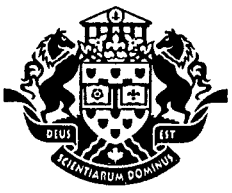

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Detection of Avian Leukosis Virus in the Laboratory and in Naturally Infected Poultry Flocks Using the Polymerase Chain Reaction

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LIST OF ABBREVIATIONS

Ab: antibody
Ag: antigen
ADOL: Avian Diseases and Oncology Laboratory
ALV: avian leukosis virus
AMV: avian myeloblastosis virus
***apo*VLDLII:** very low density apolipoprotein II gene
ART-CH: avian retrotransposon from chicken genome
ASV: avian sarcoma virus

bp: base pairs

CEF: chick embryo fibroblast
CR1: chicken repetitive element 1

DMSO: dimethyl sulphoxide
dNTP: deoxynucleotide triphosphate

EAV: endogenous avian retrovirus
EDTA: ethylenediaminetetra-acetic acid
ELISA: enzyme-linked immunosorbent assay
EM: electron micrograph
***env*:** envelope gene
***ev*:** endogenous viral gene

FAPP: filtered air, positive pressure

***gag*:** group-specific antigen gene
gsa: group-specific antigen

HBV: hepatitis B virus
HIV: human immunodeficiency virus
HPV: human papilloma virus

IgG: immunoglobulin isotype G

kD: kilodaltons

LINE: long interspersed element
LLV: lymphoid leukosis virus
LTR: long terminal repeat

PBS: phosphate buffered saline
PCR: polymerase chain reaction
***pol*:** polymerase gene
pRAV2: cloned RAV-2 genome
PrRSV-C: Prague strain of RSV (subgroup C)
PSSPD: primer specific solid-phase detection

RAV: Rous associated virus
RAV-0: Rous associated virus 0 (subgroup E)
RAV-1: Rous associated virus 1 (subgroup A)
RAV-2: Rous associated virus 2 (subgroup B)
RAV-49: Rous associated virus 49 (subgroup C)
RAV-50: Rous associated virus 50 (subgroup D)
REV: reticuloendotheliosis virus
rpm: rotations per minute
RSV: Rous sarcoma virus
RSV-29: Rous sarcoma virus 29
RT-PCR: reverse transcriptase polymerase chain reaction

SR-D: Schmidt-Rupin strain of RSV (subgroup D)
SU: surface protein

***Taq*:** *Thermus aquaticus*
TM: transmembrane protein

UV: ultraviolet

SUMMARY

Commercial poultry operations continuously test for the presence of avian leukosis virus (ALV) in their flocks. ALV is a retrovirus which causes bursal lymphomas in infected poultry as well as decreasing reproductive efficiency and overall general health of the birds. Tests currently in use for detecting ALV are ELISA (enzyme-linked immunosorbent assay)-based and detect either specific viral proteins or antibodies that are raised against ALV upon infection. There are problems with both viral protein and antibody detection leading to false positive and negative results. Some of these problems are due to the presence of endogenous retroviral elements that are naturally part of the chicken genome and are inherited in a Mendelian fashion. These endogenous viral (*ev*) genes are closely related to the exogenous forms of ALV and have been classified as being part of subgroup E in the avian leukosis group of viruses. They have a high degree of sequence similarity (> 90%) with all exogenous subgroups of ALV (subgroups A, B, C and D) at both the genetic and protein level. The similarity at the protein level is the basis for the problems of ELISA-based detection of infection; the genetic likeness introduces some complexity in developing a PCR-based test.

Regions of the ALV genome have been identified which differentiate endogenous from exogenous ALV and these areas have been targeted with PCR primers. Using semi-nested PCR amplification of the LTR (long terminal repeats), we were able to detect all four subgroups of exogenous viruses affecting chickens (A, B, C and D). Two other sets of semi-nested primers targeted within the variable regions of the *env* (envelope) gene are able to determine the subgroup, A or B (the predominant subgroups infecting North American flocks), of the infecting

virus. These sets of primers were first tested on chick embryo fibroblasts (CEF) not carrying any *ev* genes. The cells were infected with viral isolates of subgroups A (RAV-1), B (RAV-2), C (RAV-49) and D (RAV-50). The LTR primers detected all four subgroups, while the *env* primers detected only the virus of the subgroup for which they were designed.

The ultimate goal of poultry producers is to eradicate ALV from their flocks. Using the current testing procedures, this will be virtually impossible to achieve. Development of a PCR-based test will increase the sensitivity of detection and help eliminate the problems of false positives and negatives. The sensitivity of PCR detection of ALV was compared to that of the current testing methods. The LTR primers revealed that the LTR region appears to be variable in a given viral population of an infected chicken. The subgroup A-specific primers detected all birds which had tested positive for viral protein in albumen and more. It is proposed that these birds, which albumen testing missed, are responsible for maintaining ALV infection. It will now be possible to remove infected birds, which are below the threshold of detection for the commonly used tests, and thereby increase the commercial flocks' overall general health, increase production efficiency and help in eradicating ALV from commercial operations.

RÉSUMÉ

L'industrie de produits de volailles est toujours concerné de la présence du virus leucosique (ALV) dans leurs troupeaux. ALV est un rétrovirus qui cause des néoplasmes dans une proportion d'oiseaux infectés aussi bien qu'un abaissement général de la santé des troupeaux où il y a présence d'ALV. Les méthodes qu'on utilise couramment pour détecter la présence d'ALV sont basées sur la méthode d'ELISA qui détermine soit la présence de protéines provenant du virus ou des anticorps contre le virus. Les protéines peuvent être détectées dans des cellules infectées (ie. des lymphocytes) ou dans le blanc de l'oeuf quand les protéines traversent l'oviducte. Les anticorps sont detectés quand un oiseau peut élever une réponse immunisant contre le virus. Il y a des problèmes avec ces deux méthodes de détection qui menent à des résultats de faux négatifs et de faux positifs. Un de ces problèmes est la présence de gènes endogènes (*ev*) qui sont parentés des virus exogènes qui causent les problèmes de santé observés. Les *ev* ont un grand degré de similarité avec tous les virus exogènes au niveau génétique et protéinique. C'est la similitude au niveau des protéines qui peut causer des résultats de faux positifs avec les méthodes ELISA. La similitude génétique introduit de la difficulté dans le dévelopement d' un test basé sur le PCR.

Des régions du génome d'ALV ont été identifié qui sont variables quand on compare les virus endogènes aux virus exogènes. Ces régions ont été utilisé pour différencier les deux types avec le PCR. Utilisant le double PCR, des amorces ont été construits qui se dirigent soit dans la région du LTR ou dans la région de l'envelope qui designe le sous-genre du virus. Le PCR avec les amorces du LTR détecte tous les sous-genres exogènes (A, B, C et D). Les amorces qui sont

dirigés vers l'envelope détectent soit le sous-genre A ou B, les deux sous-genres responsablent pour la majorité des infections en Amérique du Nord. Tous les amorces ont été essayé sur des cellules infectées avec soit RAV-1, RAV-2, RAV-49 ou RAV-50. Les amorces pour le LTR ont pu détecter tous les variantes. Les amorces pour l'envelope ont seulement détecté les sous-genres pour lesquels ils étaient désignés.

L'industrie de volaille veut se débarasser de la présence d'ALV exogènes. Avec les méthodes couramment utilisées, ce but ne semble pas possible. Le dévelopement d'un test PCR augmentera la sensibilité de détection et aidera à éliminer les problèmes de faux résultats. La sensibilité de le PCR dans la détection de l'ADN proviral d'ALV a été comparé aux résultats des méthodes conventionnelles. Les amorces pour le LTR ont révélé la variabilité de cette région dans un même individu. Les amorces pour l'envelope ont détecté tous les individus qui étaient positifs pour des protéines d'origine viral dans le blanc d'oeuf et plus. Les oiseaux que ELISA a manqués peuvent être la source d'ALV qui empêche l'éradication. Il sera maintenant possible d'enlever ces oiseaux et ceci aidera certainement à éradiquer ALV des troupeaux commerciaux.

INTRODUCTION

The overall objective of this study was to locate areas within the genomes of exogenous avian leukosis viruses (ALV) that differ in sequence when compared to analogous regions in endogenous ALV genomes or *ev* genes. Oligonucleotide primers were then targeted against these regions and the polymerase chain reaction (PCR) technique was used to determine the exogenous ALV status, infected or not infected, of poultry. The use of exogenous virus-specific PCR testing detects infected birds which can currently be diagnosed with enzyme-linked immunosorbent assay (ELISA)-based testing as well as birds which are indeterminate or below the threshold of ELISA detection. The enhanced sensitivity of PCR over ELISA-based methods can be exploited to assist commercial poultry producers in eradicating ALV from their flocks.

ALV is a retrovirus which causes high economic losses in the poultry industry owing to its ability to induce malignancies as well as decrease overall production efficiency in infected flocks (Gavora, 1987). The economic repercussions of ALV infection have made ALV eradication a top priority for many poultry producers.

A variety of testing methods have been developed to detect ALV in infected birds including phenotypic mixing, complement fixation, immunofluorescence and virus neutralization. All of the above testing methods are time-consuming and most require the use of tissue culture, two factors which prevent their use by producers (Spencer, 1987; Mahbubani and Bej, 1994). Simpler methods currently used by commercial poultry producers to test for the presence of ALV are based on the enzyme-linked immunosorbent assay (ELISA; Spencer, 1987). ELISA is

commonly used in many diagnostic applications and is less time-consuming and easier to use than culturing methods. It can be used to detect either viral proteins or antibodies raised by a bird to fight off infection. Although ELISA detection is considered sensitive, its use has not been successful in helping to eradicate ALV infection from most flocks. A more sensitive test is required if eradication is to be achieved.

Genetic-based molecular diagnostic methods are being used more and more often but require that the genetic information of a given pathogen be known in some detail. Once the information has been gathered, the increase in sensitivity that is attained over more conventional tests can be quite dramatic. The polymerase chain reaction (PCR) is such a technique and has been shown to be both highly sensitive and specific (Mahbubani and Bej, 1994). It is being used in the diagnosis of a variety of pathogens including *Salmonella* (Hashimoto *et al*, 1995), hepatitis virus (Kaneko *et al*, 1989) and the human immunodeficiency virus (Albert and Fenyo, 1990; Sethoe *et al*, 1995). The objective of this study was to exploit the enhanced sensitivity of PCR and adapt the method to the diagnosis of ALV infection. This increase in sensitivity and specificity will aid in eradicating ALV from infected flocks.

Avian leukosis virus is a retrovirus that makes a DNA copy of its RNA genome and integrates this DNA copy into the genome of its host. Integration is required prior to viral replication (Varmus and Brown, 1989). The possibility of latent or sporadic viral infection (Garbuglia *et al*, 1995) convinced us that detecting the proviral DNA integrant and not the viral RNA genome, would eliminate a larger proportion of infected birds since the RNA genome would be present only if a bird was viraemic at the time of testing.

In contrast to what are thought of as conventional infections caused by pathogens such as those listed above, there is an extra factor that must be considered when designing primers for PCR detection of ALV. Viruses and other pathogens are more commonly thought of as being exogenous entities. Examples of viruses that come only in exogenous form are influenza virus and poliovirus. These viruses are found as viral particles in the environment where they are picked up by a host. They then go on to replicate and cause disease. ALV also exists as an exogenous virus and can be transmitted in similar ways (ie. from individual to individual through contact or from mother to offspring by *in utero* infection). There is one subgroup of ALV, subgroup E, that is transmitted in a distinct manner. The viruses in this subgroup are known as endogenous viruses or *ev* genes. These viruses are essentially genes in the chicken genome which arose when exogenous ALV stably incorporated into the germ line of the ancestors of chickens, approximately 5000 years ago (Boyce-Jacino *et al*, 1992). The high homology (>90% at both the protein and genetic level) between exogenous and endogenous forms of the virus necessitates a close look at the sequence of both to find regions of the genomes which differ between the two viral categories. *Ev* genes do not appear to be detrimental to the birds although the presence of some *ev* genes will cause immunosuppression upon exposure to exogenous ALV (Gavora *et al*, 1995a). All unselected chickens examined to date contain between five and ten different *ev* genes in their genome (Weinberg, 1980). Selection of birds with specific characteristics can change the incidence and numbers of the *ev* genes that are carried (Kuhnlein *et al*, 1989).

The following document will outline the necessary information taken into consideration when designing PCR primers that are able to distinguish between endogenous and exogenous

ALV. At the same time, it will be shown that PCR detection is more sensitive than currently used methods of detection.

Retroviral replication.

Retroviruses are single stranded, positive sense, diploid RNA viruses which have reverse transcriptase activity and, hence, have the ability to synthesize and integrate DNA copies of their genome into that of their host. The retroviral life cycle, quickly over viewed, includes virus binding to a host cell receptor which is recognized by the envelope glycoproteins protruding from the surface of the virus (see Figure 1a for electron micrograph and 1b for schematic of ALV). The viral envelope and the cellular membrane then fuse to allow entry of the viral nucleoprotein complex into the cytoplasm (Haywood, 1994). At this point, the virus-encoded reverse transcriptase that is present in infectious viral particles, produces a double-stranded DNA copy of the viral RNA genome. This proviral genome is translocated into the nucleus in association with viral enzymes and is integrated randomly into the host genome. The proviral genome can then be transcribed and translated by the host cell's machinery, producing both viral proteins and viral genomic RNA copies that are assembled to produce new viral particles. These are released from the cell by budding through the cellular membrane, where the virus picks up the host-derived viral envelope (Varmus and Brown, 1989; Cullen, 1992).

A common characteristic of retroviral replication is a high error rate when the RNA genome is reverse transcribed into the DNA proviral genome. It is estimated that reverse transcriptase replication introduces a millionfold more nucleotide substitutions (10^{-3} nucleotide substitutions per site per year) when compared to DNA polymerase-dependent DNA replication

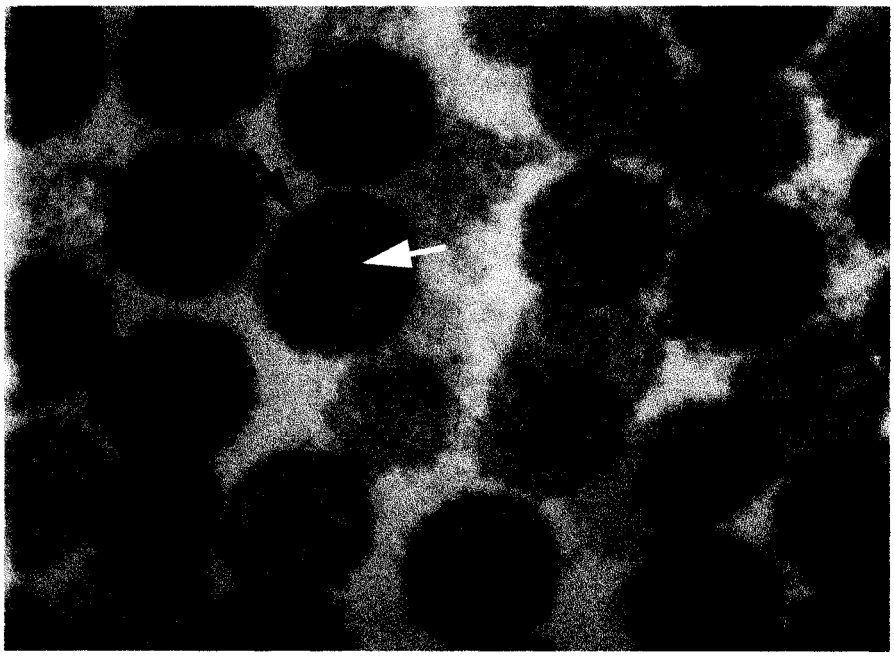


Figure 1a. Electron micrograph of a 10-day old chick embryo's stomach infected with ALV. The black arrow is indicating the host-derived viral envelope of the viral particle. The white arrow is pointing at the core of the virion where the RNA genome and its associated proteins are located. See Figure 1b for schematic depiction of the ALV viral particle. (EM taken by F. Gilka)

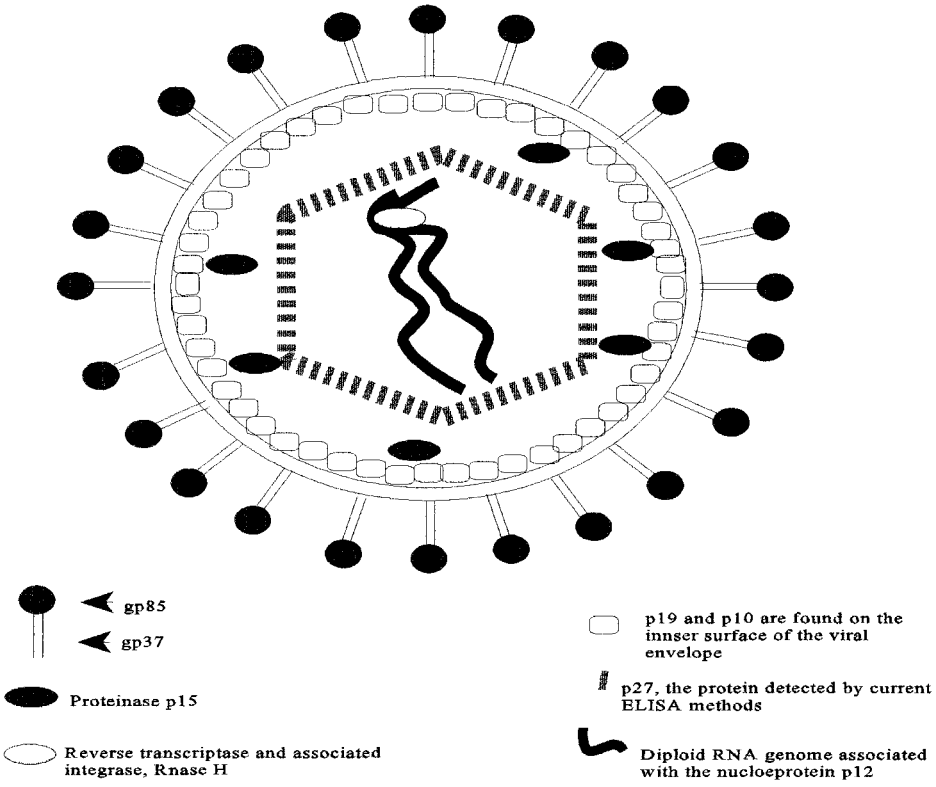


Figure 1b. Schematic depiction of an ALV viral particle (see Figure 1a for electron micrograph). The only viral protein which varies between viral subgroups is the gp85 protein used by the virus to bind to the host cell receptor. P27 is the group-specific antigen which is detected by current ELISA methods; it does not vary between endogenous and exogenous subgroups.

(10^{-9} nucleotide substitutions per site per year). This high error rate is mainly due to the lack of effective proofreading mechanisms (Darlix and Spahr, 1983; Gojobori and Yokoyama, 1985; Steinhauer and Holland, 1987). The large numbers of mutations observed in many RNA viruses can also be attributed to the short generation time for viral replication (Steinhauer and Holland, 1987). Other factors that increase variability in retroviral genomes include deletions, insertions, duplications and recombination with other viral or cellular sequences (O'Rear and Temin, 1982; Shimotohno and Temin, 1982; Dorner *et al*, 1985).

Effects of ALV on infected flocks.

There are two unrelated retrovirus groups that can infect and cause lymphomas and other neoplasms in poultry. The first group, known as lymphoid leukosis/sarcoma viruses of which avian leukosis (ALV)/lymphoid leukosis (LLV) and Rous sarcoma viruses (RSV) are members, are endemic in chicken populations (Purchase and Payne, 1984). The terms ALV and LLV are interchangeable and describe the same pathological entity. The other retroviruses are from the reticuloendotheliosis group which includes reticuloendotheliosis virus (REV) as well as spleen necrosis virus and chick syncytial virus. REV are antigenically and structurally unrelated to leukosis viruses, they more closely resemble mammalian C-type viruses, but they do cause clinical disease that can be pathologically indistinguishable from that caused by lymphoid leukosis virus (Witter and Crittenden, 1979; Aly *et al*, 1993). REV is thought to play a small role in neoplastic occurrences in commercial chicken flocks such that very little testing for REV is done on chicken populations (Kulenkamp, pers. comm.; Fadly, 1987). A PCR test has been published for the detection of REV. Testing of the primers was done on infected chick embryo fibroblasts (CEF)

and experimentally infected chickens but not naturally infected chickens (Aly *et al*, 1993).

The following will focus on the leukosis group of retroviruses that commonly infect chickens. The transformation competent viruses, such as the sarcoma viruses (ie. RSV), that carry a viral oncogene, and acute leukemia viruses, like avian myeloblastosis virus (AMV), that also carry an oncogene but are replication defective (Gonda *et al*, 1981), seem to be largely laboratory artifacts and have never been isolated from natural avian populations (Fung *et al*, 1981; Bishop, 1982).

Avian leukosis viruses are part of the avian C-type RNA tumour viruses. C-type viruses are those that bud and undergo postbudding maturation before becoming fully infectious. They are part of the subfamily *Oncovirinae* in the family *Retroviridae* (Purchase and Payne, 1984; Payne, 1992). In contrast, the human immunodeficiency virus (HIV) is part of the subfamily *Lentivirinae* (Cullen, 1992). Leukosis viruses, typically associated with infection of chickens, fall into five subgroups. Subgroups A and B are the most common cause of exogenous ALV infection in North American flocks, 80% and 20% respectively (Okazaki *et al*, 1982). Subgroups C and D have been isolated from areas of Europe and Africa (Morgan, 1973; Sandelin and Estola, 1974). Subgroup E is typified by the endogenous viral (*ev*) elements with the prototype being RAV-0 (Rous associated virus-0) which is produced by the *ev*2 locus in the genome of chickens (Astrin *et al*, 1980). Viruses of other subgroups such as F, G, H and I occur as endogenous viruses in other galliformes: pheasants harbor subgroups F and G (Fujita *et al*, 1974), partridges carry subgroup H endogenous viruses (Hanafusa *et al*, 1976), and quail carry subgroup I (Troesch and Vogt, 1985). An exogenous subgroup J virus has recently been isolated from meat-type

chicken lines (Payne *et al*, 1991). Subgroup J appears to be a recombinant between sequences of ALV and sequences from the endogenous avian retrovirus (EAV) family, another family of endogenous retroviral elements that are distantly related to the subgroup E endogenous ALV viruses found in chickens (Boyce-Jacino *et al*, 1989; Bai *et al*, 1995).

The subgroup of a virus is determined by the cellular receptor that a virus uses to gain access to the interior of the host cell (Bova and Swanstrom, 1987). ALV subgroups were determined early on in ALV research by infecting genetically defined chicken cells and looking at interference patterns and antibody neutralization (Vogt and Ishizaki, 1966).

