILTV-gI by immunofluorescence and Westernblot assays. A. Indirect Immunofluorescence Assay (IFA) using polyclonal anti-ILTV antibody to detect DF-1 cells expressing ILTV-gI after transduction and selection. B. Westernblot assays of total protein of DF-1 cells expressing ILTV-gI on passages 5 (P5), 15 (P15) and 25 (P25). DF-1 cells non-transduced were used as controls. Note two bands, suggesting different stages of glycosylation. C. IFA in SF-9 cells infected with Baculovirus expressing ILTV-gI using chicken serum after immunization with DF-1 ILTV-gI.

Table 5.1. Experimental design, vaccination protocol and ILTV challenge dose for vaccinations in SPF chickens.

	Vaccination Protocol		Challenge dose/bird[2]
	Antigen[1]	Age at vaccination (days)	
Group 1	DF-ILTV gI	1	10^2
Group 2	DF-ILTV gI	1	10^3
Group 3	DF-ILTV gI	1 and 10	10^2
Group 4	DF-ILTV gI	1 and 10	10^3
Group 5	DF-1 controls	1 and 10	10^2
Group 6	DF-1 controls	1 and 10	10^3
Group 7	None	NA[3]	NA
Group 8	None	NA	10^3

[1] Each bird received 10^7 cells in each application. Control groups received the same concentration of control DF-1 cells.

[2] Dose calculated by $TCID_{50}$ in total volume of 100µL.

[3] NA – Not applicable.

Table 5.2. Real Time PCR results for ILTV genome standardized by Chicken α-Collagen gene shown in $Log_{10}2^{\Delta\Delta Ct\ Values}$ and Average clinical signs on days 4 to 7 post-ILTV challenge.

Group	Treatment	Real Time PCR				Clinical Signs				Total Mortality[2] (%)
		5 days post challenge		7 days post challenge		Average Score				
		$Log_{10}2^{\Delta\Delta Ct}$ (Stand. Dev)	% Positive samples	$Log_{10}2^{\Delta\Delta Ct}$ (Stand. Dev)	% Positive samples	Day 4	Day 5	Day 6	Day 7	
Group 1	Vx(gI)+ILTV(10^2)[3]	$2.47^{a,b,c\ (1)}$ (2.09)	70	$1.28^{a,b}$ (1.26)	10	0.10	0.20	0.00	0.00	0
Group 2	Vx(gI)+ILTV(10^3)	4.48^{a} (1.16)	100	3.76^{a} (2.15)	86	1.44	1.13	0.43	0.00	20
Group 3	Vx(gI+gI)+ILTV(10^2)	$2.37^{a,b,c}$ (2.56)	56	0.68^{b} (1.30)	30	0.10	0.33	0.00	0.00	0
Group 4	Vx(gI+gI)+ILTV(10^3)	4.84^{a} (1.08)	100	$2.02^{a,b}$ (1.57)	78	0.30	0.78	0.11	0.11	10
Group 5	Vx(DF-1+DF-1)+ILTV(10^2)	$1.05^{b,c}$ (1.76)	40	0.23^{b} (0.91)	20	0.40	0.44	0.22	0.13	0
Group 6	Vx(DF-1+DF-1)+ILTV(10^3)	3.86^{a} (1.93)	90	$1.81^{a,b}$ (2.21)	60	0.60	0.50	0.10	0.00	0
Group 7	NoVx – No ILTV	0.0^{c} (0.42)	0	0.0^{b} (0.25)	0	0.00	0.00	0.00	0.00	0
Group 8	No Vx - ILTV(10^3)	$3.17^{b,c}$ (0.90)	100	$2.38^{a,b}$ (2.81)	40	0.60	0.80	0.00	0.20	0

[1] Different letters in the same columns denote significant differences by ANOVA Tukey test (P≥0.05).
[2] Mortality related to ILTV symptoms after challenge.
[3] Refer to Table 1 for details

Table 5.3. Indirect Immunofluorescence assay specific for ILTV gI on chicken serum at 28 days of age (pre-ILTV challenge) and at 35 days of age (7 days post-ILTV challenge).