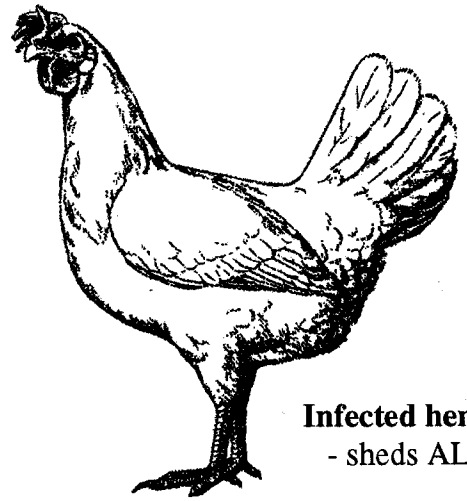
The exogenous subgroups (subgroups A, B, C and D) are oncogenic in nature and induce neoplasia at a low frequency upon infection, less than 2% in most strains (Payne, 1987). Endogenous viruses (subgroup E), even when able to form completely infectious virus, are not considered to be oncogenic (Motta *et al*, 1975; Neiman *et al*, 1975; Crittenden, 1980). The differences in the promoter regions, located in the long terminal repeats (LTR), of the leukosis viral genomes are mainly responsible for the oncogenicity of a particular virus (Payne, 1981). The other 98% of infected birds, even though they do not develop malignancies, do suffer from loss of performance in economically important traits and a 5% increase in mortality from causes other than lymphoid leukosis. Some of the economic traits that appear to be affected following ALV infection include an increase in age at sexual maturity, decreases in overall egg production, egg weight, fertility, hatchability and body weight (Gavora, 1987). The effects of ALV infection on a given strain will vary and depend on the susceptibility of the birds to a given subgroup, the mode of transmission and/or the age at which the birds are infected (Fung *et al*, 1982; Payne, 1987), as

well as the strain of virus used for infection (Baba and Humphries, 1986), and the birds' ability to raise an effective immune response. The latter depends, to some extent, on the *ev* genes that the birds are carrying (Crittenden *et al*, 1982; Gavora *et al*, 1995a).

This ALV-associated decrease in productivity has lead many commercial poultry breeders to test for the presence of the virus in their flocks and to eliminate all birds which appear to be infected. The ultimate goal is to eradicate ALV from all flocks, but the tests currently available do not appear sensitive enough to attain this goal. Although incidences of ALV have decreased since testing began, there are still sporadic outbreaks which indicate that the tests are missing some of the infected birds or that there is a reservoir, from which ALV re-emerges, that has not yet been identified.

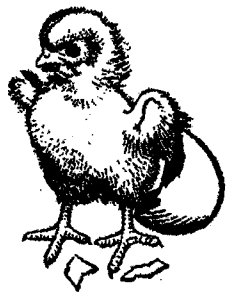
Exogenous vs. Endogenous Leukosis viruses: Transmission.

The exogenous forms of ALV are transmitted from bird to bird in one of two ways (Payne, 1987; see Figure 2). One type is termed horizontal transmission and is the one that is most commonly associated with viral spread. Infected birds shed the virus at the skin surface, in saliva and in faeces where it is picked up by flock mates either through direct contact with infected birds or by contact with fomites. How the horizontally infected birds respond to infection depends on the age of the bird, the presence of maternal antibodies, and the degree of contact exposure (De Boer *et al*, 1981). The second type of exogenous transmission is termed congenital or vertical transmission. Infected hens have been shown to shed large numbers of virus into the oviduct. As the egg passes through the oviduct, prior to egg shell formation, the virus is picked up and infects the newly developing embryo. Since the immune system of the embryo is not



Infected hen
- sheds ALV into oviduct

Congenital transmission



Congenitally infected chicks
- immunologically tolerant
- develop viremia that persists for life
- shed large amounts of virus
- major source of virus for horizontal transmission

Horizontal transmission

Pen/flock mates
- develop Ab if > 2 days of age
- seldom persistently viremic
- occasionally transmit virus congenitally

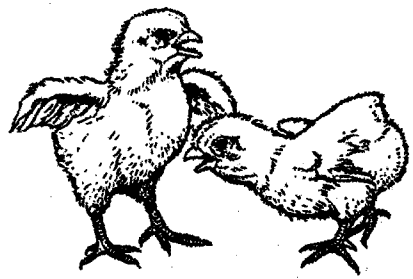


Figure 2. Diagram depicting both congenital and horizontal transmission of ALV.

mature, congenitally infected birds are never able to raise a proper immune response against the virus and will continuously shed virus, infecting their flock mates via horizontal spread (Rubin *et al*, 1962). Congenitally infected chicks are thought to be the major source of virus for horizontal transmission.

Endogenous viral (*ev*) genes are a natural part of the chicken genome and are transmitted in a Mendelian fashion. Retroviral endogenous elements have been found in all eukaryotic organisms studied to date (Hauber *et al*, 1985; Mount and Rubin, 1985; Coffin, 1985). These elements are believed to originate from infection of the germ line by a retrovirus. Once they are in the germ line and fertilization occurs, they become a stable part of the genome and are inherited like all other genes. Most endogenous retroviral elements no longer have exogenous relatives circulating in the environment. This is not the case with the family of endogenous viral elements found in poultry known as *ev* (endogenous viral) genes. These genes are closely related to exogenous ALV and have been placed in subgroup E of the ALV family. Unselected chickens will typically have between five and ten copies of *ev* genes in their genomes (Weinberg, 1980). Some of these genes can form fully infectious, subgroup E viruses (ie. *ev*2, *ev*7, *ev*12, *ev*21) while most contain deletions leaving only certain parts of the prototypical virus intact (ie. *ev*3, *ev*6 and *ev*9). The most extreme case of proviral genome deletion exists where only a solitary LTR (ie. *ev*15 and *ev*16) remains (Hayward *et al*, 1980; Hughes *et al*, 1981; Rovigatti and Astrin, 1983). *Ev* genes are thought to have been incorporated into the germ line of chickens following speciation in the *Gallus gallus* lineage since they are found only in chickens and not other *Gallus* species (Frisby *et al*, 1979). ALV-related endogenous viruses in other galliformes such as pheasant, partridge and

quail, are of different subgroups (Fujita *et al*, 1974; Hanafusa *et al*, 1976; Troesch and Vogt, 1985).

There are other groups of retroviral elements in the chicken genome which apparently do not have corresponding exogenous, pathological relatives. These are members of the EAV (endogenous avian retroviral) group of which the chicken genome contains 30 to 50 copies (Boyce-Jacino *et al*, 1992); the ART-CH (avian retrotransposon from chicken genome) group is present at more than 50 copies in the chicken genome (Gudkov *et al*, 1992) and the CR1 (chicken repetitive) elements, which are non-LTR retrotransposons or LINE-like elements (long interspersed element), are present at approximately 30,000 copies per genome (Burch *et al*, 1993). EAV are thought to have integrated into the germ line of *Gallus* long before speciation since all *Gallus* species contain copies of EAV. ART-CH elements, like *ev* genes, seem to have integrated following *Gallus* speciation since they are found only in chickens (Gudkov *et al*, 1992).

Genome of ALV.

It is the presence of the closely related *ev* genes in the chicken genome that increases the difficulty in finding PCR primers that will be specific in detecting exogenous ALV only. A comparison of the genome organization and sequence of exogenous ALV and a prototypical *ev* gene reveals very little divergence (see Figure 3 for genomic organization). All replication-competent retroviruses contain three genes: *gag*, *pol* and *env* flanked by the long terminal repeats (LTR). Some retroviruses, such as HIV (see Figure 3), contain other coding regions but ALV is

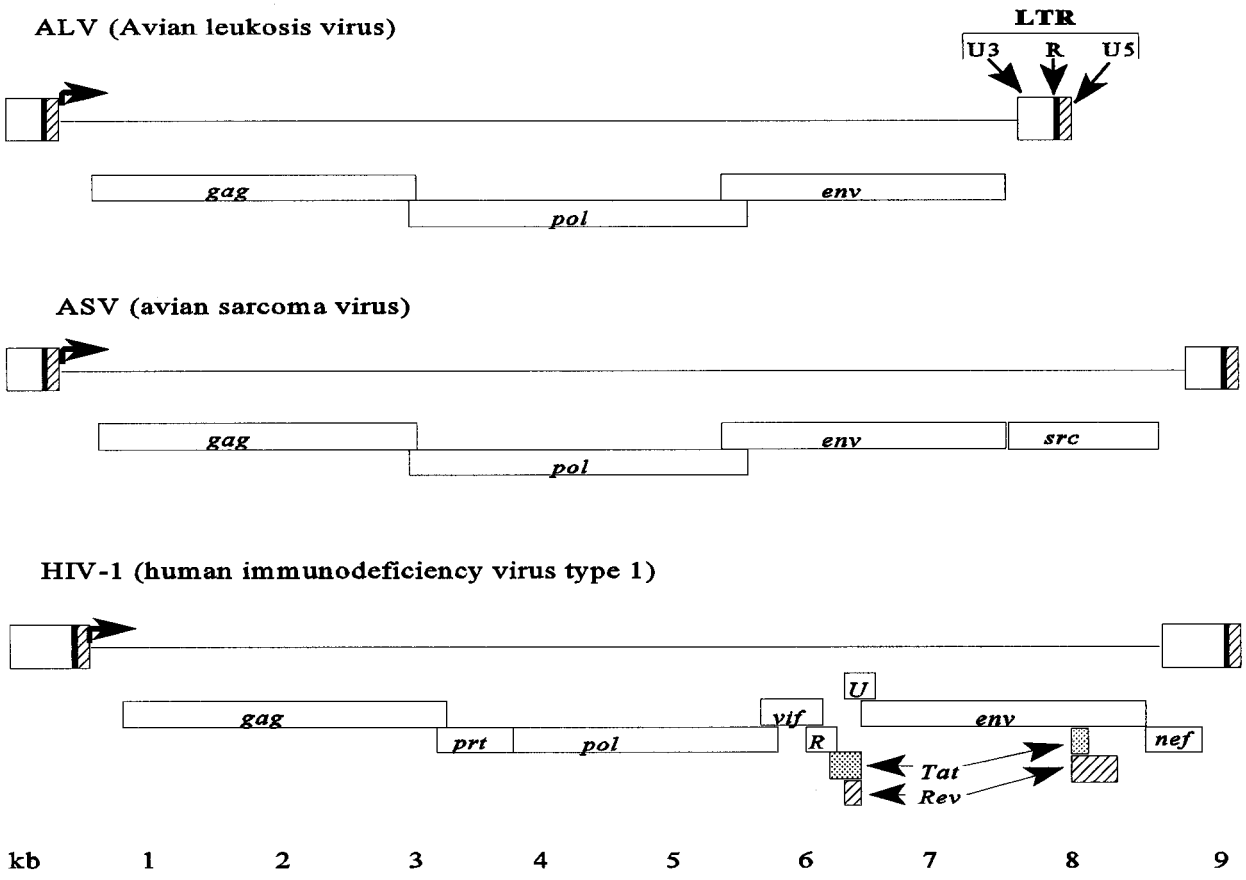


Figure 3. Genomic organization of avian leukosis virus; complete *ev* genes (ALV, subgroup E) have identical organization. The untranslated but transcribed leader region is located between the 5' LTR and the beginning of the *gag* gene. Replication competent sarcoma viruses contain all necessary viral genes (*gag*, *pol* and *env*) in addition to a cellularly-derived oncogene (ie. *src*). ALV and ASV are considered simple retroviruses in comparison to HIV which has a more complex genome encoding a number of additional viral products. The longest retroviral genomes examined have not surpassed approximately 10 kilobases.

one of the simpler retroviruses and only contains the three genes necessary for replication. The U3 region of the LTR and a region of the *env* gene that encodes the gp85 glycoprotein are the only two parts of the ALV genome which are heterogeneous when exogenous and endogenous genomes are compared (Skalka *et al*, 1979; Tsichlis and Coffin, 1980; Hishinuma *et al*, 1981).

LTR

All retroviruses contain long terminal repeats (LTR) at both the 5' and 3' ends of the integrated DNA proviral genome. The LTR can be separated into three sections, the U3 region, the repeat or R region and the U5 region (see Figure 3). If one examines the RNA genome, the U5 region is found only at the 5' end of the RNA strand whereas the U3 region is found at the 3' end; the R region is found at the termini of both the 5' and 3' ends of the RNA. Following reverse transcription into double stranded DNA and integration of the viral genome into that of the host, the U3-R-U5 configuration is duplicated and found at both ends of the proviral DNA genome forming what are known as long terminal repeats. These repeats do not encode any protein products (Czernilofsky *et al*, 1980).

The U3 region varies in length and in sequence when comparing endogenous and exogenous U3 sequences. Some regions of U3 also vary between exogenous viruses (see Figure 6a for LTR sequence comparisons). The endogenous LTR are highly conserved with only a few base substitutions and minor deletions observed (see Figure 6b). The length of U3 can be anywhere from 160 to 176 nucleotides for endogenous viral sequences and upwards of 220 nucleotides for exogenous ALV viral sequences (Hughes, 1982; Gagnon *et al*, 1995). Some acute sarcoma viruses have been found to have U3 as long as 385 bp (Mayer *et al*, 1988). The

differences in the U3 region are responsible for the increased transcription rate and enhancer activity as well as oncogenicity associated with many exogenous ALV (Cullen *et al*, 1985). Recombination of an endogenous virus replacing only its U3 region with that of an exogenous strain can increase replication 10- to 100-fold (Tsichlis *et al*, 1982). Regulatory elements in the U3 region are also important in determining the oncogenicity of the viral strain if the provirus integrates in the vicinity of a cellular proto-oncogene. The LTR will typically act as the proto-oncogene's promoter and drive its increased transcription above normal cellular levels causing transformation (Hayward *et al*, 1981; Robinson *et al*, 1982). Proviral insertion can cause cellular transformation by means other than promoter insertion. Insertion downstream of the c-*myc* gene (Nottenburg *et al*, 1987) and upstream but in the opposite orientation have also been associated with cellular transformation. Transformation caused by these configurations has been attributed to the transcriptional enhancement ability of the LTR (Payne *et al*, 1982; Khoury and Gruss, 1983).

The R region is a small stretch, 21 nucleotides in length, found at both ends of the RNA genome and later between the U3 and U5 regions at both ends of the DNA provirus. This region is used by the reverse transcriptase to 'jump' from one RNA copy to the other during reverse transcription of the RNA genome into the double stranded DNA provirus (Coffin, 1990).

The R and U5 regions of the LTR are highly conserved among all ALV subgroups (see Figure 6a; Skalka *et al*, 1979; Tsichlis and Coffin, 1980; Hishinuma *et al*, 1981). One interesting aspect of the retroviral LTR is that they are quite similar in architecture to those found in the transposable genetic elements of such diverse organisms as bacteria, yeast and Drosophila. The

most striking similarity is the presence of both direct and inverted repeats (Ju and Skalka, 1980; Shimotohno *et al*, 1980; Skalka *et al*, 1981; Swanstrom *et al*, 1981).

Leader region

The leader region is a short, approximately 380 nucleotides, untranslated stretch found at the 5' end of the genome between the LTR and the beginning of the *gag* coding region. Transcription of the provirus produces two classes of mRNA, a full length copy that is used in translation of *gag* and *pol* products (see below) and a shorter, spliced copy that is used in translating *env* products. The leader sequence is found at the beginning of both mRNA classes. It is highly conserved among all ALV subgroups (Katz *et al*, 1986).

***Gag* (group-specific antigen)**

The *gag* gene, located between nucleotides 380 and 2485 in the RSV Prague C genome (PrRSV-C; Schwartz *et al*, 1983), encodes a polyprotein weighing 76 kD. The polyprotein is subsequently processed into six viral proteins (p2, p10, p12, p15, p19 and p27). These proteins make up the internal structural core of avian sarcoma and leukemia viruses. P2 is required for virus budding from the surface of an infected cell and may be involved in activating the protease, p15 (Wills *et al*, 1994). P10 is involved in regulating the splicing of the viral RNA transcripts (Gonterak *et al*, 1993). The protease, p15, is required for processing of the Gag polyprotein into its component parts allowing viral maturation and infectivity. The matrix protein, p19, interacts with the host-derived, lipid envelope and the transmembrane glycoprotein, gp37 and may also interact with the viral RNA genome (Vogt *et al*, 1985). P27 is the major capsid protein and forms a case around the RNA genome. P27 is the viral protein which is detected with ELISA-based

testing for group-specific antigen of ALV (Smith *et al*, 1979). Many of the above proteins likely have other, as yet unidentified, roles (Wills and Craven, 1991). All of these proteins are homologous between subgroups and have been termed group specific antigens (gsa) since they are virtually indistinguishable when comparisons are made between the various subgroups of ALV (Coffin, 1980; Vogt *et al*, 1985).

***Pol* (polymerase)**

Like *gag*, the *pol* gene varies little when comparisons are made between different leukosis viruses (Coffin, 1980). There is also a high degree of homology maintained between *pol* genes of different retroviruses (Whitcomb and Hughes, 1992). The polymerase gene produces a polyprotein (160 kD), which is processed following translation but the functional enzyme remains as a holoenzyme with two subunits (α and β) that possess RNase H, endonuclease, integrase, DNA-dependent DNA polymerase and reverse transcriptase activity.

***Env* (envelope)**

The *env* gene also encodes a polyprotein, weighing approximately 90 kD, which is processed into two glycoproteins, gp85 and gp37, that form the highly glycosylated spikes protruding from the viral lipid envelope. Gp85 is the surface protein (SU) of ALV and carries the determinants that define the subgroup in which a virus is classified. These determinants are the regions of the gp85 protein which interact directly with the cellular receptor to gain access to the interior of target cells (Hunter *et al*, 1983; Bova *et al*, 1986). Five different subgroups and therefore five different gp85 variants are associated with chickens; these are subgroups A, B, C, D and the endogenous subgroup E. Other ALV subgroups have been associated with other avian

species.

Gp85 is also the target of neutralizing antibodies (Ishizaki and Vogt, 1966; Bova *et al*, 1988). The antibodies block the gp85-receptor interaction which inhibits viral entry into the host cell. The gp37 glycoprotein or transmembrane protein (TM) is linked via one or two disulphide bonds to gp85 and serves as an anchor, embedding itself into the lipid membrane (Payne, 1981). Gp37 is conserved between subgroups while gp85, due to its biological function, has regions of variability between subgroups, flanked by the protein 'backbone' which is conserved between subgroups (see Figure 7). The conserved regions are more than 90% homologous between subgroups. The homology drops to between 40 and 60% when the variable regions are compared. If comparing the variable regions within the same subgroup, the homology is again on the order of 90% (Bova and Swanstrom, 1987). This means that the variable regions of gp85 can be used to distinguish between exogenous subgroups and the subgroup E, *ev* genes.

Cellularly-derived oncogene in sarcoma viruses.

Figure 3 also gives a schematic depiction of a transformation competent leukosis viral genome. The predominant difference between transformation defective and transformation competent leukosis viruses is the presence of an oncogene in the genomes of the transforming viruses which is absent in the nontransforming genomes. It is generally accepted that transforming leukosis viruses are formed when a nontransforming viral genome recombines with one of a number of normally occurring cellular sequences encoding proto-oncogenes (Wang *et al*, 1978; Bishop, 1982). Some examples of proto-oncogenes that have been picked up by retroviruses include c-*src* (the viral homologue is known as v-*src;* Rous sarcoma virus is the

prototypical virus carrying this oncogene; Takeya and Hanafusa, 1982, 1983), c-*myc* (v-*myc* is carried by myelocytomatosis viruses; Payne *et al*, 1982), and c-*myb* (v-*myb* is carried by avian myeloblastosis virus; Klempnauer *et al*, 1982). There are many more examples of retroviruses carrying other oncogenes. Papers by Varmus (1987) on viral and cellular oncogenes and Kung and colleagues' paper (1991) on cellular oncogenes both cover a variety of examples. These proto-oncogenes are typically, although not always, protein kinases which are used to phosphorylate proteins, an important means of regulating protein activity (Bishop, 1982; Kitamura *et al*, 1982). Other types of oncogenes that have been picked up by sarcoma viruses include nuclear proteins, growth factors and GTP-binding proteins (Mayer *et al*, 1988).

As mentioned previously, it is thought that avian sarcoma viruses, such as the Rous sarcoma viruses, are largely laboratory artifacts . Even the original Rous strains, which were first studied at the turn of the century, were passaged many times from one bird to another before the viruses, with which we work today, were isolated. Lerner and Hanafusa (1984) postulated that the viruses which Rous passaged from bird to bird were, in fact, replication defective and that they picked up a cellular oncogene at the expense of losing one of their viral genes, typically a portion or all of the *env* gene. Only a few transforming sarcoma viruses contain an oncogene as well as all of the necessary viral genes to remain replication competent (ie. Schmidt-Ruppin RSV and the Prague strains of RSV). Replication defective viruses require the presence of a helper virus to provide the viral genes that they are missing in *trans*. Dutta *et al* (1985) later examined RSV-29, a strain which has had the least number of passages since its original isolation from a Rous tumour and showed that it was replication defective. The viruses, which caused the original

tumours, were probably typical, transformation-defective ALV and not recombinant sarcoma viruses. The frequent passages allowed recombinants, carrying the oncogenes, to be preferentially isolated since they tended to be more oncogenic and induced tumour formation much more quickly than did the leukosis viruses (Bishop, 1982)

Oncogenesis.

Rous sarcoma virus (RSV) and other viruses which transform cells in culture, encode a viral oncogene which induces neoplasms rapidly. Cell culture transformation and the rapid onset of malignancies following infection are directly due to the presence of the virally encoded oncogene. ALV, unlike RSV, does not cause *in vitro* cell transformation or cytopathic effects, nor does it encode a viral oncogene. Even without a viral oncogene, in a small percentage of cases ($\leq$2%), infection with ALV will lead to tumour formation (Payne, 1987). Development of the tumour takes longer than those caused by RSV, a few weeks for oncogene-carrying retroviruses compared to four to eight months for ALV (Neiman *et al*, 1975; Graf and Beug, 1978; Neiman *et al*, 1980; Fung *et al*, 1982). The small percentage of ALV-induced tumours indicates that the transformation event occurs at a low frequency. The fact that different tumours in the same individual are clonal growths also suggests that transformation occurs rarely (Ewert and De Boer, 1988).

Although ALV can infect a variety of different cell types, the most common site of transformation is the bursa of Fabricius leading to a B cell neoplasia termed lymphoid leukosis (Purchase and Burmester, 1978). The bursa of Fabricius is where the B lymphocytes of chickens differentiate (Van Alten and Meuwissen, 1972; Glick, 1977). The immature B lymphocytes, when

still found in the bursa prior to peripheralization, appear to be the primary targets of transformation of ALV. Other malignancies that arise at a low frequency due to transformation of cells other than those in the bursa include, nephroblastomas, sarcomas, erythroblastosis and osteopetrosis (Schmidt *et al*, 1982; Kung and Maihle, 1987).