		28 days of age (pre-challenge)				35 days of age (7 days post challenge)			
		Dilutions				Dilutions			
	Treatment	1:50	1:100	1:500	1:2000	1:50	1:100	1:500	1:2000
Group 1	Vx(gI)+ILTV(10^2)[3]	10/10 (100)	**5/10[2] (50)**	0/10 (0)	0/10 (0)	10/10 (100)	**9/10 (90)**	0/10 (0)	0/10 (0)
Group 2	Vx(gI)+ILTV(10^3)	NT[4]	NT	NT	NT	7/7 (100)	**6/7 (85)**	0/10 (0)	0/10 (0)
Group 3	Vx(gI+gI)+ILTV(10^2)	10/10 (100)	9/10 (90)	**1/10 (10)**	0/10 (0)	10/10 (100)	10/10 (100)	**3/10 (30)**	0/10 (0)
Group 4	Vx(gI+gI)+ILTV(10^3)	10/10 (100)	**8/10 (80)**	0/10 (0)	0/10 (0)	9/9 (100)	9/9 (100)	**5/9 (55)**	0/10 (0)
Group 5	Vx(DF-1+DF-1)+ILTV(10^2	0/10 (0)	NT	NT	NT	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
Group 6	Vx(DF-1+DF-1)+ILTV(10^3	NT	NT	NT	NT	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
Group 7	NoVx – No ILTV	0/8 (0)	NT	NT	NT	0/8 (0)	0/8 (0)	0/8 (0)	0/8 (0)
Group 8	No Vx - ILTV(10^3)	0/5 (0)	NT	NT	NT	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)

[1] SF-9 insect cells were infected with BacV-gI and fixed 3 days later (See material and Methods for details).
[2] Number of positive samples/number of samples tested. Positivity percentage shown in parenthesis. End point IFA-positive dilutions are shown in bold.
[3] Refer to Table 1 for details
[4] NT – Not tested

Table 5.4. Geometric mean titer and range of titers of chicken sera measured by virus-neutralization (VN) before and after ILTV challenge.

Group	Treatment	28 days of age (Before Challenge)			35 days of age (7 days post-challenge)		
		Number of samples	Geometric mean titer[1]	Range of titers	Number of samples	Geometric mean titer[2]	Range of titers
Group 1	Vx(gI)+ILTV(10^2)[3]	10	2.00	0-2	10	2.00[b]	0-8
Group 2	Vx(gI)+ILTV(10^3)	NT[2]	NT	NT	7	14.85 [a,b]	0-32
Group 3	Vx(gI+gI)+ILTV(10^2)	10	0.20	0-2	10	6.00 [b]	2-16
Group 4	Vx(gI+gI)+ILTV(10^3)	10	0.20	0-2	9	27.77 [a]	2-64
Group 5	Vx(DF-1+DF-1)+ILTV(10^2)	10	0.20	0-2	10	2.00 [b]	0-16
Group 6	Vx(DF-1+DF-1)+ILTV(10^3)	NT	NT	NT	NT	NT	NT
Group 7	NoVx – No ILTV	8	0	0	8	0.25 [b]	0-2
Group 8	No Vx - ILTV(10^3)	5	0	0	5	1.20 [b]	0-4

[1]Titers are expressed as the reciprocal of the highest serum dilution that neutralized virus.
[2] Different letters in the same columns denote a significant difference by ANOVA Tukey test (P≥0.05).
[3] Refer to Table 1 for details
[4] Not Tested.

CHAPTER 6

CONCLUSION

Vaccination is still one of the most effective ways to prevent losses from viral infection in commercial poultry, in particular infection by viruses that are difficult to eradicate. Vaccine production methods available today rely mostly on the use of embryonated eggs or primary or secondary cell cultures derived from embryonated eggs. These eggs must be SPF (specific pathogen free), which makes them very expensive and one of the most costly components of vaccine manufacturing. Inactivated vaccines are largely used in poultry to increase antibody levels in commercial egg layers and broiler breeders. The production of inactivated vaccines also relies on embryonated eggs. Due to large demand and a limited number of sources of SPF eggs, a future shortage of embryonated eggs cannot be discarded and alternative ways of vaccine production should be explored. Methodologies to express recombinant proteins intended for poultry vaccine production have been reported in the literature, but most of them are based on heterologous systems.

Aiming to develop a new system for vaccine production, we designed the hypothesis that immunogenic proteins from avian viruses could be stably expressed in avian cell lines and used for vaccine preparation without purification. The methodology would include the development of a novel avian retroviral system, based on Avian Leukosis viruses (ALV) with unique biological features and the stable expression of exogenous proteins in avian cell lines.

First, the complete proviral genomes of three natural recombinant replicating avian leukosis viruses (PDRC-1039, PDRC-3246 and PDRC-3249) isolated from commercially produced contaminated Marek's Disease vaccines were fully sequenced. All three viruses were shown to be extremely similar to each other at the nucleotide level (>99.3%), demonstrating that most likely they share the same ancestor. Nucleotide and amino acid sequence analyses and comparison against previously known endogenous and exogenous ALV sequences revealed that the LTRs, *gag*, *pol* and TM (gp37) of PDRC-1039, PDRC-3246 and PDRC-3249 are highly similar to corresponding sequences in endogenous viruses. Thus most of the contaminant ALV genomes have an endogenous origin, with the gp85 coding region of the envelope gene being the sole exception, as this region in all three contaminant viruses was highly similar to the gp85 region of exogenous ALV-A. The LTR sequences of the contaminating ALVs were found to be most similar to LTR sequences of endogenous viruses. This is the most likely reason why these viruses have shown non-oncogenic properties in White Leghorn SPF chickens.

These viruses most likely are a product of recombination between an exogenous virus (ALV-A) and endogenous ALV sequences. A variety of relatively frequent recombinatorial and mutational events are known to occur in retroviruses including avian retroviruses, mainly because of the diploid nature of their genomes and the lack of a proof reading mechanism during reverse transcription. Our results show that almost the entire genomes of the vaccine contaminant viruses are closely related to endogenous ALV-E (*ev* loci) and only part of the SU protein of the envelope gene is closely related to an exogenous virus (ALV-A). Our search for *ev* loci in the total genomic DNA from the original contaminated vaccine vials revealed 5 *ev* loci in each vaccine. The embryos used for vaccine production may have been produced by a common source SPF egg company and may have been carriers of a variety of *ev* loci while the phenotypes

of endogenous virus particles expressed in the vaccine substrates may have varied with the embryo pools used. The chicken embryo fibroblasts from all three vaccines showed an identical pattern for *ev* loci.

Despite the presence of replication-competent endogenous virus sequences in the vaccines, the only possible mechanism for the vaccine contaminants to acquire the exogenous ALV-A SU sequences would be by coinfection between ALV-E and ALV-A. We hypothesize that homologous recombination between sequences from endogenous ALV and exogenous ALV-A may have taken place. Our results support this theory, since only part of the SU coding sequence is similar to ALV-A and the rest of the viruses still resemble mostly ALV endogenous virus sequences.

In our second study, we constructed an ALV vector system based on the PDRC-1039 isolate. This system, termed pBZ, allows for the efficient transduction of exogenous genes into chicken cells. The pBZ system does not contain any region of homology between the delivery vector and the helping vectors, which provide the necessary proteins for virus assembly in *trans.* This absence of homology among the pBZ system plasmids reduces greatly the possibility of recombination events during virus assembly and reverse transcription. This is a major advantage of the pBZ vectors compared to previously reported ALV vectors. In addition, the pBZ system is the first ALV-based vector that contains 13 restriction sites in the multicloning site, allowing substantial flexibility for construction of recombinant viruses. Our novel ALV-based pBZ system includes eight different vectors. The diversity of designs of the pBZ system may facilitate future studies. Also, the expression of a gene of interest in the pBZ vector system is always associated with the expression of a drug selectable marker, which normally renders a higher level of expression of the gene of interest. The objective of developing a new retroviral vector based on