If ALV does not encode an oncogene, how does it induce neoplasms in infected tissues? The LTR of ALV acts as a strong promoter and has characteristics of eukaryotic transcriptional promoters (Tsichlis and Coffin, 1980). If a copy of the retrovirus is integrated in proximity to a cellularly encoded proto-oncogene (ie. c-*myc*), increased expression of the proto-oncogene above normal cellular levels, driven from the LTR, can lead to transformation of the cell and tumour formation (see Kung *et al*, 1991 for a review of retroviral mutagenesis and oncogenesis). The LTR seems to be the important factor in the induction of oncogenesis since deletion of any or all remaining parts of the retroviral genome can still lead to tumour formation as long as at least one LTR is present (Fung *et al*, 1981; Westaway *et al*, 1984). Many of the tumours caused by c-*myc* transformation have been analysed and show that most of the viral genome is deleted with only a functional LTR remaining to drive increased expression of the oncogene (Payne *et al*, 1981). This may be the reason that the immune system is not able to effectively combat the malignancy since there are no viral products expressed on the tumour cells (Payne *et al*, 1981; Westaway *et al*, 1984; Robinson and Gagnon, 1986). The cellular proto-oncogene that has almost exclusively been implicated in ALV transformation of the bursa is the c-*myc* gene (Schubach and Groudine, 1984; Linial and Groudine, 1985).

It has been shown that integration of a provirus into a cell does not necessarily mean that

the provirus will be expressed. Flamant *et al.* (1987) have shown that virtually every cell type, of embryos infected at one day of age, will have integrated provirus in them. By *in situ* hybridization, eight days later, they showed that regulation of the proviruses was tissue specific. Infection of chickens of a susceptible line leads to spread of the virus and viral integration into most tissues. Welt and coworkers (1977) demonstrated viral protein synthesis in all tissues examined (spleen, bursa, liver, kidney, testis, ovary and lung) of 18-day old, line 15I susceptible birds which had been infected with Rous-associated-virus-1 (RAV-1) at two days of age. Viral proteins continued to be synthesized in all the tissues examined up until the birds were nine weeks of age. At ten weeks of age, only the lungs and ovary continued to show viral protein synthesis even following an immune response. This could explain why seemingly non-viremic dams are able to shed virus through the oviduct, thereby infecting their eggs. Infected birds that were less than ten days of age showed no viral protein synthesis in any of the tissues examined. This fact prevents detection of ALV in many infected birds with methods that rely on detecting viral protein expression such as those currently used. Although ALV preferentially transforms bursal cells, viral replication that does not lead to transformation, is possible in many other tissue types (Spencer *et al*, 1984).

There appear to be bursa-specific proteins that bind to the LTR of integrated ALV that leads to bursal transformation (Ruddell *et al*, 1988; Smith *et al*, 1994). Studies have shown that genetic resistance to lymphoid leukosis can reside directly in the bursal target cell. Bursal cells from genetically resistant chickens were transferred into a susceptible strain of birds whose bursal lymphocytes had been depleted; the opposite was also done where bursal cells from susceptible

chickens were transferred into resistant chickens. The birds were then infected with the RAV-1 strain of avian leukosis virus. The genetically resistant birds with bursal lymphocytes from the susceptible strain developed lymphoid leukosis whereas the susceptible birds who had been given lymphocytes from the resistant strain did not (Purchase *et al*, 1977). Another study that indicated the necessity of the bursal environment for the development of lymphoid leukosis tumours showed that both chemically (Purchase and Gilmour, 1975) and physically bursectomized chickens were persistently viremic yet they did not develop lymphoid leukosis. Instead, they developed osteopetrosis and other ALV-related neoplasms. The development of these other neoplasms may be due to immunologic incompetence where B lymphocytes are not available, due to the absence of the bursa, to raise an effective immune response against newly arising malignant cells (Purchase and Gilmour, 1975).

Immunity to ALV.

Mechanisms of resistance to ALV can occur at different steps in the infection process. Chickens lacking the gene(s) encoding the subgroup-specific cell receptor(s), used by the various subgroups to gain access to the interior of the cell, will not become infected (Ishizaki and Vogt, 1966, Payne and Biggs, 1966; Dorner and Coffin, 1986). The only cellular receptor that has been identified is the one used by subgroup A viruses. It is a member of the low density lipoprotein receptor family. One would expect this receptor to be expressed on a large number of different cell types (Südhof *et al*, 1985; Young *et al*, 1993; Bates *et al*, 1993; Zingler *et al*, 1995) explaining the large cell tropism.

The ability of the provirus to integrate into the host genome is another step that can be

responsible for determining susceptibility or resistance to a retrovirus (Goff, 1992). Proper transcription of the provirus can also be a factor of whether a bird will become viremic or not (Jähner and Jaenisch, 1980; Jaenisch *et al*, 1981; Flamant *et al*, 1987). Even if a bird becomes infected, overt disease may not manifest itself due to an effective immune response being raised, preventing virus spread and/or tumour formation (Rubin *et al*, 1962). The absence of host cellular proteins necessary in the induction of lymphoid leukosis is another means of resistance to the effects of ALV (Purchase *et al*, 1977; see above for details).

Infection of a bird that has a mature immune system leads to the production of subgroup-specific antibodies. Only cells that are expressing viral antigen will be recognized as infected by the immune system and destroyed. Cells that are not expressing antigen but still have an integrated provirus will not be cleared. Differential expression in various tissues as well as the same tissue over time has been documented by Welt's group (1977) and Flamant *et al* (1987). The titers of group-specific antibodies that are raised depend on the *ev* genes that are present in a given bird. Many *ev* genes will also produce gsa and the presence of these genes will prevent antibody formation against exogenous-derived gsa; the homology will prevent recognition by the immune system and the exogenous-derived gsa will not be recognized as foreign (Crittenden *et al*, 1982, 1984; Smith and Fadly, 1988). It also takes much longer for birds to raise adequate titers of antibody against ALV when compared to other viral infections due to the tolerizing effect of producing related self-proteins from *ev* (Smith and Fadly, 1988, Kuhnlein *et al*, 1993). This is especially true of *ev* that produce complete subgroup E virus (ie. *ev*12, *ev*21).

*Ev*21 is maintained in many commercial lines due to its genetic linkage with the sex-linked,

slow-feathering gene used to economically sex chicks (Bacon *et al*, 1988). Birds which carry *ev*21 tend to have a higher incidence of lymphoid leukosis when compared to birds not carrying *ev*21 (Smith and Fadly, 1988; Chambers *et al*, 1993/4). It has been shown that the immune response of these birds to ALV is greatly impaired (Harris *et al*, 1984; Gavora *et al*, 1995a).

Chicks that have been congenitally infected, or horizontally infected prior to maturation of their immune system (< 2 days), are even more tolerant to ALV such that a proper immune response, necessary to clear the virus, is never raised. These birds become chronic carriers, shedding the virus in large amounts and infecting their flock-mates (Payne, 1981). The presence of maternal antibodies at birth may decrease the effect somewhat (Fadly and Smith, 1991).

Very little is known of the cellular immune response of chickens to ALV, but ALV has been shown to immunosuppress the birds that they infect. This likely causes the increased mortality seen in ALV-infected flocks since the birds cannot fight other pathogens effectively (Smith and Fadly, 1988).

History of ALV research.

Extensive laboratory work has been done on avian oncogenic viruses such as avian leukosis virus (ALV) and the closely related Rous sarcoma viruses (RSV). When Ellerman and Bang (1908) and later, Peyton Rous (1910, 1911), described transplantable tumours via cell-free filtrates at the turn of the century, it was the first time that transformation, leading to tumour formation, could be ascribed to a transmissible agent (Rous, 1911). Crude isolations of the virus soon followed from the Rous group in the Laboratories of The Rockefelller Institute for Medical

Research (Rous, 1911, 1914; Lange, 1914; Tytler, 1913) but controversy over whether or not the tumours were legitimate neoplasms continued for at least two decades. It was not until the early 1960's that it was postulated and later shown by Temin that avian sarcoma virus (ASV), an RNA virus, was integrated into the host genome as a DNA provirus (Temin, 1963a, 1963b, 1964a, 1964b, 1964c, 1967). Since then, much work has been done on studying the virus and its life cycle in the laboratory setting as well as the occurrence of ALV in the field and in commercial poultry operations.

Even with all of this research, ALV still causes problems in the poultry industry, both with the incidence of lymphoid leukosis and losses in production. With the development of a vaccine for the herpes virus which causes Marek's disease, a disease which can have similar pathological consequences as those caused by ALV (Fadly, 1987), the presence of ALV is now the most economically important cause of neoplastic disease in chickens. Commercial poultry breeders currently spend large sums of money screening their flocks for potentially infected birds to prevent outbreaks. Their ultimate goal is to eradicate ALV from their flocks but current testing procedures are not able to meet this goal and a more sensitive testing method is required.

Current detection methods.

Commercially available diagnostic kits to detect ALV in flocks are based on conventional viral diagnostic methods such as ELISA (enzyme-linked immunosorbent assay; Wisdom, 1976) detection of viral proteins or antibodies (see Figure 5). Either the p27 (gsa) core protein from various tissues or antibodies in serum of infected birds are detected (Spencer, 1987). Although antibody and viral protein detection have been successfully used for many viral infections, their

usefulness in detecting every different type of viral infection is not a *sine qua non*. The sensitivity and reliability of detecting these products is highly questionable with what is currently known about ALV infection. P27 detection can lead to both false positives and negatives. The former is due to the presence of *ev* genes in the chicken genome and their ability to produce p27. The latter is due to the fact that p27 is not necessarily expressed in all infected tissues all of the time (Spencer *et al*, 1984; Flamant *et al*, 1987). One question that still remains is why p27 detection works at all. Free P27 is apparently found in albumen (Spencer *et al*, 1976) yet, theoretically, it is not a protein which should be found there since it does not contain a leader sequence thought to be necessary for protein secretion.

Antibody detection will not detect congenitally infected chicks since these birds produce few, if any, antibodies upon being exposed to ALV. Their immune system is not yet mature and they 'see' exogenous ALV as self (Rubin *et al*, 1962; Payne, 1981; Smith and Fadly, 1988). The reliability of antibody detection is still questionable and does not appear to be highly trusted by the industry (Kulenkamp, pers. comm.). Whether a bird is ever able to completely clear ALV from its system following infection is still unanswered. If clearance is possible, the presence of antibody may not necessarily mean that the bird carries infectious virus. Another disadvantage of basing ALV infection on antibody status is the time lag that occurs between exposure to ALV and the build up of detectable titers of antibody.

The above methods are quite reliable for detection of viruses which replicate to high numbers or that elicit a strong humoral immune response in the host but these methods are useless for viruses that do not have these characteristics. Researchers turned to the detection of viral

nucleic acids for the diagnosis of non-cultivatable viruses with low replication efficiencies and that did not induce an adequate humoral immune response. Nucleic acid hybridization was initially used but with the advent of the polymerase chain reaction, the sensitivity of nucleic acid detection was greatly increased. The inability of the traditional, protein-based methods to eradicate ALV from commercial flocks requires more sensitive means of detection be developed. The use of the polymerase chain reaction in detecting ALV proviral integration may be useful in identifying infected birds which are being missed by conventional ELISA.

Detection with the polymerase chain reaction (PCR).

The general principles behind the amplification of a specific nucleotide sequence that occurs during the polymerase chain reaction are quite simple. Two oligonucleotide primers are chosen which flank the sequence of interest. The primers are chosen so that each anneals to a different complementary nucleotide strand and are oriented such that amplification by a thermostable enzyme occurs only in the region between the two primers. Following a number of cycles which include denaturation of complementary strands, annealing of the primers to their respective sites and extension of the primers by a thermostable enzyme such as *Taq* (*Thermus aquaticus*) polymerase, there is an exponential accumulation of the target sequence since each cycle essentially doubles the amount of target sequence present in the preceding cycle (Saiki *et al*, 1988).

Primer design requires many factors be considered. The ability of a set of primers to efficiently amplify a specific product depends on such factors as the kinetics of association and disassociation of the primer and its complementary template, the stability of any mismatches,

secondary structures that can be present in either the primers or the template, as well as primer dimer formation if there is 3' complementarity between two primers.

Redundancies can be incorporated when designing primers. This is especially important when one is attempting to detect variants. Retroviruses are known to produce many variants, even in one infectious cycle (Steinhauer and Holland, 1987; Bieth and Darlix, 1992). This is magnified in the case of an HIV infection where spatial variants were isolated around primary sites of infection in tissue, reminiscent of plaques when growing virus on agar plates (Cheynier *et al*, 1993). This is due to the lack of proofreading capability and low fidelity of the reverse transcriptase encoded by the retroviral genome (Steinhauer and Holland, 1987).

PCR tests are presently being developed for clinical diagnosis of many important human viral diseases such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), and human papilloma virus (HPV) as well as being extensively used in genetic analysis of virtually every studied species (Clewley, 1989). This is due to its ease of use and high specificity and sensitivity (Mahbubani and Bej, 1994).

It is estimated that approximately one in ten thousand circulating lymphocytes is infected with HIV (Simmonds *et al*, 1990). It is assumed that the proportion of ALV-infected cells is much higher in poultry although the question has not been addressed in any detail. Even though no definite quantitative experiments have been done, the large number of cell types which can be infected due to the apparently ubiquitous nature of the receptors used by the various ALV subgroups and the many tissues in which it is found leads one to believe that many more cells

would be infected. *In situ* hybridization assays would also need to be done to determine the average number of integrated ALV genomes per infected cell in a naturally infected population.

Finding a small number of copies of a specific sequence in a large background of DNA is one of the problems of nucleic acid-based detection. All avian erythrocytes are nucleated and contain a copy of the avian genome. The size of the diploid, avian genome is approximately 1.3 billion base pairs (Cheng, 1994). A typical size for an integrated ALV genome is less than ten kilobase pairs. If only a small fraction of a given cell population is infected, PCR may not be sensitive enough to detect it with only a single round of amplification. It was assumed from the outset that a more sensitive method would be required to detect ALV in a naturally infected host which did not appear pathologically infected. Another consideration was that the testing method had to be relatively simple and cost-effective if it were to be used in a commercial application. A semi-nested PCR method was devised that permits large signal amplification of ALV-related products that can then be visualized on ethidium bromide stained gels.

The following details the steps taken to detect ALV in both experimentally infected samples and naturally infected birds using a semi-nested PCR technique. The sensitivity of PCR compared to traditional ALV detection methods is discussed as well as its potential in helping eradicate ALV from commercial flocks.

MATERIALS AND METHODS

Primer design and synthesis.

Published sequences of endogenous and exogenous ALV/ASV were compared to determine the best sites to target for discrimination between endogenous viral elements and exogenous ALV infection. Three sets of primers were chosen to screen samples for exogenous ALV infection. The first set (LTR1, LTR2A and LTR2B) targets the LTR region of the provirus (see Table 1 for all primer sequences, orientation and coordinates with respect to the PrRSV-C genome; Schwartz *et al*, 1983). LTR1 and LTR2A are used in the first round of PCR whereas LTR1 and LTR2B are used in the second, semi-nested round (see Figure 4 for schematic of semi-nested PCR). The second set of primers (ENVAB1A, ENVA1B and ENVA2) target subgroup A proviruses in the *gp*85-envelope region of their genomes. ENVAB1A and ENVA2 are used in the first round of PCR while ENVA1B and ENVA2 are used in the second round. Using similar considerations, a third set of primers (ENVAB1A, ENVB1B and ENVB2) were designed to target subgroup B proviruses in the *gp*85-region of the envelope gene. Notice that the same primer, ENVAB1A, was used for both subgroup A and B primary amplifications since it targets a conserved region of *env* when different subgroups are compared.

Redundancies for each primer were included only if a variant had been shown to have the sequence. For example, for LTR1, no variant was seen which contained the sequence 5'-TGTAGTCTTATGCAATACTCCTGTAAT-3' such that this primer was not synthesized or incorporated into the LTR1 primer mix. Bases where redundancies were incorporated are in parenthesis (see Table 1).

Table 1. Primers used for the amplification and detection of exogenous ALV. Oligonucleotide names as well as DNA strands which they target, sequence of primers and subgroups of ALV on which they anneal are indicated. The region of the ALV genome in which the primer anneals is also given as well as the position of the primer with respect to the conventional numbering of the Prague subgroup C Rous sarcoma virus.

Oligonucleotide designation and DNA strand on which it primes	Sequence (5'→3')	Subgroups on which primer will anneal[d]	Region of priming (position with respect to the conventional numbering of PrC-RSV [a])
LTR1 (+)	TGTAGTCTTATGCAATACTC(T/C)TGTA(G/A)T	A,B,C,D	U3 of LTR (9060-9086)
LTR2A (-)	AGCCTTCTGCTTCAT(G/T)CA(G/T)GTG	A,B,C,D,E	U5 of LTR (75-96)
LTR2B (-)	ATC(A/G)ACGGTCCG(A/G)CCATCAACC	A,B,C,D	U5 of LTR (36-57)
ENVAB1A (+)[b]	TCTACACAGTCAGCCACCTC	A,B,C,D,E	gp85 of *env* (5324-5343)
ENVA1B (+)	TCAGC(C/T)GACTTTACTGGCGGT	A	vr1 of gp85 (5456-5476)
ENVA2 (-)	GCGTGGC(C/T)TCCTGTCTA	A	hr1 of gp85 (5683-5699)
ENVAB1A (+)[b]	TCTACACAGTCAGCCACCTC	A,B,C,D,E	gp85 of *env* (5324-5343)
ENVB1B (+)	GGACGAGCCATCAGAACTA	B	vr2 of gp85 (5548-5566)
ENVB2 (-)	GGACCAATTCTGTCTCATTTG	B	hr2 of gp85 (5846-5863)[c]

[a] Schwartz *et al*, 1983; published sequence of the **RNA** genome of the Prague C strain of RSV.

[b] Same primer used for both subgroup A and subgroup B first round amplification.

[c] There is a three-base insertion in this region in subgroup B viruses when compared to subgroup C.

[d] Based on published sequences of viral isolates belonging to these subgroups.

Primers were compared to sequences of both chicken and viral genomes contained in the MicroGenie Sequence Analysis Program database (Spinco Division, Beckman Instruments, Inc., Palo Alto, CA; Queen and Korn, 1984) as well as analysed by the OLIGO Primer Analysis Software (National Biosciences, Plymouth, MN) which indicates possible secondary structures in primers, stability, primer pair compatibility and primer-dimer formation (Breslauer *et al*, 1986; Freier *et al*, 1986). All primers were synthesized on an Applied Biosystems 392 DNA/RNA Synthesizer (Perkin Elmer Co., Applied Biosystems Division, Mississauga, ON) by the cyano-phosphoramidite method, according to specifications. All primers were resuspended in sterile distilled water following purification through G50-Sephadex (Maniatis *et al*, 1982) and drying by vacuum centrifugation (SpeedVac SC100, Savant Instruments , Farmingdale, NY). Primers were quantified by measuring their absorbency at or near 260 nm by scanning between 200 and 320 nm on a Spectronic Genesys 5 Spectrophotometer (Milton Roy Company, Rochester, NY).

PCR Conditions.

Taq DNA polymerase with accompanying 10x concentrated buffer was supplied by either Promega (Madison, WI) or Boehringer Mannheim Canada (Laval, PQ). The Promega buffer contained 500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C) and 1% Triton X-100. Accompanying 25 mM magnesium chloride was added to the reaction to a final concentration of 2.0 mM for all first-round reactions and 1.5 mM for all second-round reactions. The Boehringer Mannheim buffer contained 500 mM KCl, 100 mM Tris-HCl (pH 8.3 at 20°C) and 15 mM $MgCl_2$. $MgCl_2$ was added up to a final concentration of 2.0 mM for all first round reactions. The lack of 1% Triton X-100 in the Boehringer Mannheim buffer did not seem to affect amplification.

The change in supplier of *Taq* from Promega to Boehringer Mannheim part way through the project was due to the inability of Promega to continue supplying *Taq* polymerase. Deoxynucleotide triphosphates from Pharmacia (LKB Biotechnology Group, Baie d'Urfe, PQ, Canada) were diluted to form mixtures consisting of all four dNTPs at a concentration of 1.25 μM each. Small aliquots of the dNTPs were stored at -20°C since dNTPs are unstable and degrade quickly under repeated freezing and thawing (Charlieu, 1994).

To increase the sensitivity of the assay, a semi-nested PCR method was used whereby a first round of PCR is performed with a pair of primers targeted to either the LTR region or to the *env* region of the proviral genome. A small amount of the product from the first round of PCR is then transferred into a second PCR reaction mixture containing one of the primers used in the first round and a second, different primer whose complementary sequence is internal to the first round PCR product (see Figure 4). The products from the primary amplification are not visible, or just faintly so , depending on the level of infection. Bands are clearly visible in positive samples using semi-nested PCR methodology.

Primary PCR reaction samples, regardless of primer pairs, all contained 10 μl of DNA sample, 0.625 units of *Taq* polymerase (Promega or Boehringer Mannheim), 0.2 mM each dNTP, 0.8 μM each primer, 50 mM KCl, 10 mM Tris-HCl, 2.0 mM $MgCl_2$ and either no Triton X-100 if using the Boehringer Mannheim buffer or 0.1% Triton X-100 if using the Promega buffer. Samples consisted of a total volume of 25 μl. For the second, semi-nested round of amplification, a 2 μl aliquot of the first round amplification was added to a second PCR mixture containing similar concentrations as noted above except for the fact that only 1.5 mM of $MgCl_2$ was present

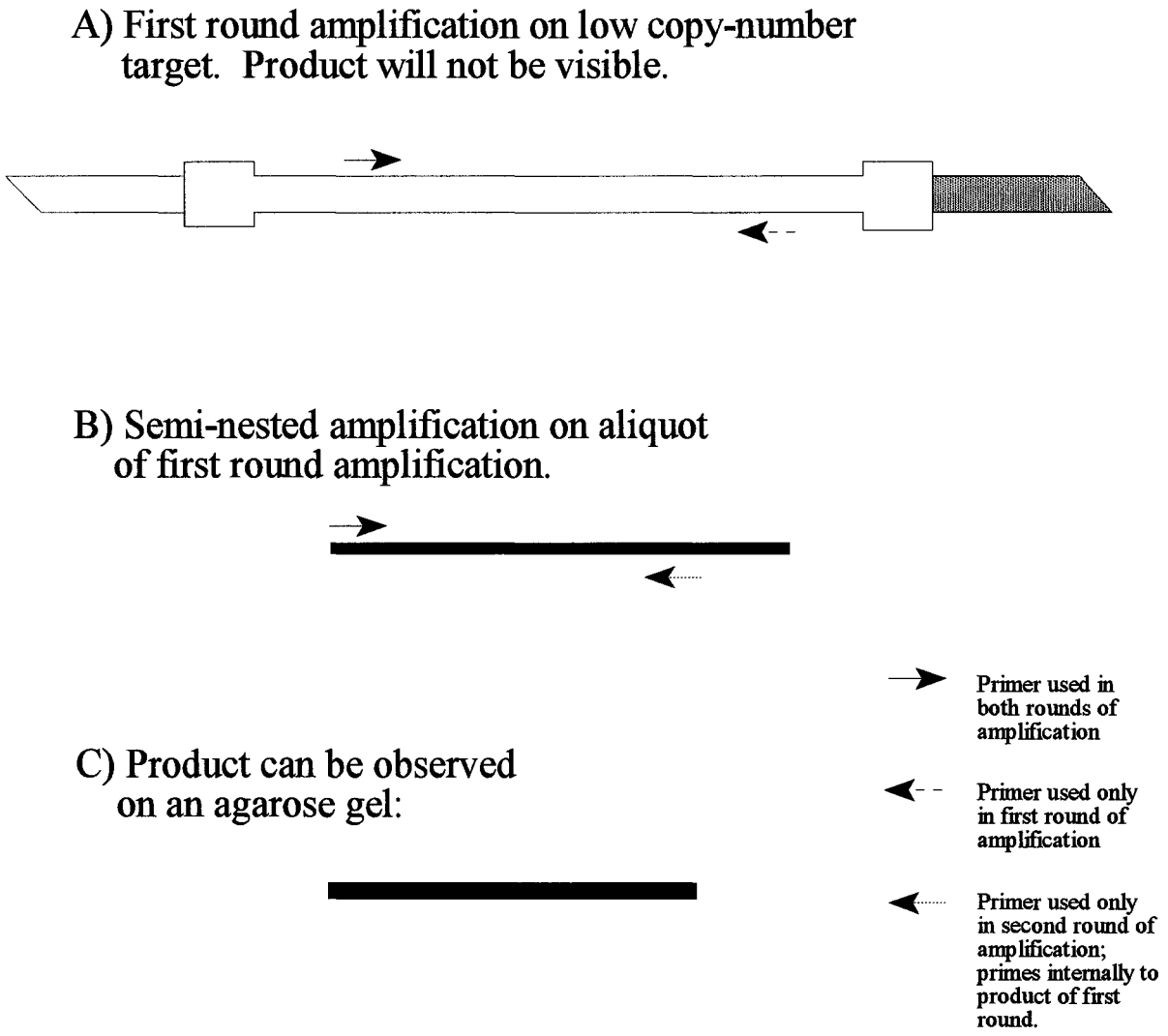


Figure 4. Schematic representation of the steps taken for semi-nested PCR amplification of a low copy-number target. A) The first round of amplification is performed much like a typical PCR reaction. For very low copy-number targets, there is not enough amplified product to visualize on an agarose gel. B) A small aliquot of the first PCR reaction is taken and placed in a second PCR mixture containing one of the primers used in the primary amplification paired with a second primer which anneals to the product of the first round. C) This produces enough product to be visualized on an agarose gel stained with ethidium bromide and UV illuminated.

and one of the primers had been replaced with one that would prime internally to the product of the first round amplification.

All PCR reactions were performed in a PTC-200 DNA Engine (MJ Research Inc., Watertown, MA) thermocycler. 'Touchdown' PCR was used during all PCR amplifications to decrease false priming and spurious amplification (Don *et* al, 1991).

First round PCR: LTR primers (LTR1 & LTR2A).

DNA was amplified in 34 cycles preceded by a 1 minute denaturation step at 96°C. Two of the 34 cycles consisted of denaturation at 94°C for 30 seconds, primer annealing at 66°C for 30 seconds and chain elongation at 72°C for one minute. The next two cycles consisted of denaturation at 94°C for 30 seconds, primer annealing at 64°C for 30 seconds and chain elongation at 72°C for one minute. The last 30 cycles consisted of denaturation at 94°C for 30 seconds, primer annealing at 62°C for 30 seconds and chain elongation at 72°C for one minute. All PCR amplifications ended with a 10 minute extension step at 72°C.

Semi-nested PCR: LTR primers (LTR1 & LTR2B).

DNA was amplified in 36 cycles preceded by a 1 minute denaturation step at 96°C. Two of the 36 cycles consisted of denaturation at 94°C for 30 seconds, primer annealing at 68°C for 30 seconds and chain elongation at 72°C for one minute. The next two cycles consisted of denaturation at 94°C for 30 seconds, primer annealing at 66°C for 30 seconds and chain elongation at 72°C for one minute. Another two cycles consisted of denaturation at 94°C for 30 seconds, primer annealing at 64°C for 30 seconds and chain elongation at 72°C for one minute.

The last 30 cycles consisted of denaturation at 94°C for 30 seconds, primer annealing at 62°C for 30 seconds and chain elongation at 72°C for one minute.

First round PCR: ENVA primers (ENVAB1A & ENVA2).

DNA was amplified in 38 cycles preceded by a 1 minute denaturation step at 96°C. Two of the 38 cycles consisted of denaturation at 94°C for 1 minute, primer annealing at 66°C for 5 seconds and chain elongation at 72°C for one minute. The next two cycles consisted of denaturation at 94°C for 1 minute, primer annealing at 64°C for 5 seconds and chain elongation at 72°C for one minute. The following two cycles consisted of denaturation at 94°C for 1 minute, primer annealing at 62°C for 5 seconds and chain elongation at 72°C for one minute. Another two cycles consisted of denaturation at 94°C for 1 minute, primer annealing at 60°C for 5 seconds and chain elongation at 72°C for one minute. The last 30 cycles consisted of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 5 seconds and chain elongation at 72°C for one minute.

Semi-nested PCR: ENVA primers (ENVA1B & ENVA2).

DNA was amplified in 38 cycles preceded by a 1 minute denaturation step at 96°C. Two of the 38 cycles consisted of denaturation at 94°C for 1 minute, primer annealing at 63°C for 5 seconds and chain elongation at 72°C for one minute. The next two cycles consisted of denaturation at 94°C for 1 minute, primer annealing at 61°C for 5 seconds and chain elongation at 72°C for one minute. The following two cycles consisted of denaturation at 94°C for 1 minute, primer annealing at 59°C for 5 seconds and chain elongation at 72°C for one minute. Another two cycles consisted of denaturation at 94°C for 1 minute, primer annealing at 57°C for 5

seconds and chain elongation at 72°C for one minute. The last 30 cycles consisted of denaturation at 94°C for 30 seconds, primer annealing at 54°C for 5 seconds and chain elongation at 72°C for one minute.

First round PCR: ENVB primers (ENVAB1A & ENVB2).

DNA was amplified in 39 cycles preceded by a 1 minute denaturation step at 96°C. Two of the 39 cycles consisted of denaturation at 94°C for 1 minute, primer annealing at 58°C for 5 seconds and chain elongation at 72°C for one minute. The next two cycles consisted of denaturation at 94°C for 1 minute, primer annealing at 56°C for 5 seconds and chain elongation at 72°C for one minute. The last 35 cycles consisted of denaturation at 94°C for 30 seconds, primer annealing at 54°C for 5 seconds and chain elongation at 72°C for one minute.

Semi-nested PCR: ENVB primers (ENVB1B & ENVB2).

DNA was amplified in 39 cycles preceded by a 1 minute denaturation step at 96°C. Two of the 39 cycles consisted of denaturation at 94°C for 1 minute, primer annealing at 58°C for 5 seconds and chain elongation at 72°C for one minute. Another two cycles consisted of denaturation at 94°C for 1 minute, primer annealing at 56°C for 5 seconds and chain elongation at 72°C for one minute. The last 35 cycles consisted of denaturation at 94°C for 30 seconds, primer annealing at 54°C for 5 seconds and chain elongation at 72°C for one minute.

PCR of cloned pRAV2 and *in vitro* infected CEF.

All primers were initially tested on the cloned subgroup B isolate, pRAV2 (Ju *et al*, 1980; Smith and Crittenden, 1986). Semi-nested PCR was not immediately performed on cloned

material but rather primer pairs were put through a single round of PCR to see if the proper product size was obtained or to make sure that no product was visible in the case of the subgroup A-specific primers.

Following this initial testing, pRAV2 was diluted into chicken DNA which was ALV-free (both endogenous and exogenous), in ten-fold increments, down to a final concentration of one pRAV2 copy for every 10 000 chicken genome copies; 10 µl of the 1/10 000 pRAV2:chicken genome dilution contained five pRAV2 copies, on average. Concentrations for pRAV2 and chicken DNA was determined spectrophotometrically and genome equivalents were calculated by converting the known size, in bases, of the pRAV2 genome into grams (1 base = 325 Daltons = 1.65×10^{-24}g). Semi-nested PCR amplification was performed on these samples and the 1/10 000 dilution was used as a positive control throughout the remainder of the project. A proportion of all samples which gave negative results with the exogenous ALV-specific primers were tested with PCR primers that were expected to give positive results and served as controls for the presence of amplifiable material. Samples of birds not carrying any *ev* genes (strain EV00 used to produce CEF) were amplified with a set of primers which target the apoVLDLII gene (Gavora *et al*, 1995b) present in every chicken (van het Schip *et al*, 1983). Samples of birds known to carry *ev* genes (strains 07 and 08) were amplified with primers that amplify a portion of the LTR of all RAV-type endogenous viral elements (*ev*; Benkel *et al*, 1992).

Viral stocks of RAV-1, RAV-2, RAV-49 and RAV-50, used to infect cultures of chick embryo fibroblasts (CEF; see below for CEF preparation) were originally obtained from ADOL (Avian Diseases and Oncology Laboratory, USDA, East Lansing, MI) although they have been in

L. Spencer's (Animal Disease Research Institute, Agriculture and Agri-food Canada) possession for a number of years.

Chick embryo fibroblast (CEF) preparation and virus inoculation.

Potential parents for the production of CEF cultures were tested with the exogenous ALV LTR primers. Parents were housed in a FAPP (filtered air, positive pressure) building (Drury *et al*, 1969; Grunder *et al*, 1975) and were slated as being free of Marek's disease virus, *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. Previous testing using p27 detection of ALV in egg albumen (Spencer *et al*, 1976; Smith *et al*, 1979) also had birds designated ALV-free (Spencer *et al*, 1984; Gavora *et al*, 1995a). Only exogenous ALV-LTR-negative birds were mated to produce embryos. Eggs were collected daily and placed under nitrogen gas to prevent further development until the necessary number of eggs had been collected. The eggs were then placed in 42°C, humidified incubators for 11 days. On the eleventh day, eggs were candled to confirm fertility of the egg and viability of the embryo.

CEF cultures were prepared from 11 day old, EV00 White Leghorn chick embryos. These birds were developed at ADOL (USDA, East Lansing, MI) and are free of RAV-type endogenous elements. They have the phenotype C/E, which means that they are susceptible to subgroups A, B, C and D of leukosis viruses but not subgroup E (Astrin *et al*, 1979). The embryos were processed under aseptic conditions. Embryos were handled and processed individually, not pooled, to verify and ascertain the absence of any exogenous ALV using PCR detection as well as p27 detection in the cell culture supernatant.

Eggs were disinfected with 5% Wescodyne (West Chemical Products of Canada Ltd., Ville d'Anjou, PQ) solution and allowed to dry before the egg shell above the air cell was cracked and removed to expose the embryo (see Freshney, 1987 for general procedures for tissue culture preparation). Embryos were decapitated and the bodies rinsed with warm 1x PBS (phosphate buffered saline). The embryos were then forced through 20 ml syringes into 125 ml Erlenmeyer trypsinization flasks. This effectively disaggregated the chick embryonic tissue. The tissue was washed with prewarmed PBS at 37°C with the use of a magnetic stirrer until the fluid was free of blood and cellular debris. This was followed by at least four-10 minute trypsinization (0.05% trypsin in PBS) treatments. The cells and debris obtained from the first trypsin treatment were discarded; the cells from all following treatments were collected by straining the liquid portion of the contents of the flasks through a funnel lined with four layers of cheese cloth into 50 ml Falcon tubes placed on ice and containing one millilitre of calf serum which helps inactivate the trypsin. The Falcon tubes containing the cells were then centrifuged (International Equipment Co., IECCR-6000 centrifuge) at 4°C, at 1000 rpm (rotations per minute) for five minutes or until the cell pellet was firmly packed but not compacted. The supernatant was poured off and the cells resuspended in F10-199 complete medium (Gibco BRL, Life Technologies, Gaithersburg, MD) to a live cell count, using trypan blue dye exclusion, of 10^6 live cells per millilitre. Thirty millilitres or 3.0×10^7 cells were added to 100 mm diameter tissue culture dishes (Nunc Plastics, Copenhagen, Denmark). Plates were placed in a 37°C- 5% CO_2 - humidified incubator (NAPCO Model 6300) and the cells permitted to adhere and grow for three days.

On the third day cells were harvested as follows: Complete media in the plates was

removed by suction. Cells were gently rinsed with prewarmed (37°C), fresh media. This too was suctioned off and 10 ml of trypsin-versene (0.2 g/l EDTA•4Na in PBS) was gently added and swirled to cover the cells. The trypsin-versene was immediately removed and the plates placed in a 37°C incubator for approximately five minutes with frequent observation so as to not over-trypsinize. The EDTA in the versene chelates free Ca^{2+} and Mg^{2+} which are required by the cells to adhere to the plate. When cells were observed to be disassociating from the plate bottom and each other, 10 ml of complete media was added and the cells mechanically disaggregated by pipette action. The cell suspension was then transferred to a 15 ml conical tube that was placed on ice. The tubes were centrifuged for five minutes at 1000 rpm at 4°C until cells were firmly packed but not compacted. Supernatant was poured off and the cells resuspended in 5% DMSO media (5% dimethyl sulphoxide, 15% calf serum in complete medium) to a final concentration of 10^7 cells per millilitre. Aliquots of 1.5 ml were dispensed into cryotubes and the samples were frozen in the vapour phase of nitrogen for 1 hour. The samples were then lowered into the nitrogen tank for long-term storage.

Both the primary cells and the harvested cells were tested for ALV with the exogenous LTR primers (see PCR methodology). Once the cells were confirmed to be ALV-free, the frozen cells from individual embryos were thawed and plated onto 96-well tissue culture plates. Two nonsibling embryos were used for each viral isolate, all samples were done in duplicate and only one virus isolate was inoculated per plate. Plates were placed in a CO_2 incubator overnight. The following morning, 10-fold serial dilutions of the viral stocks (RAV-1, RAV-2, RAV-49 and RAV-50) were prepared and 20 µl of each dilution was added to individual wells. Cells were

placed in a CO_2 incubator for six days. On the sixth day, cells were frozen and thawed three times. The supernatant was collected and tested for p27 (gsa) by ELISA (see below). The cells were taken, DNA was extracted (see below for extraction method used) and PCR was performed for exogenous ALV detection.

Nucleic acid extraction.

All extractions were done according to the method of Higuchi (1989) with modifications detailed below.

DNA extraction from infected CEF.

Cells were collected after three cycles of freeze-thawing. The freeze-thaw cycles were performed to obtain supernatant for the detection of p27 by ELISA (see below). The resulting cells were placed in 500 µl of Higuchi lysis buffer (1989) containing 0.32 M sucrose, 10 mM Tris-HCl with a pH of 7.5, 5 mM $MgCl_2$, and 1% Triton X-100. The samples were then centrifuged at approximately 13 000 rpm for one minute and the supernatant poured off. The nuclear pellet was resuspended in 500 µl of Higuchi extraction buffer (50 mM KCl, 10 mM Tris-HCl with a pH of 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin and 0.9% Tween 20; the original recipe for the extraction buffer contains 0.45% each of Nonidet P40 and Tween 20) to which was added 0.03 mg/ml of Proteinase K (Boehringer Mannheim). Samples were incubated at 55°C until the pellet was completely digested, normally between one to three hours. The samples were then placed at 95°C for 10 minutes to deactivate the Proteinase K and then frozen or used immediately for PCR. Ten microlitres from each sample was used for PCR amplification.

Three different tissue/cell types were collected and their DNA extracted to determine the best source of DNA for detection of integrated ALV in infected poultry. Feather pulp, leukocytes and whole blood were all tested to determine the best, noninvasive sampling site.

DNA extraction from feather pulp.

Feather pulp samples were collected and frozen at -70°C until processed. Feather pulp from one feather was thawed for each bird and the pulp squeezed aseptically out of the quill into PBS containing 0.5% trypsin. Pulp was incubated at 37°C for at least one hour to begin dissociating cells from the tissue mass. Samples were spun and the supernatant poured off. The cellular pellet was then resuspended in 500 µl of Higuchi (1989) lysis buffer, mixed, centrifuged at approximately 13 000 rpm for one minute and the supernatant poured off. A second aliquot of lysis buffer was added and centrifugation and discarding of supernatant was repeated. The pellet was then resuspended in 500 µl of Higuchi (1989) extraction buffer to which was added 0.05 mg/ml of Proteinase K (Boehringer Mannheim). Samples were incubated at 55°C for approximately three hours until the pellet was completely digested. The samples were then placed at 95°C for 10 minutes to deactivate the Proteinase K and then frozen or used immediately for PCR. Ten microlitres from each sample was used for PCR amplification with exogenous ALV primers.

DNA extraction from blood.

Blood samples for both leukocyte isolation and whole blood were collected by wing venipuncture using Vacutainer brand tubes (Becton Dickinson, Rutherford, NJ) containing EDTA(K_3).

DNA extraction from leukocytes.

A quick method of enriching for peripheral blood leukocytes was devised rather than using the more labour-intensive Ficoll-Hypaque method. Heparinized micro-hematocrit capillary tubes (Fisher Scientific Co., Nepean, ON) were filled with approximately 40 µl of fresh, whole blood and the tubes were spun on a Haemofuge (Baxter Diagnostics Co., Canlab Division, Mississauga, ON) for 30 seconds. Spinning for longer periods of time essentially compacts both erythrocytes and leukocytes but a quick, 30 second spin draws the erythrocytes to the bottom and leaves the leukocytes at the interface. The leukocytes can then be collected by using a gel-loading tip to reach the cells. From an erythrocyte to leukocyte ratio of 1000:1 for whole blood (Sturkie and Griminger, 1986), this method can lower the ratio down to 1-3 erythrocytes for every one white blood cell. These ratios were determined by observing and counting cells in a haemacytometer. The collected cells were then extracted by the Higuchi method (1989) as described above, resuspending in a final volume of 250 µl extraction buffer.

DNA extraction from whole blood.

Blood was collected in Vacutainer brand tubes containing EDTA(K_3). Blood was either frozen at this point or an aliquot immediately extracted for its DNA. Five microlitres of fresh or thawed blood was transferred to 500 µl of Higuchi (1989) lysis buffer, mixed, centrifuged at approximately 13 000 rpm for one minute and the supernatant poured off. Lysis buffer was added a second time if the pellet retained traces of haemoglobin. Otherwise, the pellet was resuspended in 250 µl of Higuchi (1989) extraction buffer to which was added 0.03 mg/ml of Proteinase K (Boehringer Mannheim). Samples were incubated at 55°C for at least 1 hour or until the pellet

was completely digested. The samples were then placed at 95°C for 10 minutes to deactivate the Proteinase K and then frozen or used immediately for PCR.

Detection in a naturally infected flock: Comparison with antigen and antibody detection.

Previous attempts to eradicate exogenous ALV from strains 07 and 08, White Leghorn chickens housed at the Green Belt Farm (Agriculture and Agri-food Canada, Nepean, ON) have been unsuccessful with currently used methods of ALV detection. Strain 07 has a broad genetic base and has been maintained at the facility since 1958, without selection. Strain 08 was derived from strain 07 in 1969 and has since been selected for economically important traits such as high egg production (Gowe and Fairfull, 1980).

An albumen sample as well as a serum and whole blood sample were each taken from 144 birds of strains 07 and 08. All sampling was performed on the same day. The albumen samples were tested for p27 using ELISA (see below) and the serum samples were tested for antibodies against subgroups A and B ALV (see below). The whole blood was taken and extracted according to the Higuchi method outlined above and all 144 blood samples were tested with all three primer sets (LTR, subgroup A envelope and subgroup B envelope primers). All PCR reactions were done in duplicate. Results from the various testing methods were then compared.

Repeatability.

Eighty-five birds from the next generation of the above described birds, strains 07 and 08, were bled twice with an interval of two weeks between bleeds, to see whether PCR detection of ALV was repeatable. Blood samples from the two bleeds were processed separately. PCR

amplification was also performed at completely different times. Both LTR and *env*-specific amplifications were performed on all samples and done in duplicate.

Analysis of PCR products.

All PCR products were analysed by running 8 μl of a PCR sample on 1.5 % agarose gels (SeaKem® GTG Agarose, FMC BioProducts, Rockland, ME) stained with ethidium bromide and visualized by UV fluorescence.

The molecular weigth marker, ΦX174 digested with *Hae*III, was used for all gel analysis of PCR products. The corresponding weights from the largest to the smallest bands of ΦX174 are: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 bp, respectively.

Precautions.

Pre- and post-PCR manipulations were carried out in separate locations to limit the likelihood of contamination from PCR carry-over. The area that was used to set up the PCR reactions was illuminated with UV light when not in use. All stocks were aliquoted into small volumes to minimize the number of times a specific tube was handled. When transferring the PCR reaction products of the first round amplification to the second, extreme precaution was taken in opening the first round reaction tubes and their subsequent handling during the transfer. Sample handling was tested periodically by performing semi-nested PCR on a total number of 48 known negative and positive samples only. The technique was fine tuned until all negative samples consistently came out negative. Negative (water controls and uninfected CEF) and low copy number positive controls were used throughout the experiment.

Sequencing.

Sequencing of PCR products was done to confirm that it was indeed exogenous ALV that was being amplified in both laboratory infected CEF and naturally infected flocks. Cycle sequencing using dye-labelled terminators (ABI PRISM Dye Terminator Cycle Sequencing Core Kit with AmpliTaq®DNA Polymerase, FS from Perkin Elmer-Cetus, Norwalk, USA) was performed mainly on second round amplification products. PCR products were column purified using Centricon-100 microconcentrators (Amicon Division, W.R. Grace & Co., Beverly, MA) to remove unincorporated dNTPs and unused primers. The purified products were then quantified by visualizing on 1.5% agarose gels stained with ethidium bromide, prior to sequencing. Thirty to 90 nanograms of template was used for each individual sequencing reaction. Sequencing was performed in a final reaction volume of 15 µl rather than the 20 µl recommended by Perkin Elmer (5.5 µl of reaction premix was used, rather than 8 µl and the volume was made up to 15 µl, rather than 20 µl). All other amounts and volumes were as recommended by Perkin Elmer. Cycle sequencing was performed on the GeneAmp PCR System 9600 (Perkin Elmer) with the following parameters: DNA was amplified in 25 cycles preceded by a 1 minute denaturation step at 95°C. All 25 cycles consisted of a 96°C denaturation step for 15 seconds, followed by a 2 second annealing step at 52°C and ending with a 4 minute extension step at 60°C. Sequencing products were ethanol precipitated and dried, then resuspended in loading buffer recommended for the ABI 373 sequencer.