the recent isolate ALV PDRC-1039 was accomplished, and we successfully transduced DF-1 cells with all eight novel ALV vectors. DF-1 cells could be clonally selected and these clones expressed green fluorescent protein (GFP) for several passages. No detectable production of infectious virions was observed either in transfected or transduced cells, thus the system could be useful for the introduction of exogenous genes into avian cells in which the continuous production of virus is unacceptable.

Finally, we demonstrated that the pBZ retroviral vector system could be used to stably transform DF-1 cells with the purpose of expressing exogenous proteins of viral origin. We chose to stably express the ILTV gI protein, which was successfully achieved in DF-1 cells. The antigenicity of the recombinant ILTV gI protein expressed in DF-1 cells was tested on a vaccine preparation. SPF chickens were injected subcutaneously with the ILTV gI-expressing DF-1 cells. All injected birds seroconverted to ILTV gI, and an anamnestic response was observed in chickens vaccinated twice with the same vaccine. The ILTV gI-specific antibody levels increased seven days post-challenge only in vaccinated groups of chickens, demonstrating the priming effect of the vaccinations.

We also tested the ability of anti-ILTV IFA positive antibodies to neutralize ILTV replication in cell culture. The pre-challenge serum did not neutralize the USDA ILTV strain. However, ILTV gI-vaccinated birds challenged with higher doses of ILTV had neutralizing antibodies in their serum seven days post-challenge. It has been previously proposed that humoral responses may exert a very small role, if any, in protecting birds against ILTV challenge. However anti-ILTV serum was able to neutralize ILTV *in vitro* in cell cultures. Our results support the hypothesis proposing that anti-ILTV gI antibodies detectable by IFA and VN do not correlate with protection *in vivo*.

The main goal of this research was to demonstrate that proteins of viral origin could be stably expressed in cell cultures using the novel ALV-based pBZ retroviral vector system and that proteins thus expressed can induce an immune response in chickens. The results clearly showed that viral proteins can be expressed in sufficient levels to potentially be used as antigens in vaccine preparation.

However, the results also showed that the choice of the protein to be expressed in this system can be a major factor for the efficacy of the vaccine. Additional to the research shown here, we also studied the possibility of expressing chicken infectious anemia virus (CIAV) proteins using the same system. The VP1 ORF was amplified by PCR from a CIAV field isolated and inserted in the vectors pBZ1.0, pBZ2.0, pBZ3.0, and pBZ4.0. Expression of CIAV protein was detected after transfection (HEK-293T cells) and transduction (DF-1 cells). However, after selection, the isolated cell clones were all negative for CIAV-VP1 protein. In order to map the region responsible for the silencing of the VP1 expression, eight different truncated forms of VP1 were constructed by PCR. CIAV VP1 is 499 amino acids long and it has a nuclear localization signal (NLS) within the first 50 amino acids of the N-terminus. Truncated forms included deletions of the first 15, or 30, or 50, or 100 or 150 amino acids of the N-terminus. Also three constructs were designed to have either the 1-150, 150-300 or 300 to 449 amino acid positions. All eight contructs were tested in the pBZ vectors (1.0, 2.0, 3.0 and 4.0). None of the recombinant viruses rendered a cell clone expressing the respective truncated form of CIAV VP1. The reasons for this failure were not investigated but it is evident that this expression system may not be successful for all proteins.

Nevertheless, the pBZ system could be an alternative method for the vaccine industry to explore production of inactivated vaccines, especially for viruses that are difficult to propagate in

laboratory conditions. An additional application of the system described could be the expression of specific viral receptors in cell lines that are ordinarily non-permissive for viral replication, which would allow the propagation of viruses that would otherwise be difficult or impossible to propagate in specific cell lines.