P27 detection.

To detect gsa (group-specific antigen, p27) of ALV, egg albumen samples were collected

and diluted one to one in PBS. The diluted albumen samples were then placed into wells of 96-well microtitration plates coated with rabbit anti-p27 IgG (Life Sciences Inc., St. Petersburg, Florida) which was raised by immunizing rabbits with chromatographically purified p27 of avian myeloblastosis virus (AMV; Smith *et al*, 1979); the antibody is therefore polyclonal in nature (see Figure 5). The wells were washed and rabbit anti-p27 IgG coupled to horse radish peroxidase was added followed by the addition of the horse radish peroxidase substrate, azino di-[3-ethyl-benzthiazoline sulfonate]-H_2O_2 which produces a colour change that can be spectrophotometrically read at 414 nm (for more detail see Spencer, 1987).

Antibody detection.

To detect antibody against both subgroup A and subgroup B ALV, the Flockchek® :ALV-Ab (IDEXX Laboratories, Inc., ME, USA) test kit was used. Chicken serum samples were collected and processed according to IDEXX's specifications. Absorbency measurements were taken at 650 nm with a BioRad Model 3550 microplate reader (BioRad, Mississauga, ON) and sample to positive (S/P) ratio values were calculated [(sample reading - negative control mean)/(positive control mean - negative control mean)]. Samples with S/P ratios less than or equal to 0.4 were considered negative; samples with S/P ratios larger than 0.4 were considered positive and these birds were identified as having been exposed to ALV of either subgroup A or subgroup B. Antibody to subgroup E ALV, if present, is not detected with this kit. Validity of the test is based upon the condition that the difference between the positive control mean ($PC_{\bar{x}}$) and the negative control mean ($NC_{\bar{x}}$) be greater than 0.075. The negative control mean should also be less than or equal to 0.150.

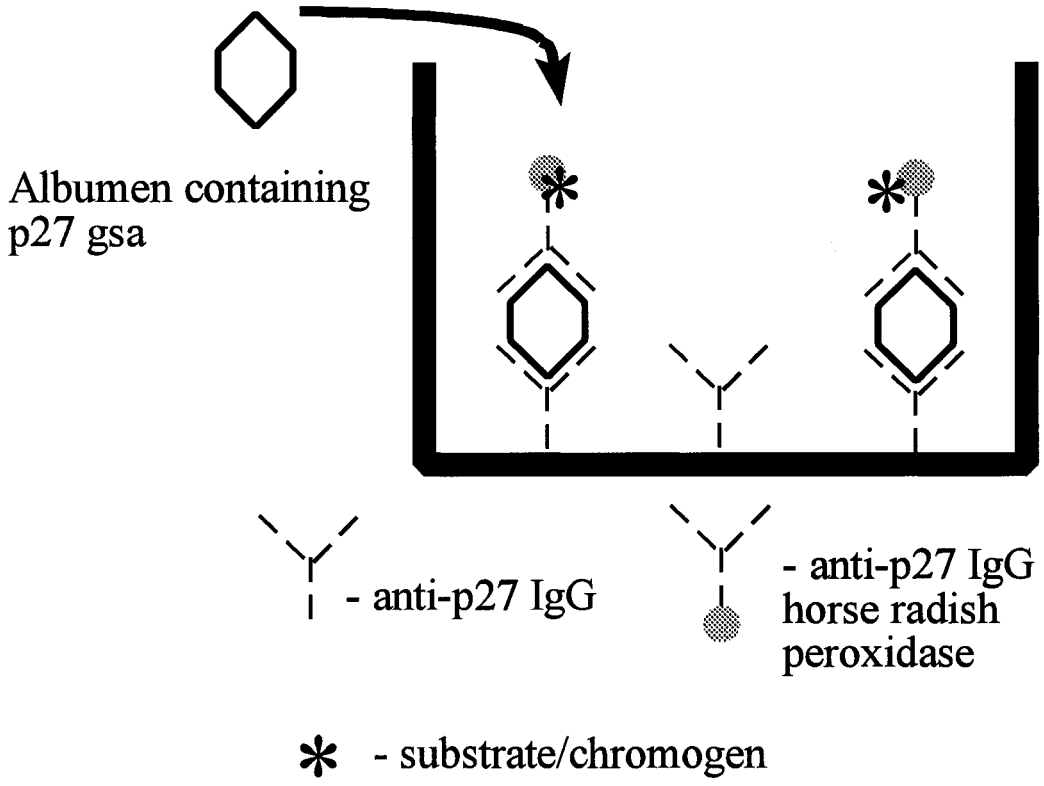


Figure 5. Schematic of ELISA detection of p27 in egg albumen. Antibodies to ALV found in infected birds' serum is detected in a similar manner but viral particles, rather than antibodies, are bound to the solid-phase of the microtiter plates. The serum is then added, followed by anti-chicken IgG conjugated to horse radish peroxidase.

RESULTS

PCR amplification of exogenous ALV regions.

The presence of endogenous viral (*ev*) genes in the chicken genome introduces an atypical level of complexity in the design of reliable primers for the detection of exogenous ALV. This is in contrast to PCR detection of most other retroviral infections (ie. HIV) since there are no related endogenous viral elements known to exist in the genomes of the hosts of these viruses. Regions of exogenous ALV genomes targeted with PCR primers must first of all be conserved between the exogenous viral isolates that one wishes to detect. These regions must also be different enough from the RAV-type endogenous viral genes such that they will not be amplified. Comparing published sequences of exogenous subgroups A, B, C and D with endogenous viral genes of subgroup E, it is apparent that there are very few regions that can be targeted to discriminate between endogenous and exogenous ALV. Most of the regions of the genome of exogenous ALV are more than 90% similar to homologous regions in *ev* elements (Coffin, 1980). The regions of high homology include all of the *gag* and *pol* regions (Coffin *et al*, 1978) as well as the portion of the *env* gene coding for gp37. The R and U5 regions of the LTR and the leader region (Hackett *et al*, 1991) are also highly conserved between exogenous and endogenous ALV (see Figure 6a, sequence comparison of LTR). This leaves only the U3 region of the LTR and the region of *env* coding for gp85 as potential targets for exogenous-specific primers (see Figure 6a and 7 for sequence comparison of U3 region and gp85 region, respectively). Even in these regions, there are subregions of either divergence, between exogenous variants, and homology, between exogenous and endogenous variants, which leaves only a few specific stretches of

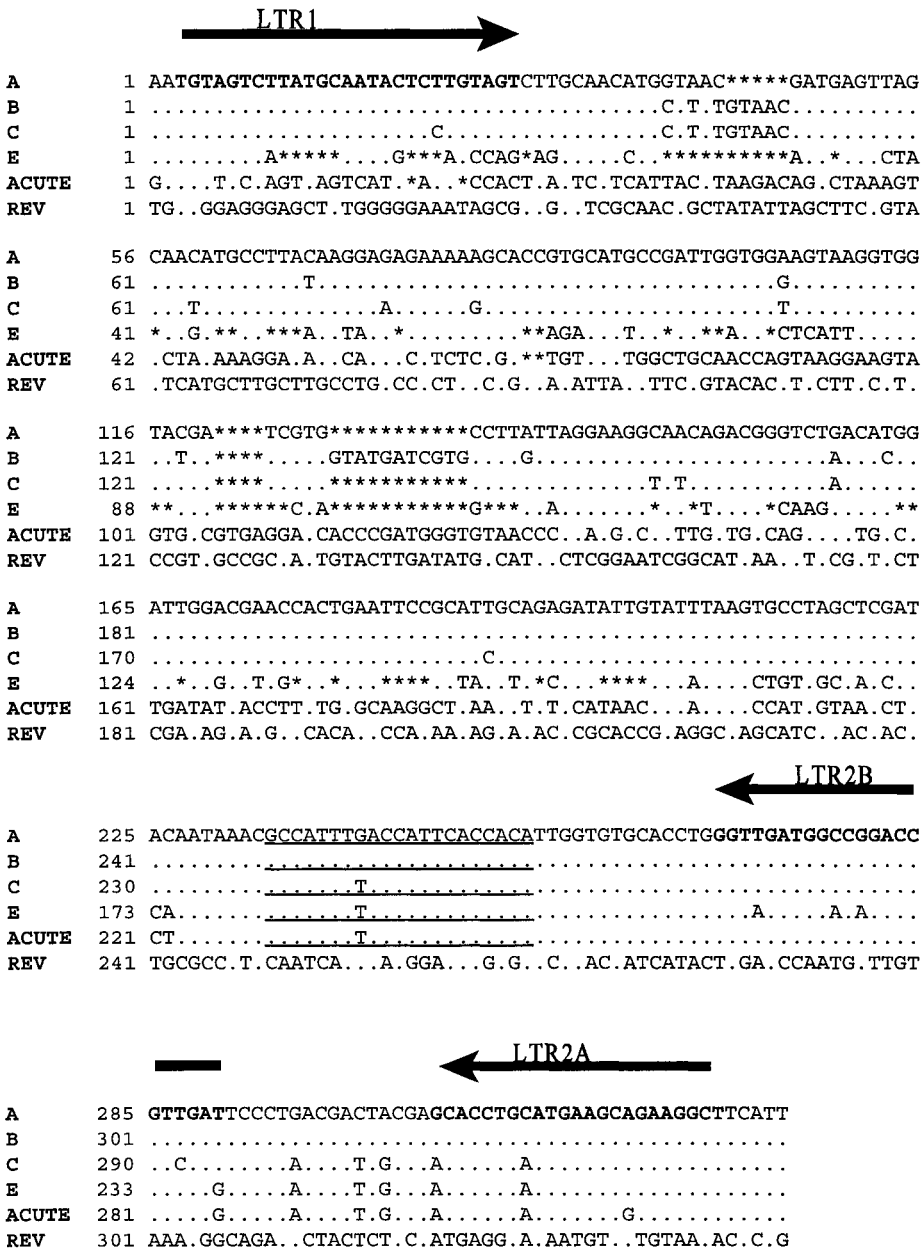


Figure 6a. Examples and sequence comparison of the LTR (long terminal repeats) of avian retroviruses. The letter or word in front of the sequence describes the subgroup or type of retrovirus with which the LTR was associated when sequenced; the LTR sequence is not subgroup-specific. Subgroups A, B, C and the acute retroviruses are all exogenous avian leukosis/sarcoma viruses (ALV/ASV). Subgroup E is the subgroup in which endogenous viral (*ev*) elements are classified; all *ev* genes have similar LTR (see Figure 6b). REV (reticuloendotheliosis virus) is another retrovirus that infects poultry but is not related to ALV. Only a portion of the U3 region of REV is given. The sites of exogenous-specific, LTR-primer binding are indicated by arrows and bold type. The R region is underlined; all sequence upstream of R is considered the U3 region, downstream sequence is the U5 region. Sequences are from: A (Swanstrom *et al*, 1981); B (Bizub *et al*, 1984); C (Schwartz *et al*, 1983); E (Hughes, 1982); acute (Fujinami sarcoma virus sequenced by Shibuya and Hanafusa, 1982), and REV (Shimotohno *et al*, 1980). Refer to Table 1 for all primer sequences.

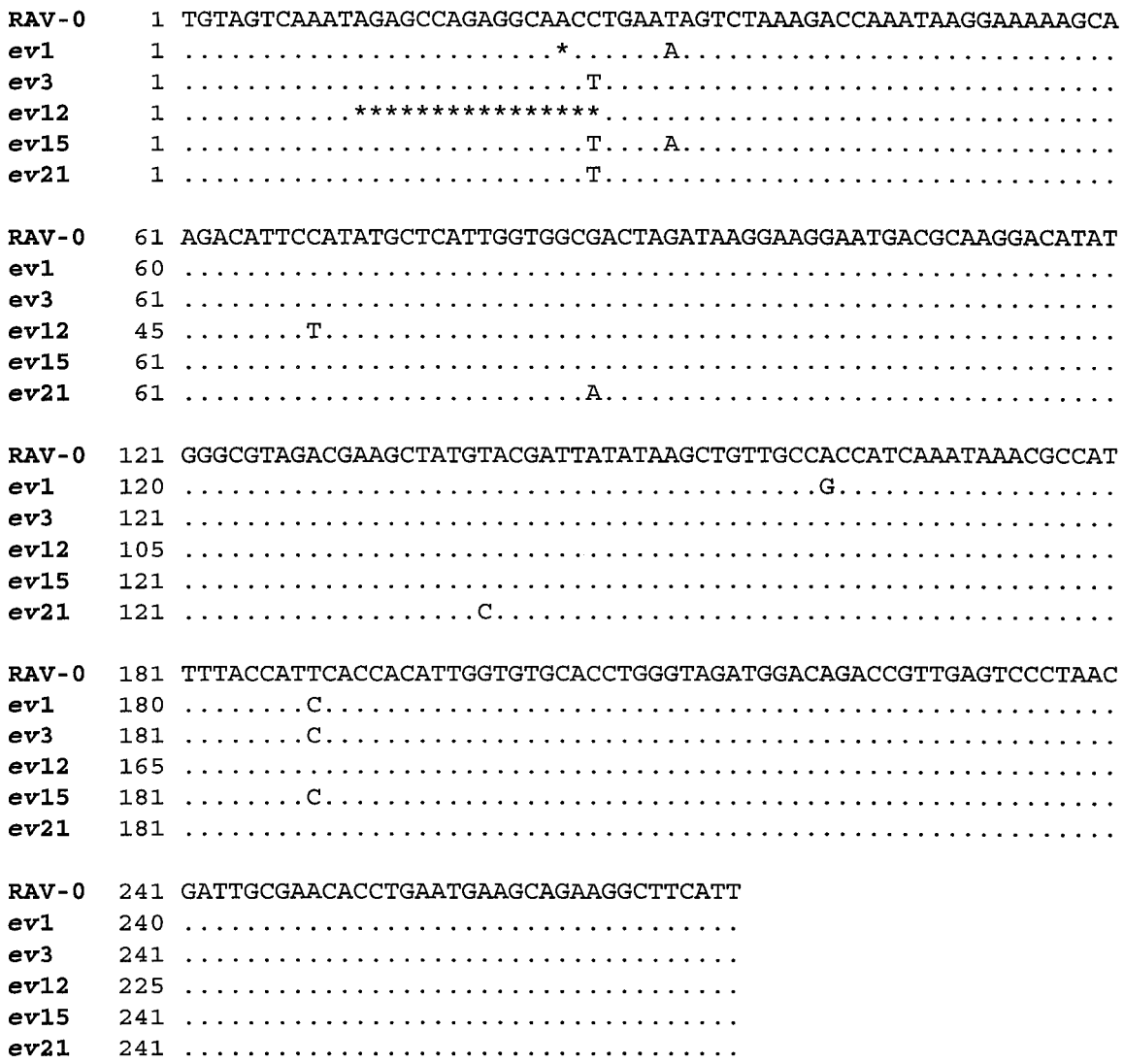


Figure 6b. LTR (long terminal repeat) sequences of some of the endogenous viral (*ev*) genes that are closely related to exogenous avian leukosis retroviruses (ALV). All *ev* LTR that have been sequenced to date differ little from the above examples (Gagnon *et al*, 1995). The primers designed for detection of exogenous ALV LTR should not produce a product in the presence of *ev* genes. The RAV-0 sequence is from Hughes (1982); *ev*1 (Hishinuma *et al*, 1981) and *ev*21 (Levin and Smith, 1991). The LTR sequences of *ev*3, 12, and 15 have not been published (Benkel *et al*, in prep.).

nucleotides that can be reliably used.

A small stretch at the 5' end of the U3 region appears to be conserved between the exogenous ALV subgroups that typically infect chickens and divergent enough from the *ev* sequences; a primer was designed to target this location (LTR1; see Table 1 for sequence of primer and Figure 6a for site of priming). This primer will not recognize many of the laboratory strains of acute ASV since the LTR of these viruses tend to be heterogeneous and only a few of the published sequences of LTR associated with acute sarcoma viruses have homology to the LTR1 primer (see Figure 6a for an example of an acute ASV-LTR sequence). The downstream primer chosen for the first round of amplification in the LTR region (LTR2A; see Table 1 for sequence of primer and Figure 6a for site of priming) was targeted to the 3'-end of the U5 region of the LTR which is highly conserved between all subgroups. Although this primer will anneal to endogenous viral genes in the chicken genome, the lack of an upstream partner will not allow an amplified product to be produced when the primer anneals to *ev* genes (see below for one exception). The same upstream primer (LTR1) was used for the semi-nested, second round of amplification coupled with LTR2B which primes internally to the product of the first round of amplification (See Figure 4 for schematic of primer design for semi-nested amplification). The nested primer will only anneal and produce a product that can be visualized on an ethidium bromide-stained agarose gel if an amplified product produced by LTR1 and LTR2A is present in the reaction. It will not anneal to products formed by the interaction of LTR2A with *ev* genes since LTR2A and LTR2B prime on the same sense strand of the proviral genome.

Similar strategies were used to design the subgroup-specific primers for subgroups A and

B detection. The same upstream primer (ENVAB1A; see Table 1 for primer sequences and Figure 7 for sites of primer annealing) was used in both first round amplifications of subgroups A and B. This primer anneals to a region of gp85 that is conserved between both endogenous and exogenous subgroups (Dorner *et al*, 1985; Bova *et al*, 1986). The same arguments as those used above can be utilized to explain the lack of product when ENVAB1A interacts with *ev* genes. The downstream primer for detection of subgroup A proviruses (ENVA2) targets the hr1 region of gp85 (see Figure 7). This portion of gp85 encodes the amino acids that form one of the domains of the gp85 glycoprotein that makes close contact with the subgroup A receptor found on host cells, allowing the virus to gain access to the interior of a cell (Dorner *et al*, 1985; Bova *et al*, 1986; Dorner and Coffin, 1986). There is between 40 and 60% nucleotide sequence identity in the variable regions of the different subgroups of ALV and greater than 90% identity in the intervening, conserved domains (Bova *et al*, 1986; 1988; Bova and Swanstrom, 1987). The subgroup A-specific downstream primer (ENVA2) is used again in the semi-nested amplification paired with ENVA1B which primes in the vr1 region of gp85 (see Figure 7). Vr1 is another region of gp85 which is biologically important for either virus/cell receptor interaction or possibly membrane fusion (Hunter *et al*, 1983). The downstream primer for detection of subgroup B provirus (ENVB2) targets the other region of gp85, hr2, which encodes the amino acids that form a second domain of the gp85 glycoprotein that interacts directly with the subgroup-specific receptor on the host cells, in this case, the as yet unidentified subgroup B receptor (Dorner *et al*, 1985; Bova *et al*, 1986; Dorner and Coffin, 1986). The semi-nested upstream primer anneals to a region of gp85 which is only variable in subgroup B viruses, vr2 (Dorner *et al*, 1985). The biological reason for this variability is not known.

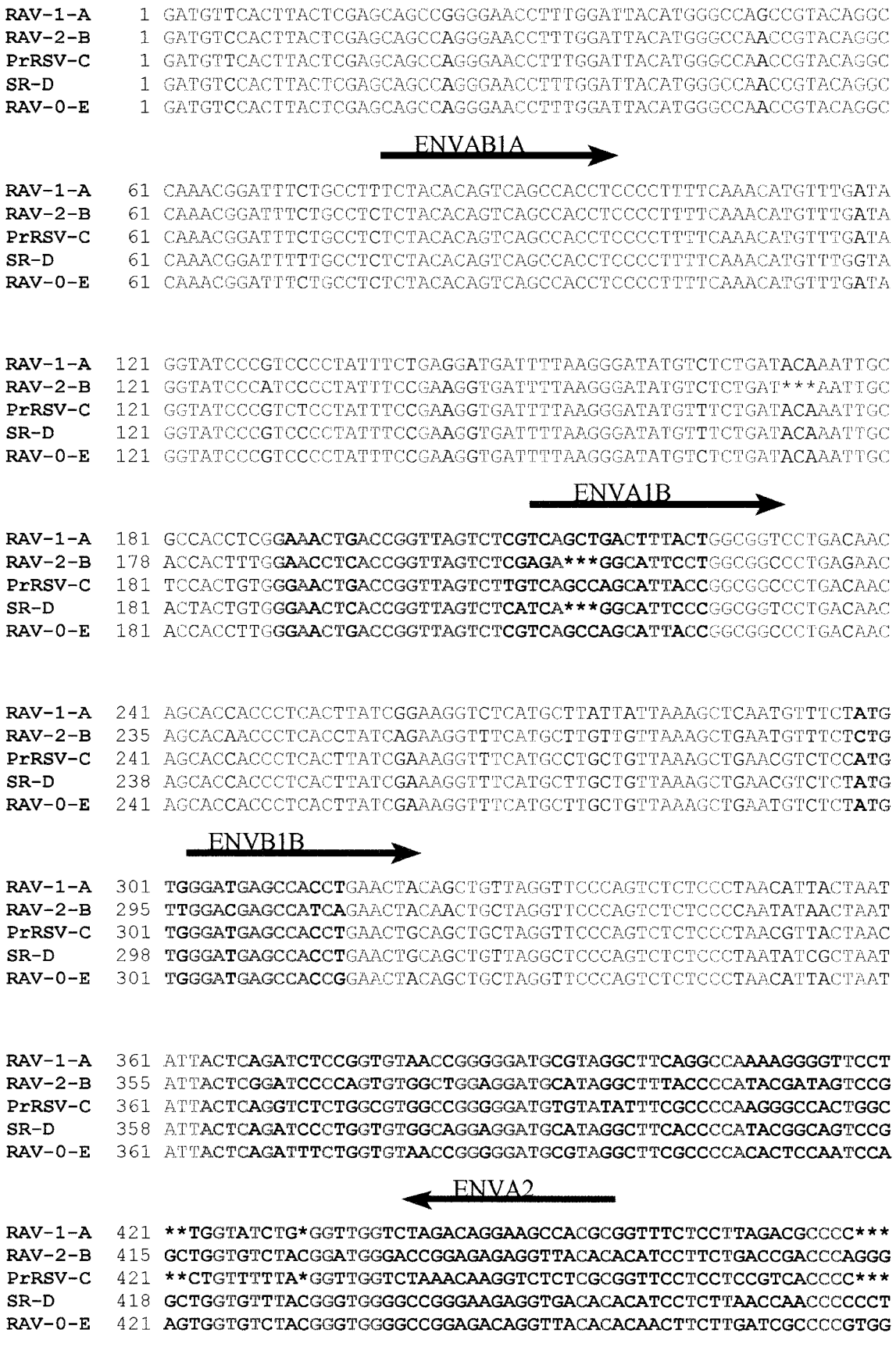


Figure 7. Continued on next page...

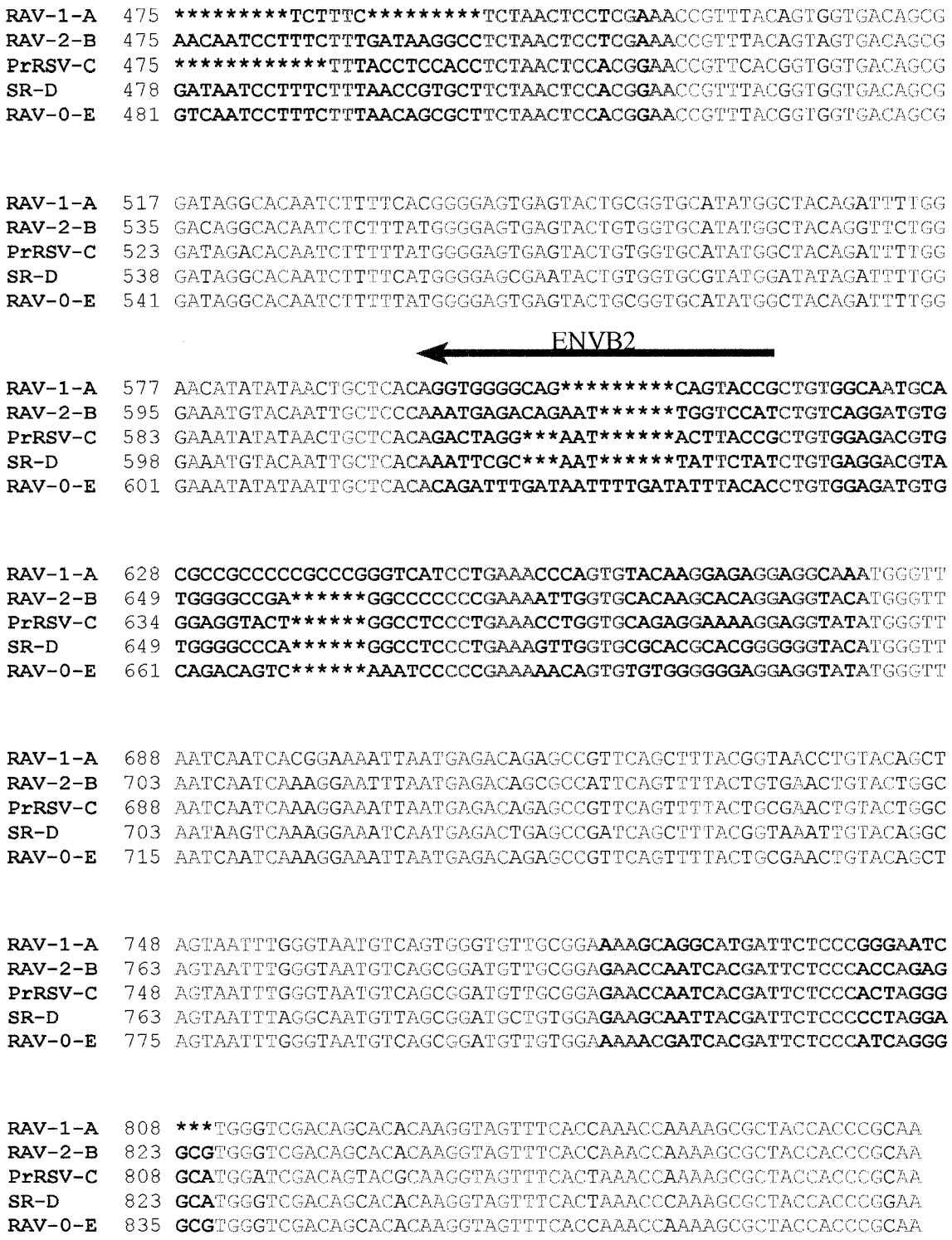


Figure 7. Sequence comparison of the entire gp85 region of the *env* gene. Viral isolates, subgroups and base numbers are indicated. Regions in bold are the variable regions (vr1, vr2, hr1, hr2 and vr3) encoding the domains of gp85 which interact with the subgroup-specific host cell receptors; as described by Bova *et al*, 1988. Primers used for detection of both subgroup A and subgroup B, exogenous ALV are indicated; the primer denoted as ENVAB1A is used in both reactions. Bases that are conserved in all subgroups are in red, bases that have been found to vary are in black or blue.

LTR amplification of pRAV2 and infected CEF.

The first round LTR primers (LTR1 in combination with LTR2A) gave a product of the appropriate size, approximately 340 bp, when tested on pRAV2 copy DNA of a subgroup B ALV. The second round LTR primers (LTR1 and LTR2B), when used in a single round reaction, also gave a product of the appropriate size when tested on pRAV2, approximately 300 bp. The addition of various amounts of chicken DNA to pRAV2, down to a ratio of one pRAV2 copy for every 10 000 DNA genome copies, did not result in nonspecific amplification. Both first round and second round products were sequenced to verify exogenous ALV-specific amplification (results not shown).

The primers were next tested on CEF (chick embryo fibroblasts) which had been infected with ALV strains of either subgroup A (RAV-1), subgroup B (RAV-2), subgroup C (RAV-49), or subgroup D (RAV-50). These subgroups are those that have been found to most commonly infect chicken populations although subgroups C and D have never been detected in North American flocks (Payne, 1987). Chickens not carrying any *ev* genes (Astrin *et al*, 1979), which were housed in a FAPP (filtered-air, positive pressure) building and were believed to be pathogen free (Spencer *et al*, 1984; Gavora *et al*, 1995a), were mated to produce embryos from which CEF were prepared. The viral stocks were inoculated onto these cells in 10-fold serial dilutions down to a dilution of 10^{-10}. Negative controls in the form of uninfected cells were included. The results were often positive when PCR detection of exogenous LTR was performed on the uninfected controls. It was initially assumed that this was due to PCR carryover when performing semi-nested PCR but it soon became clear that the cells were carrying at least the

LTR of exogenous ALV. Previous testing of the FAPP facilities by ELISA detection of the group-specific antigen (gsa), p27, indicated that the FAPP house was ALV-free. Past cases of birds dying of lymphoid leukosis-like lesions had been attributed to a nonviral etiology (Crittenden *et al*, 1979). From the PCR results, the suspicion that the FAPP house might indeed have a low level infection of ALV lead to the entire population being tested, again by the method of p27 detection in egg albumen. Of the 430 females which were tested, three had ELISA readings higher than the cutoff value of 0.2 optical density units with the specific values being 0.53, 0.268 and 0.239. In the past, the low number of positive birds plus the fact that the ELISA readings were not excessively high would lead to the conclusion that it was endogenous gsa that was being detected. *Ev*-derived p27 cannot explain two of the ELISA readings obtained here since the bird which had a reading of 0.53 was of strain EV00 and did not carry any *ev* genes. The female with a reading of 0.268 was of strain EV06. The EV06 strain only carries the *ev6* gene and no other ALV-related endogenous viral elements (Gavora *et al*, 1991). The *ev6* gene does not produce p27 since its upstream LTR has been deleted as well as all of *gag,* which is the region of the genome that codes for p27. Part of the *pol* gene is also missing in *ev6* (Crittenden, 1991). The last reading of 0.239 was obtained from a bird of strain EV12 which can produce fully infectious subgroup E viral particles (Crittenden, 1991). The positive ELISA result in the case of this bird might be explained by the presence of endogenous-derived p27. A review of the mortality records for the birds housed in the FAPP facilities from the year 1992 to 1994 showed that four birds had died of lymphoid leukosis (LL) in 1992, seven had died of LL in 1993 and five had died in 1994. All of these deaths had been attributed to non-ALV lymphomas.

To obtain truly negative birds for production of uninfected CEF, all males and females of strain EV00, housed in the FAPP house, were tested with the exogenous LTR-specific primers. Of a total of 41 birds tested, 16 (39%) were positive with the LTR primers. One of these birds was the female that had tested positive for gsa in albumen with a reading of 0.53 (see above). Four exogenous LTR-negative females were pedigree-mated to negative males, and eggs were collected for CEF production from 11 day-old embryos. The primary and harvested cells from each individually processed embryo (n=8) were tested with exogenous LTR primers. One embryo gave a positive reading in the harvested cells and was discarded.

The other embryos, two non-sibling embryos per viral isolate, were plated onto 96-well microtitration plates and inoculated with serial dilutions of RAV of subgroups A, B, C and D. PCR amplification with the exogenous LTR primers gave similar results with all four subgroups (see Figure 8 for products formed in both the first round and semi-nested round of amplification on serially diluted RAV-2, 10^{-1} to 10^{-10}, inoculated onto CEF). All uninfected controls remained negative throughout the remainder of the experiment. Table 2 compares the sensitivity of primary amplification, semi-nested amplification and p27 ELISA detection of CEF infected with either subgroup A (RAV-1), subgroup B (RAV-2), subgroup C (RAV-49) or subgroup D (RAV-50) ALV. Samples were collected on the sixth day following viral inoculation. The primary amplification would consistently detect viral dilutions down to 10^{-4} for RAV-1 and RAV-2, 10^{-3} for RAV-49 and 10^{-5} for RAV-50. Comparing these results to ELISA detection, obtained when the cells were freeze-thawed and the supernatant collected and tested for the presence of p27: ELISA was able to detect dilutions of RAV-1 down to 10^{-4}, RAV-2 down to 10^{-3}, RAV-49 down

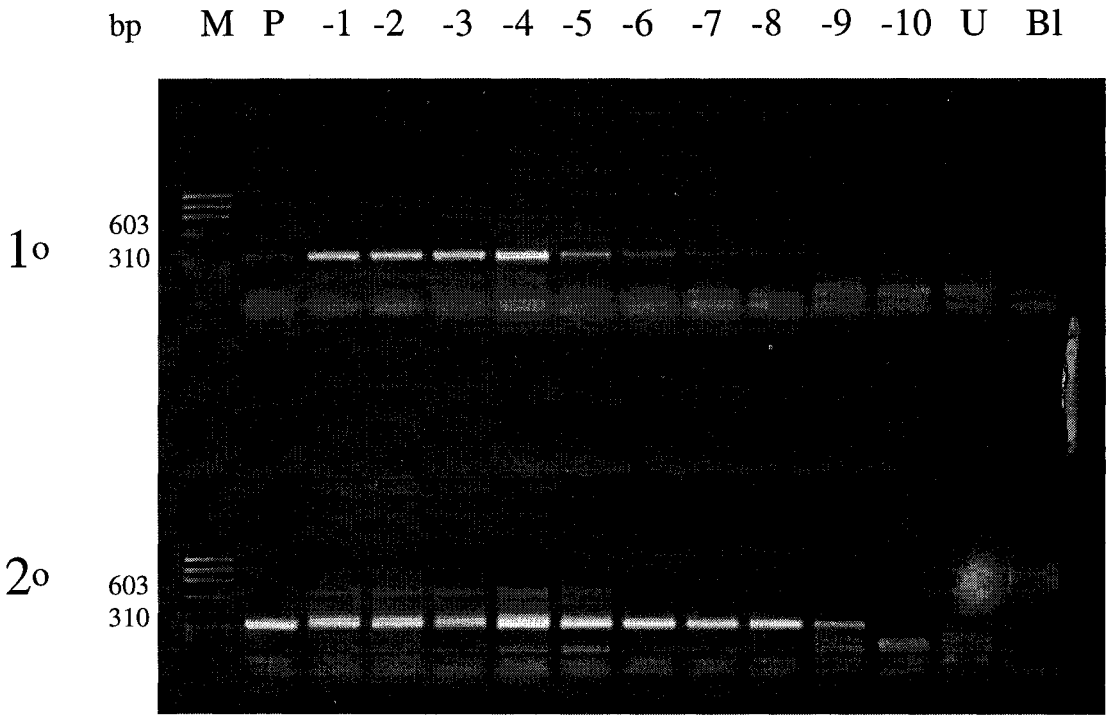


Figure 8. Comparison of sensitivity between a single round of amplification (1o) and semi-nested amplification (2o). PCR was performed with the LTR-specific primers on CEF infected with various subgroups of exogenous ALV. Only the RAV-2 isolate results are shown. The primary amplification consistently detected viral dilutions down to 10^{-4}. A second, semi-nested amplification allowed product to be consistently visualized in viral dilutions down to 10^{-8}. Product was sporadically seen in the lower dilutions. Similar results were obtained for the subgroup-specific primers. Viral dilutions are indicated. M=molecular weight marker, PhiX173 *Hae* III; P=pRAV-2 (positive control); U=uninfected CEF; Bl=water blank.

to 10^{-2} and RAV-50 down to a dilution of 10^{-3}. Semi-nested amplification of DNA extracted from infected CEF was able to detect provirus in dilutions of 10^{-8} for RAV-1, RAV-2 and RAV-50. RAV-49 was detected down to a dilution of 10^{-6}. Primary amplification was similar or slightly more sensitive than p27 detection whereas the use of a second round of PCR increased the sensitivity at least 10^{4}-fold when compared to ELISA detection. RAV-49 consistently gave poorer results for all methods of detection. Sequencing of the LTR product of RAV-49 did not indicate any major alterations in the LTR sequence that might cause less efficient amplification. The most reasonable explanation is that there were less infectious particles in the RAV-49 stock compared to the other three viral isolates. The exact number of infectious particles in a given stock is difficult to determine for ALV since they do not lyse cells, nor do they cause any cytopathic effects when cultured (Payne, 1987).

Table 2. Comparative sensitivities between ELISA detection of p27 in cell culture supernatant, primary amplification using exogenous-specific LTR primers and semi-nested amplification using exogenous-specific LTR primers. CEF were inoculated with ALV isolates of subgroups A (RAV-1), B (RAV-2), C (RAV-49) and D (RAV-50) in 10-fold serial dilutions down to 10^{-10}.

	Viral dilution down to which ALV was detected			
Detection method	RAV-1	RAV-2	RAV-49	RAV-50
ELISA-gsa[a]	10^{-4}	10^{-3}	10^{-2}	10^{-3}
primary PCR[b,d]	10^{-4}	10^{-4}	10^{-3}	10^{-5}
semi-nested PCR[c,d]	10^{-8}	10^{-8}	10^{-6}	10^{-8}

[a] Cells were freeze-thawed three times and supernatant was collected and tested for p27-gsa.

[b] DNA extracted from cells was amplified using exogenous-specific LTR primers: LTR1 and LTR2A.

[c] A 2 µl aliquot was taken from the primary amplification and amplified using LTR primers: LTR1 and LTR2B.

[d] All PCR products were visualized on ethidium bromide-stained gels.

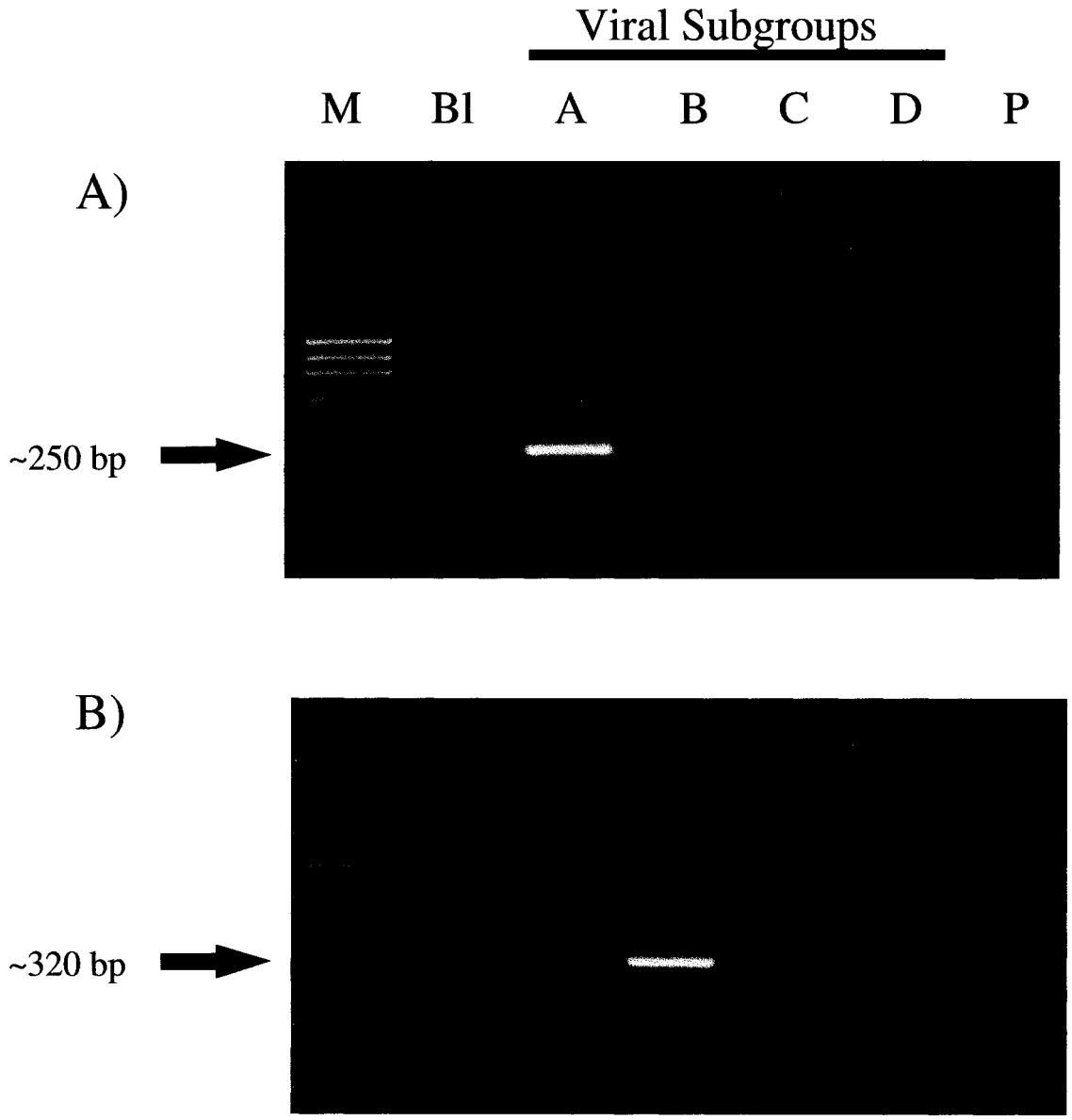


Figure 9. A) Amplification of the four exogenous ALV subgroups (A, B, C and D) with subgroup A-specific primers. M=molecular weight marker, PhiX174; Bl=water blank; A=RAV-1; B=RAV-2; C= RAV-49; D=RAV-50 and P=pRAV2. Only the subgroup A isolate produces a product. B) Amplification with the subgroup B-specific primers. As expected, the RAV-2 isolate and the cloned DNA of RAV-2 produce a visible product.

Subgroup A and subgroup B detection in infected CEF.

Figure 9 shows the specificity of the primers targeted to the *env* region of either subgroup A or B. Non-specific amplification was not observed in CEF infected with viruses of other subgroups. The sensitivity of the subgroup A and B primers were identical to those obtained for the LTR-specific primers on RAV-1 and RAV-2, subgroups A and B, respectively. Sequencing of the products verified their origin (results not shown).

Source of DNA for ALV detection.

Three different tissue types were tested to determine the best source of DNA for detection of ALV in infected birds: feather pulp, peripheral leukocytes and whole blood. It was necessary to choose a site that would not be too traumatic to the birds and relatively simple to collect, especially when sampling large numbers.

Electron microscopy had previously shown feather pulp to be a site in which viral particles were observed in intercellular spaces. Immunofluorescent detection of gsa had also shown p27 to be present in the basal epidermal cells of the developing feather (Spencer *et al*, 1984). From this information, and the fact that feather samples are relatively easy to obtain from birds, feathers that had not yet dried and still contained feather pulp were collected from birds that had tested positive for ALV by ELISA detection of p27 in their albumen.

Semi-nested PCR detection of DNA extracted from feather pulp was not reliable. Although some of the PCR reactions performed on the same feather pulp sample would occasionally be positive, it was obvious that either the cells of the pulp did not contain large

numbers of integrated provirus or that the DNA was degraded to such an extent that PCR on these samples was no longer feasible. A look at the condition of the DNA extracted from feather pulp samples did show more extensive degradation when compared to DNA extracted from tissue with less connective tissue such as blood (results not shown) although the DNA was still of sufficient size that PCR should have worked if there were sufficient numbers of integrated provirus. More gentle methods of extraction that would have limited the amount of degradation of the DNA from feather pulp were not attempted since it would have been too time consuming for the screening of large numbers of birds.

Since the most common target cells for transformation by ALV are immature B lymphocytes in the bursa of Fabricius (Purchase and Burmester, 1978), it seemed logical to next isolate DNA from peripheral blood leukocytes (PBML), on the order of which 30% are B lymphocytes (Albini and Wick, 1974; Sturkie and Griminger, 1986). Unlike mammals, approximately half of the buffy coat from avians is comprised of thrombocytes which are functionally analogous to mammalian platelets (Lucas and Jamroz, 1961). Thrombocytes are derived from the same cell lineage as erythrocytes (Sturkie and Griminger, 1986). This essentially reduces the percentage of B lymphocytes in avian buffy coat to 15%. The thymus appears to be resistant to ALV proviral integration (Ewert and De Boer, 1988; Baba and Humphries, 1986) such that the T lymphocytes in the buffy coat (approximately 35% of the total buffy coat volume) would not be expected to carry large numbers of integrated ALV. PCR detection of ALV, using the LTR-specific primers on DNA extracted from buffy coat samples was successful and gave similar results to those obtained on DNA extracted from whole blood (see below).

Based on the above results, it was thought that enrichment for leukocytes from whole blood would be the simplest means of obtaining DNA containing large enough numbers of proviral inserts for PCR detection of ALV to be reliable; until the results of two groups (Baba and Humphries, 1986; Ewert and De Boer, 1988) were uncovered. From their results, it appeared that the tissues or cell types with the largest number of integrated proviruses were in fact the bone marrow and erythrocytes. The bone marrow is the site of erythrocyte development and maturation (Janeway and Travers, 1994). Upon DNA extraction from whole blood of infected birds that had tested positive for the LTR of exogenous origin in leukocytes, it was evident that red blood cells did in fact carry integrated provirus. Figure 10 depicts representative results when both LTR-specific and subgroup A-specific primers were used on DNA extracted from whole blood samples of infected birds.

PCR vs. ELISA-based detection.

Testing the primers on cells infected with laboratory ALV strains was a necessary preliminary step but is not sufficient in showing the usefulness of the primers in detecting natural isolates. To begin testing the designed primers on naturally infected flocks, 144 birds of a flock comprised of strains 07 and 08, in which ALV eradication has not been successful using traditional methods (Spencer and Fairfull, 1993), were tested with all of the designed exogenous-specific primers as well as for gsa in albumen and antibodies to subgroups A or B in their serum. Sampling for all tests was done on the same day. Table 3 summarizes the results.

Of 144 birds tested, 52 (36.1%) were negative with all four tests (p27 in albumen and antibodies in serum by ELISA, exogenous LTR and exogenous envelope regions by PCR). 4.2%

(n=6) were positive for antibody in their serum yet had no evidence of gsa or proviral integration. Forty two (29.2%) were only positive with the LTR-specific primers; there was no evidence of gsa, antibody or either subgroup A or B envelope genes. Fourteen of the birds (9.7%) were both

Table 3. Detection of gsa (p27) in albumen, antibodies (Ab) to ALV of subgroups A or B in serum, exogenous LTR of provirus, and exogenous gp85 of either subgroups A or B in a sample (n=144, mature females only) of a flock of birds resistant to ALV eradication.

Group	Profile of Test results[a,b]	% of birds (n/144)
1	gsa-, Ab-, LTR-, *env*A-[c]	36.1 (n=52)
2	gsa-, Ab+, LTR-, *env*A-[c]	4.2 (n=6)
3	gsa-, Ab-, LTR+, *env*A-[c]	29.2 (n=42)
4	gsa-, Ab+, LTR+, *env*A-[c]	9.7 (n=14)
5	gsa+, Ab-, LTR-, *env*A+[d]	0.7 (n=1)
6	gsa-, Ab+, LTR-, *env*A+[d]	2.1 (n=3)
7	gsa-, Ab-, LTR+, *env*A+[d]	5.6 (n=8)
8	gsa+, Ab+, LTR-, *env*A+[d]	0.7 (n=1)
9	gsa+, Ab-, LTR+, *env*A+[d]	2.1 (n=3)
10	gsa-, Ab+, LTR+, *env*A+[d]	5.6 (n=8)
11	gsa+, Ab+, LTR+, *env*A+[d]	4.2 (n=6)

[a] None of the birds tested positive with subgroup B primers.
[b] χ^2 results: Ab vs. gsa, P = 0.004; Ab vs. *env*, P = 0.012; gsa vs. *env*, P = 0.075.
[c] Birds that should be maintained.
[d] Birds that should be culled.

antibody positive and LTR positive. One of the birds (0.7%) had detectable levels of p27 in the albumen and also had a positive result for the subgroup A-gp85 protein. This bird was not positive for either antibody or LTR. Three of the birds (2.1%) were antibody positive and subgroup A positive. Another 5.6% (n=8) of the birds contained both exogenous LTR and

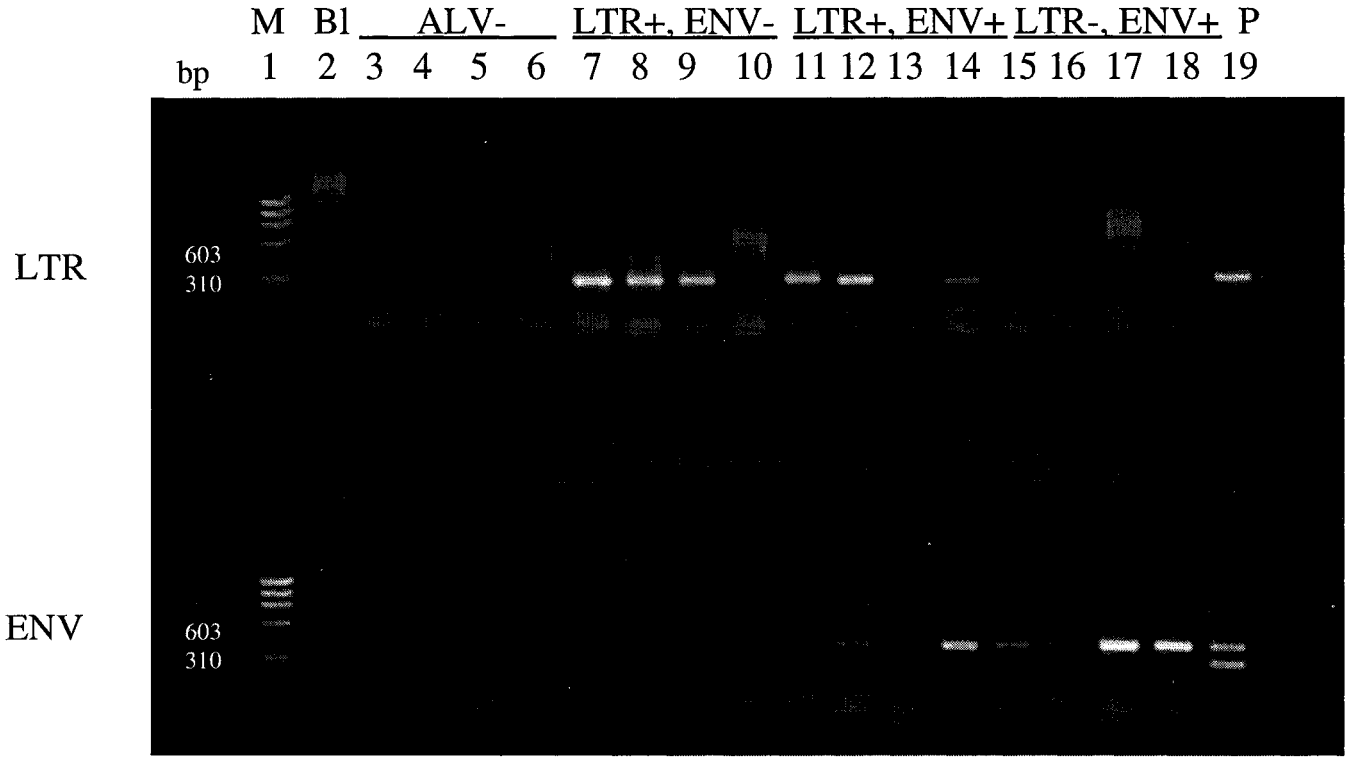


Figure 10. Representative samples of PCR results obtained when strains 07 and 08 birds were tested with both the exogenous, LTR-specific primers (top row) and the subgroup-specific primers (bottom row). Sample results are duplicates of eight different birds. The one bird that did not test positive with the *env* primers in duplicate is included (see row two, well numbers 13 and 14). The results of many LTR amplifications were +/- when done in duplicate. This leads us to believe that the LTR region is highly variable between proviruses within the same infected individual. The *env* amplification was repeatable within the same sample except for the one noted exception. M=molecular weight marker, PhiX174 *Hae* III; Bl=water blank; P=CEF infected with RAV-1 (subgroup A). The extra band seen in the *env* amplification of P appears to be due to the high copy numbers of RAV-1 in the experimentally infected cells. This band only appeared in the higher viral dilutions.

subgroup A envelope regions yet they were not detected by either conventional ALV testing method. One bird (0.7%) was positive with all tests but the LTR. Three (2.1%) were positive for all except antibody. Another 5.6% (n=8) were negative for gsa but positive by all other testing procedures. Only six (4.2%) were positive with all four testing methods. All samples were tested in duplicate for both LTR and subgroup-specific amplifications. The LTR primers did not consistently yield a product when used on an apparently infected bird. A bird that yielded a +/- result when amplified twice with the LTR primers gave both duplicate samples positive (+/+) when amplified with the subgroup-specific primers; there was only one sample which gave a +/- result with both the LTR and the *env* primers (see Figure 10). The use of DMSO or glycerol in the primary reaction to enhance LTR amplification (Smith *et al*, 1990), did not resolve this discrepancy. Changing the PCR cycling profile also did not change the LTR results' outcome.

The samples which tested positive by subgroup A-specific amplification and did not read positive ($\leq$0.2) for gsa in albumen, did not have significantly higher ELISA readings (student's t test) than the samples that tested negative by all four testing methods.

Concordance of test results.

Table 4 summarizes the concordance of the different test results in pair-wise comparisons between gsa detection, antibody detection and PCR detection with subgroup A-specific primers. Comparing p27 detection in albumen to subgroup-specific PCR detection, 86.8% of the samples were concordant, both testing methods indicated that the bird was either negative or positive for ALV. Nineteen of the birds (63.3% of birds positive with either PCR or gsa) were positive by PCR only. None of the birds were positive for gsa only. Of the positive birds for either antigen

Table 4. Pair-wise comparisons of p27 (gsa), antibody (subgroups A and B) and PCR (gp85-directed) detection of exogenous avian leukosis virus (ALV) in 144 birds sampled from strains 07 and 08. There have been problems eradicating ALV from these strains using protein-based testing methods such as p27 and antibody detection. All samples (egg albumen, serum and whole blood) were collected on the same day.

				Of the positives			
Test Comparison	Number of samples	% positive[1]	%Concordant (- & +)	% Concordant (+ only)	% Antigen +ve only	% Antibody +ve only	% PCR +ve only
Ag vs Ab	144	29.2 (n=42)	75.7 (n=102+7)	16.7 (n=7)	9.5 (n=4)	73.8 (n=31)	NA
PCR vs Ab	144	34.7 (n=50)	77.8 (n=94+18)	36.0 (n=18)	NA	40.0 (n=20)	24.0 (n=12)
PCR vs Ag	144	20.8 (n=30)	86.8 (n=114+11)	36.7 (n=11)	0 (n=0)	NA	63.3 (n=19)

Ag = p27 group specific antigen detection by ELISA in egg albumen
Ab = antibodies against subgroups A or B detected by ELISA in serum
PCR = polymerase chain reaction detection of exogenous subgroup A gp85 ; subgroup B was not detected
NA = not applicable
[1] Samples tested positive by either method.

detection or PCR detection (n=30), 36.7 % (n=11) were positive by both methods of testing.

Comparing antibody detection in serum to subgroup-specific amplification, 77.8% of the samples gave identical results with both testing methods; 8.3% of the birds were positive by PCR amplification only and 13.9% were positive for antibody only. Of the positive birds by either antibody detection or PCR detection (n=50), 36 % (n=18) were positive by both methods of detection.

When a comparison was made between antibody and antigen detection, 75.7 % of the samples were concordant and were either positive or negative with both testing methods, 2.8 % (n=4) were positive by antigen detection only and 21.5 % (n=31) were positive by antibody detection only. Of the positive birds by either antigen or antibody detection (n=42), only 16.7 % (n=7) were positive with both testing methods (see also calculated χ^2 results for test comparisons, Table 3). Similar discrepancies have been observed in results from other groups and in our own previous experiments (Fadly *et al*, 1981; Gavora *et al*, 1995a).

Repeatability of LTR- and *env*- specific PCR tests.

A total of 85 birds were bled twice with an interval of two weeks between bleeds. The samples were then individually processed and tested for both exogenous LTR and *env* and the results compared to determine repeatability. The subgroup A-specific results were 100% repeatable whereas out of 85 samples tested and retested with the LTR, five were positive on the first sampling date and negative on the second sampling date. All samples that were negative on the first sampling date remained negative for both primer sets.

Confirmation of exogenous ALV amplification.

A number of PCR products from various samples were sequenced to confirm that amplification was due to the presence of exogenous ALV and not spurious amplification. The LTR product (~ 300 bp) of the positive control (pRAV-2) was found to be 100% homologous to the RAV-2 published sequence (Bizub *et al*, 1984). The RAV-1 isolate had an LTR sequence similar to that of the RAV-2 published sequence with as many as six individual base substitutions and one base insertion. Unless otherwise stated, only one strand was sequenced so that all observed substitutions cannot be guaranteed due to the *Taq* polymerase incorporation error rate. Sequencing was performed to confirm the exogenous ALV origin of PCR amplification, not to compare sequenced products. The RAV-2 isolate's LTR was quite dissimilar to the published sequence with ten base substitutions, two single-base insertions and an extra 11-base copy of a tandem repeat (5'-GTGGTATGATC'-3') which was found only twice in the published sequence (Bizub *et al*, 1984). The RAV-49 isolate, representing subgroup C was also very similar to the RAV-2 published sequence with as many as seven base substitutions and one base insertion. When the LTR of RAV-49 was compared to the published sequence of PrRSV-C (Schwartz *et al*, 1983), there were 15 base substitutions, one 1-base insertion and an extra copy of the 11-base tandem repeat mentioned above. There is no published LTR sequence associated with a subgroup D isolate. The RAV-50 isolate LTR, when compared to the RAV-2 sequence, had seven base substitutions, a 1-base insertion not found in any other isolates and an extra copy of the 11-base tandem repeat.

Sequencing of the envelope portions amplified with subgroup A-specific primers indicated,

in all cases, that the variable regions were almost identical to the variable regions of subgroup A published sequences (Bova *et al*, 1988). A few single base mismatches were observed in both the conserved and variable regions. In all cases, the identical mismatch was identified in one or more different subgroups for which sequences have been published (subgroup B, Bova *et al*, 1986; subgroup C, Schwartz *et al*, 1983; subgroup D, Bova *et al*, 1988), raising the possibility that the variability at these bases is not a subgroup characteristic.

***Ev* gene amplification.**

In a total of 229 different birds tested with exogenous LTR primers, five samples produced a small product that was not the size expected for exogenous LTR (approximately 250 bp compared to 300 bp for exogenous amplified product; see Figure 11a). The smaller product was isolated, purified and sequenced with both the upstream and downstream primers to determine its source. Sequencing results revealed it to be amplification of the LTR of an endogenous viral (*ev*) element (see Figure 6b for examples of *ev* LTR). Figure 11b indicates the site of priming of LTR1 in relation to the *ev* gene. The last four bases of LTR1 prime to the first four bases of the U3 sequence of all *ev* genes. As previously stated, both LTR2A and LTR2B have high homology with existing *ev* elements.

Theoretically, four bases of annealing, even though they are at the 3' end of the primer, will not stabilize the primer enough at the temperatures at which PCR is performed to be able to form a product. A more plausible explanation for amplification of an *ev* gene to occur is that the upstream flanking region, the 16 bases immediately preceding the beginning of the 5'-U3, have some homology with the remainder of the LTR1 primer. The upstream region of the 3'-U3, which

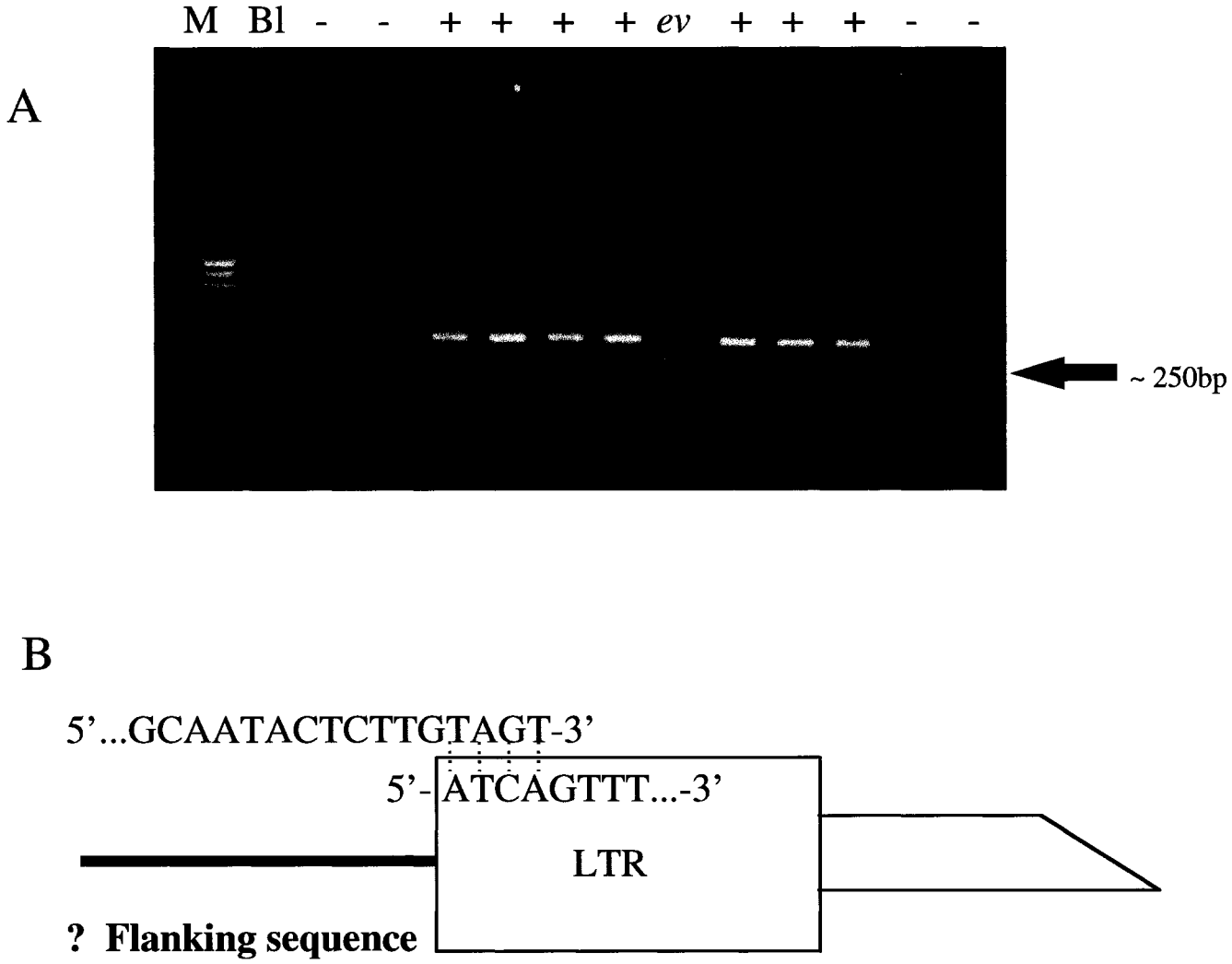


Figure 11. A) While using the LTR-specific primers, occasionally a smaller product would appear. See sample marked *ev*. Sequencing of this product revealed it to be amplification of the LTR of an endogenous viral element (*ev*) closely related to exogenous ALV. B) The upstream primer (LTR1) has four 3' terminal bases which are homologous to the first four bases of all *ev* LTR. *Ev* genes whose flanking sequences are known, do not have high homology with the remainder of the LTR1 primer. This raises the possibility that four bases of homology are enough to occasionally form a product when using nested PCR. The two downstream primers used in semi-nested amplification, are highly homologous to both exogenous and endogenous ALV. M= molecular weight marker; Bl=water control; - = LTR-; + = exogenous LTR+.

is part of the ALV genome, is known and does not show homology to the LTR1 sequence (Hughes, 1982). This would also explain bird-to-bird variation. Of the known upstream flanking sequences, *ev*2 and *ev*3 are the two *ev* genes which have the highest similarity between their upstream flanking sequences and LTR1 but the calculated melting temperatue should still be below 30°C. In theory, LTR1 should not anneal to these *ev* genes with the PCR conditions used. *Ev*2 has four homologous, consecutive base pairs containing three GC interactions which are also very close to the 3'-end giving greater stability to the 3' end of the primer. *Ev*3 has homology to the first six bases of LTR1. This greater homology for the 3'-end of LTR1 might explain product formation and all samples that produced the endogenous-derived product were tested for the presence of both *ev*2 and *ev*3 (see Benkel *et al*, 1993 for *ev*3 PCR diagnostics; test for *ev*2 is unpublished). None of the birds tested positive for either *ev*2 or *ev*3 using the specific PCR tests. Birds were also tested for *ev*7, 9 (complete tests for *ev*7 and *ev*9 are unpublished) and 15 (Benkel and Smith, 1993) whose upstream flanking sequences have even less homology to LTR1 than those of *ev*2 and 3. All endogenous-specific PCR testing gave negative results when used on the samples that had produced the smaller band with the exogenous LTR primers.

DISCUSSION

Latency.

The inability of protein-based methods of detection to eradicate ALV from commercial flocks requires that a more sensitive and specific test be devised. The ELISA tests, designed to detect either viral protein products or antibodies raised against ALV, can give either false positives (ie. p27 production from *ev*) or are not sensitive enough to detect low levels of gsa and antibody, resulting in false negatives. They are also not able to detect latent ALV infection. Latent viral infections have only been documented for the subgroup E, endogenous ALV loci (Rovigatti and Astrin, 1983; Smith, 1986) but there is no reason why it should not occur for exogenous ALV infection. Latent infection has been confirmed for HIV (Garbuglia *et al*, 1995) and herpes simplex virus infection (Perng *et al*, 1994) and has been associated with many other oncogenic viruses (Fredricks and Relman, 1996). A bird that is latently infected carries viral genetic material but the genetic material is not transcribed and viral protein products are not produced. This may be due to the cell type in which a provirus has integrated or the site of integration of the provirus. Many areas of cellular genomic material are not transcriptionally active at a given time (Winslow *et al*, 1993). If the proviral genome is not transcribed and translated, an immune response will also not be raised since there are no foreign, viral proteins present that can be recognized by the immune system. If the latency can be overcome, the host will become viremic and go on to transmit the virus to other individuals before a neutralizing immune response is raised. If latency is shown to play a role in ALV infection, continuous testing of a flock with the ELISA-based methods will be required to detect infected birds since many will

be missed during any given testing period.

Latency in ALV infection still needs to be experimentally demonstrated. Birds that are negative for both antibody and antigen but are positive with both the LTR and *env*-specific PCR tests (see Table 3) should be identified and isolated. The birds, or tissue culture samples obtained from birds carrying proviral inserts, would then be stressed (ie. UV irradiation or other environmental or chemical stressors) to promote induction of ALV replication. If it is shown that ALV infection can be latent, nucleic-acid based detection of proviral inserts will be the only way to eradicate ALV from infected flocks. A test based on the polymerase chain reaction will likely be the simplest and most cost-effective means of ALV proviral detection.

The presence of endogenous retroviral elements in the chicken genome, closely related to the exogenous group of ALV retroviruses, poses some difficulty in designing PCR primers that will differentiate between endogenous and exogenous viral genomes. The high mutation rate generated by the mode of replication of retroviruses (O'Rear and Temin, 1982; Shimotohno and Temin, 1982; Darlix and Spahr, 1983; Gojobori and Yokoyama, 1985; Dorner *et al*, 1985; Steinhauer and Holland, 1987) also introduces more difficulties in choosing a proper target for reliable PCR amplification. The target must be highly conserved within the subgroups one is interested in identifying. A close look at both exogenous and endogenous ALV sequences shows that there are only two regions of the ALV genome which can be targeted to distinguish between the two. These regions are found in either the U3 region of the LTR (see Figure 6a) or the region of the *env* gene that encodes the surface glycoprotein, gp85 (see Figure 7). Primers targeting both of these regions were used and results compared to the traditional ALV testing methods.

LTR region is not subgroup-specific.

Due to the relatively small number of ALV/ASV isolates that have been sequenced, it has been largely assumed that a specific LTR sequence will always be associated with a specific viral isolate or a given subgroup. There is no biological reason why this should be so. When the LTR products of the laboratory isolates were sequenced and analyzed it was shown that the RAV-1 isolate had an LTR that was similar to the published RAV-2 sequence (Bizub *et al*, 1984) whereas the RAV-2 isolate's LTR was quite different from the published sequence (see results). In the infected flock, it was again shown that a RAV-2-like LTR was associated with a subgroup A infection. Variation in the LTR between different RAV-2 isolates has been documented by Westaway and colleagues (1984). They found 20 out of 350 positions to vary between different sequences of RAV-2-derived LTR.

Variability within the U3 region of exogenous LTR.

Many of the published sequences of exogenous ALV contain a stretch of bases (approximately 35 bp) at the 5'-terminus of the U3 region of the LTR which has been found associated with subgroups A, B and C and was shown, during this project, to be present in a subgroup D isolate (RAV-50, see Table 2). This region differs in the endogenous ALV-related LTR (see Figure 6a and 6b). This same stretch was also shown to vary between exogenous isolates, particularly in cases of acute retroviruses (Shibuya and Hanafusa, 1982). The remainder of the U3 region is either too variable between exogenous subgroups or has high homology to the endogenous subgroup and cannot be used to reliably distinguish between exogenous and endogenous ALV (see Figure 6a).

The primer that was chosen to differentiate between endogenous and exogenous ALV proviruses (LTR1, see Figure 6a) targets a portion of the short conserved stretch at the 5'-terminus of the LTR of exogenous ALV. This site is the only region of the ALV genome which can be used to detect all four exogenous subgroups without amplifying *ev* genes. There is no published sequence of an LTR associated with a subgroup D virus but the LTR primers were as efficient in detecting RAV-50, a subgroup D laboratory isolate, as they were in detecting the other three subgroups (see Table 2). The downstream primers were chosen for their conserved nature between all subgroups, the stability of primer binding and the size of the resulting products.

Although the LTR is required for viral replication, there are relatively few regions which are known to interact directly with viral or host proteins. Regions that are an absolute requirement are conserved within both endogenous and exogenous LTR (ie. R and U5). This limits the constraints on sequence variability in the U3 region (Cullen *et al*, 1985; Roth *et al*, 1989). From our results, this variation appears to occur at a relatively high frequency. Detection of exogenous ALV with LTR-specific primers did not yield consistent results when samples were tested in duplicate (see Figure 10). This was in contrast to the subgroup A-specific primers where only one sample did not produce the same result in duplicate reactions (see Figure 10; the sample was not retested to determine if the negative result was due to a failed reaction). This can be explained if only a proportion of the infected cells contain LTR regions that have the LTR1 target sequence and the remaining proviral integrants encode altered U3 regions that lack the target sequence. Many of the acute sarcoma viruses have such LTR (Rushlow *et al*, 1982; Kitamura *et al*, 1982; Shibuya and Hanafusa, 1982; Nunn *et al*, 1984; Kan *et al*, 1984; Neckameyer and

Wang, 1985; see Figure 6a for an example of an acute viral LTR). It is proposed that the acute retrovirus-like LTR make up a proportion of the LTR of any infective cycle of ALV. When some of the acute retroviruses were isolated, it was by chance that they contained variants of the LTR different than those typically associated with non-oncogenic ALV. The exact mechanisms inducing this variation and the frequency of occurrence are not known. Out of 30 birds that tested positive with the subgroup A-specific primers, 20% (n=5) of them did not test positive with the LTR primers. If the viruses that initially infected these birds had LTR variants, the likelihood of a revertant occurring might be too low to detect with the LTR-specific primers. The likelihood of the opposite occurring, a bird being infected with a virus that has an LTR homologous to the LTR1 primer and producing LTR variants which would not be detected by the primer is conceptually more likely.

Many labs are currently trying to develop PCR-based detection methods for ALV but very few publications have resulted. Shuman and Heath (1991) described the use of LTR-targeted primers in the detection of laboratory ALV isolates in cells and experimentally infected birds but no further results have unfolded. From our results, basing ALV detection solely on the LTR region of the genome does not appear to be the solution. Other labs may simply not have given up on targeting the LTR due to its presumed ability to detect all four major subgroups involved in infection of chicken flocks.

Results suggest that an ALV-infected individual contains a number of variants of ALV proviruses or quasispecies (Eigen *et al*, 1981; Steinhauer and Holland, 1987). The initial infecting virus(es) would act as the master copy, producing more viruses that would spread throughout the

host. The high error rate and recombination rate of retroviruses would produce a number of different proviral inserts, many of which would be defective, nonproductive and/or have altered LTR. Only the proviruses which maintained an active LTR with all necessary viral genes required for replication would be able to produce fully infectious virus. All other insertions would be either dead-end integrations or would require a helper-virus to supply missing viral products in *trans* (Scheele and Hanafusa, 1971).

ALV *in vivo*.

If the above steps occur during retroviral replication, results of the antigen, antibody, PCR comparison can be interpreted (see Table 3). Eleven different test profiles or groups were obtained when 144 birds were tested. These birds were from Agriculture and Agri-food Canada strains 07 and 08; two strains in which it has been difficult to lower the incidence of ALV infection. What follows is an interpretation of what is occurring *in vivo* in an ALV-infected flock:

Group 1 (see Table 3): Birds that are negative with all four tests (36.1% of 144 birds) have either never been infected or they were able to raise an effective immune response early on in infection and cleared all infected hematopoietic cells containing exogenous proviral inserts. The probability of a bird being infected without having some hematopoietic cells infected is considered to be low (Ewert and De Boer, 1988).

Group 2: The birds which are solely antibody positive (4.2%), are either antibody false-positives or are more likely efficiently fighting off an ALV infection. Antibody testing is not considered as reliable as antigen testing in determining viraemia in a bird (Kulenkamp, pers.

comm.). Culling birds which are exclusively antibody-positive may be an objectionable practice since these birds appear to have cleared the virus. It is proposed that birds such as these will become negative with all four tests once antibody titers decrease unless the birds continue to be exposed to ALV products which stimulate an antibody response. As long as they do not show proviral integration, these birds should be maintained.

Group 3: The birds which are LTR-positive (29.2%) presumably carry only defective proviral integrants and would not be considered infectious unless the birds became superinfected with a helper virus that would supply the missing genes in *trans* (Scheele and Hanafusa, 1971). This helper virus would then be detected with subgroup-specific primers. PCR testing would not establish whether only a solitary LTR remains or whether the provirus still has *gag* and *pol* portions of its genome due to the homology of the *gag* and *pol* genes of exogenous and endogenous ALV. Although considered unlikely, the other possibility is that these birds are infected with a different subgroup other than subgroups A or B. None of the birds tested positive with the subgroup B-specific primers. Subgroups C and D have never been detected in North American flocks (Morgan, 1973; Sandelin and Estola, 1974).

It would be interesting to see whether or not hematopoietic stem cells in the bone marrow of LTR-positive birds are infected or if it is more mature erythrocytes which contain provirus. ALV requires a target cell which is actively replicating to both integrate and begin viral replication (Temin, 1967; Humphries and Glover, 1981). Although avians have nucleated, mature erythrocytes, they are not targets of infection for ALV. The presence of a proviral insert in the genome of a mature erythrocyte will not allow viral particles to be produced since the nuclei of

these cells are inactive with no transcription of gene products occurring. Only cells in the less mature stages of erythrocyte development and the stem cells can be targets for ALV infection and allow viral replication to proceed since the cells in these stages of erythrocyte development still have the ability to actively replicate (Ewert and De Boer, 1988).

It is proposed that if large numbers of the hematopoietic stem cells in the bone marrow are infected, the birds will never lose their positive LTR status. If more mature erythrocytes are infected, the birds will eventually become LTR-negative provided that superinfection does not occur. The average life span of avian erythrocytes ranges between 28 and 35 days, this is much shorter than the average life span of human erythrocytes whose life span ranges between 50 and 60 days. This difference is due to the higher body temperature of avians (42°C) and the higher metabolic rate (Sturkie and Griminger, 1986). The LTR-positive birds could be tested again, after 35 days, to determine their LTR status. If they were still positive, the stem cells would be considered infected if superinfection had not occurred (ie. gp85 positive). If the more mature erythrocytes were carrying the proviral integrants, the cells carrying proviruses would have had time to reach the end of their life cycle and died, leaving birds which are LTR negative (ie. group 1 birds). The repeatability results whereby five birds tested positive for exogenous LTR on the first bleed and were negative on the second bleed, although not conclusive, indicates that ALV clearance is possible.

Group 4: The antibody positive, LTR positive birds (9.7%) can be explained by combining the last two scenarios. The bird is effectively fighting off an ALV infection and all that remains are defective proviruses. The antibody titers should disappear once all cells carrying

proviral integrants that produce foreign protein are eliminated. As with other antibody profiles, antibodies against ALV will remain in the serum some time after clearing an infection (Janeway and Travers, 1994). The length of time depends on the extent and type of infection and the memory B cells which are involved.

All of the above birds are subgroup A-gp85-negative. It would be ill-advised to remove any of these birds from the flock since they appear to have either never been infected or they have been able to clear replication-competent proviruses from their system. These birds make up 79.2% of the birds sampled.

The remaining 20.8% of the birds (see Table 3) should be removed from the flock since they tested positive for the subgroup A-gp85 region and in most cases also tested positive for either viral antigen or antibody as well as the LTR.

Group 5: One bird (0.7%) was positive for both group-specific antigen and the subgroup A-specific PCR test but negative for both antibody and LTR. This is when it became apparent that the LTR of exogenous ALV might be more variable than is desired for it to be solely relied upon as a target for PCR detection. The bird consistently tested positive for the gp85 region of *env* yet not one sample tested positive for the LTR. The fact that all samples tested positive with the *env*-specific primers means that a large proportion of the cells contain ALV provirus. The presence of p27 in the albumen indicates that provirus was transcriptionally active and that this bird was more than likely infectious. The lack of an apparent immune response would necessitate culling of this bird since it would be a source of infection for other flock mates. If the bird was

congenitally infected, an immune response would never be raised (Rubin *et al*, 1962).

The virus which originally infected this bird was likely an ALV variant that carried an altered LTR sequence that was not recognized by the LTR1 primer. No detectable revertants, back to the more common RAV-like LTR, were ever detected following repeated testing.

Group 6: Three birds (2.1%) were antibody-positive, gp85-positive. These birds would have been missed if only albumen testing had been performed. The birds also appeared to carry variants of the LTR which could not be detected with the LTR-specific primers. These birds may be able to eventually clear ALV but until then, they must be considered as infectious.

Group 7: Eight birds (5.6%) were both LTR and gp85-positive without being positive for either antigen or antibody. These are the birds that either harbor a latent ALV infection or the birds have such low levels of virus that immune responses are not elicited and ELISA is not able to detect exogenous viral proteins in albumen. Birds like these should be examined further to see if they ever do produce detectable viral protein products and/or antibody and monitored closely to see if they ever become viremic. If this is confirmed, the usefulness of the subgroup-specific primers will be greatly enhanced.

Groups 8-11: The birds in the four remaining test profile groups (12.6%; n=18) were all positive for subgroup A, gp85 and were also positive for either antigen (n=3) or antibody (n=8). Seven were positive with all three test types. It is the presence of gp85 which should worry the industry. Even if there are regions of *gag* or *pol* which are missing in the provirus, the presence of a full transcript of the *env* gene could be used by other defective ALV in *trans* (Scheele and

Hanafusa, 1971).

More epidemiological experiments and testing of the PCR primers is required to confirm the stages of ALV infection outlined above. Although latency seems a rational means of avoiding the immune system, it has not been demonstrated with ALV. Latency would also explain the sporadic outbreaks frequently seen in flocks.

The quasispecies distribution (Steinhauer and Holland, 1987) in an infected bird also appears consistent in explaining ALV infection. The variation that results allows new genetic combinations to be tested as well as aid the virus in evading the immune system. HIV is one of the best examples of the variation that can be found in a given retroviral population (Cheynier *et al*, 1993).

Does non-ALV induced lymphoid leukosis occur?

In the past, cases of lymphoid leukosis have been documented where ALV was not detected and therefore attributed to a non-viral etiology (Crittenden *et al*, 1979). Crittenden and his colleagues looked for the presence of ALV in such cases by probing DNA from leukosis-like tumours with full length copies of ALV cDNA. It was later shown that a majority of tumours have proviral insertions in proximity to the c-*myc* gene that have a large proportion of the viral genome deleted and all that remains is a functional LTR (Payne *et al*, 1981; Westaway *et al*, 1984; Robinson and Gagnon, 1986). A full-length copy of ALV, like that used by Crittenden's group (1979), would not detect a solitary LTR if it were present (Benkel and Smith, 1993; pers. obs.). The DNA from the suspected tumours would have to be probed with an LTR-specific

fragment to remove all possibility of ALV etiology.

Birds housed in the FAPP building were thought to be ALV-free (Gavora *et al*, 1995a). Testing of a portion of these birds with PCR indicated that exogenous ALV was present. The fact that three birds tested positive with ELISA detection of p27 in albumen also indicated that ALV was present in the FAPP facilities. Past cases of lymphoid leukosis-like lesions had been attributed to a non-ALV etiology. With the above results, this supposition has to be reexamined. The DNA from tumours of birds housed in the FAPP facilities that have lymphoid leukosis-like malignancies should be probed with an LTR-specific probe to determine whether ALV was at the basis of the observed leukosis. This would remove all doubt of a non-ALV etiology.

Applications.

The use of PCR to determine ALV status of a flock will ultimately depend on the cost of testing. ELISA-based testing is relatively cheap and simple to perform and has been used for a number of years. PCR completely supplanting ELISA detection seems unlikely in the near future especially if a simple alternative to nested amplification is not devised. Initially, PCR detection of ALV will probably be used as a confirmatory test for indeterminate or questionable ELISA readings. This practice would not have removed nearly 15% of the birds in this study that only tested positive by PCR but had negative readings with ELISA-detection of gsa in albumen.

The use of these primers to detect ALV infection in commercial flocks will require more testing prior to full implementation but one can visualize the use of the subgroup-specific primers to eliminate all potentially viremic birds. A virus must carry the *env* region of the ALV genome to

be infectious or it requires the presence of a helper virus which supplies the *env* gene products in *trans* (Scheele and Hanafusa, 1971). Since subgroup A and B ALV are the only subgroups known to cause problems in North American chicken populations, 80% of infections attributed to subgroup A and the other 20% attributed to subgroup B (Okazaki *et al*, 1982), one could envision first screening entire populations with the subgroup A-specific primers and eliminating all birds that carry the subgroup A glycoprotein. A portion of the population could be sampled with the subgroup B primers to determine whether it is present in the population. If it was present, then all remaining birds would also have to be tested. If not, continuous screening of the birds with the subgroup A primers would be conducted until there was no longer any evidence of subgroup A infection. The next generation would then be tested for subgroup A and LTR-specific primers to confirm the absence of subgroup A infection and also show that no other exogenous subgroups were present in the population. Proper and strict husbandry would also have to be maintained to keep any new sources of ALV from entering the flocks.

A flock that is known to be infected with a subgroup B virus will first have to be located and the subgroup-B specific primers tested *in vivo* since the flock in this study appeared not to harbor it (Gavora *et al*, 1995b). The possibility of other subgroups must not be overlooked. Subgroup C primers have been designed but not yet tested. Primers that are specific for subgroup D do not appear feasible. Subgroup D is closely related to subgroup B and does not contain unique regions, when compared to the subgroup B sequence, that could be reliably targeted with PCR primers (Bova *et al*, 1988). Two different PCR tests would have to be performed in order to confirm a subgroup D isolate; one test would determine that it is either a

subgroup B or D isolate and a negative result with the subgroup B-specific primers would indicate a subgroup D outbreak.

The ability of retroviruses to recombine and mutate means that a constant vigil will have to be maintained. Isolates may arise that would not be detected by currently available primers. These isolates would have to be quickly identified and sequenced so that new primers could be designed before the isolates had a chance to spread. Recombination between different retroviral types (ie. ALV and REV) has been observed by at least two groups. Smith and colleagues (1985) reported a recombinant between MC29, an acute myelocytomatosis virus, and ring-neck pheasant virus which is part of the REV group of avian retroviruses. Another group (Bai *et al*, 1995) reported a new subgroup of avian retrovirus that appears to be a recombinant between a sarcoma virus and the endogenous retroviral element, EAV. These recombination events are enhanced *in vitro*, but they also indicate that they are likely occurring *in vivo*.

Site of sampling.

The observation of viral particles in feather pulp does not mean that there are large numbers of integrated proviruses in the same tissue. Spencer and colleagues (1984) showed that there were indeed viral particles in the intracellular spaces in feather pulp but gsa was not detected in high amounts in the cells. Instead, large amounts of p27 was detected in the basal epidermal cells. Viral particles likely bud from the basal epidermal cells and migrate to the surface to be shed. The viral particles observed by Spencer *et al* (1984) were presumably viruses that were migrating and about to be shed. Since we are detecting proviral inserts and not viral particles containing viral genomic RNA, the presence of viral particles at a sampling site will not be

detected with the present method. Reverse transcriptase PCR (RT-PCR) would be required to detect the RNA retroviral genome. A bird would also have to be viremic at the time of sampling to be detected with RT-PCR. False negatives would be a problem with ALV-RNA genome detection, particularly if latent infection occurs, resulting in similar problems as those observed with ELISA-based detection.

The preferred sites of ALV transformation are the immature B lymphocytes found in the bursa of Fabricius. From this information, it was assumed that a high proportion of circulating B lymphocytes of an infected bird would carry proviral inserts. This does not appear to be the case. Both Baba and Humphries (1986) and Ewert and De Boer (1988) have shown that young (< 8 weeks of age), infected birds will have proviral DNA in peripheral lymphocyte (PBML) populations. Little, if any, proviral inserts were detected in PBML of older birds. ALV proviruses continued to be detected in lymphocytes located in the bursa as well as the bone marrow and erythrocytes. The strain 07 and 08 birds which were used for this experiment were mature hens. If the above observations are true, detection of ALV when using DNA extracted from enriched PBML was probably due to the presence of provirus in the erythrocytes of the processed samples. The method used to isolate buffy coat was quite crude and erythrocytes were still present in high numbers (≥ 50% of total cell numbers).

Specificity of PCR.

The variability of retroviral genomes persuaded us to include redundancies in the design of the primers. Primers were made to include only sequences which had been published rather than all possible sequence combinations. Depending on where the redundancies in the primers were

located, it is debatable whether they added increased sensitivity or yield to the PCR test especially following the amplification of an endogenous viral element with low homology with the LTR1 primer (see above results). Kwok *et al* (1990) have shown that single internal mismatches have no significant effect on product yield when amplifying a region of the *gag* gene of human immunodeficiency virus type 1 (HIV-1) whereas yields varied according to the mismatched base at the 3'-terminal end. It is widely assumed that a 3'-terminal mismatch will not allow amplification but, according to Kwok, amplification still occurs although at a lesser efficiency. The efficiency depends on the 3'-terminal mismatch with a thymidine mismatch allowing for greater amplification than other mismatched bases. A 3'-terminal mismatch cannot be depended on to distinguish between isolates, especially if one is using semi-nested amplification.

Amplification of *ev*.

The inability to locate a specific *ev* gene with flanking regions homologous to the LTR1 primer leaves the degree of PCR specificity unanswered. Either the *ev* gene that is causing product formation has not yet been sequenced in its flanking regions or PCR specificity is not as reliable as previously thought. Results obtained raise the possibility that four bases at the 3'-terminal end of a primer are sufficient to allow detectable product amplification provided that amplification begins in the very early cycles and the use of nested PCR yields sufficient amounts of a product to be visualized on an agarose gel. If the latter is the case, the use of PCR to accurately distinguish between variants with single-base mismatches (Charlieu, 1994) seems highly unlikely. The low incidence of the endogenous-derived LTR band and the marked difference in product size should not diminish the usefulness of the chosen primers in detecting

exogenous ALV.

For all of the advantages of PCR, it is not a perfect system; amplification of an *ev* gene with the exogenous-specific LTR primers illustrates this well. False-negatives and positives and cross contamination between samples can occur quite easily even when using the utmost care. False-negatives can be avoided by choosing areas in the target genome which are highly conserved and by using more than one set of primers to amplify different regions of the same genome sample (Clewley, 1989).

False-positive results will sometime occur in single-round amplifications but the problem is magnified when using semi-nested and nested PCR. Sources of contamination leading to false-positive results include carry-over products from previous PCR amplifications, high concentrations of cloned DNA often used for positive controls, and cross contamination between samples; the latter are an important factor when performing nested PCR. Contamination from other sources such as materials and surfaces contacted when collecting samples or during their preparation prior to amplification can also be responsible (Clewley, 1989; Yap *et al*, 1994).

Most of the above sources of contamination can be minimized, if not eliminated, by using some general precautions. In any laboratory performing PCR, the pre- and post-PCR operations should be spatially separated. Different areas and instruments should be used for nucleic acid isolation, PCR set up, amplification and product analysis. Reagents should be aliquoted into small working volumes such that if contamination of a reagent occurs, only a small volume need be discarded. When setting up PCR reactions, the use of plugged tips or positive displacement

pipettes is highly recommended to prevent any aerosol carry-over. Master mixes should be prepared whenever possible and all stock reagents should be removed from the work area prior to opening any samples containing DNA. Positive controls should have the minimum concentration of DNA to obtain a positive result; highly concentrated, positive controls can be a major source of cross-contamination. The use of containment hoods with a UV light source to cross-link any contaminating DNA can also be helpful. When performing nested PCR, the way one opens and handles the tubes when transferring amplified products from a first round sample to a second round sample is also important. The less one manipulates the tubes, the better. The use of multiple negative controls interspersed throughout the test samples can help in identifying possible problems (Clewley, 1989; Yap *et al*, 1994; Fredricks and Relman, 1996). All of the above precautions were used throughout the development of PCR detection of ALV.

Alternatives to nested amplification.

The desire of commercial companies to implement PCR technology into ALV monitoring convinced us to develop a relatively simple, quick and inexpensive method. For these reasons, nested amplification was chosen over single round amplification followed by hybridization of a complementary exogenous probe. There are currently many labs and biotech companies working on methods to increase PCR sensitivity without having to perform nested PCR or hybridization. The following are but a few examples of methods that might simplify detection of ALV with PCR without having to perform nested amplification.

Many of the techniques being developed combine PCR methodology with colorimetric detection. One such technique is known as primer specific solid-phase detection (PSSPD;

Khudyakov *et al*, 1994) whereby PCR products from a single round of amplification are captured on a solid-phase using oligonucleotide probes attached to the surface. During PCR amplification, substrates for colorimetric detection (ie. streptavidin conjugated to horse radish peroxidase) are incorporated into the PCR product. Capture of the product on a solid-phase (ie. microtiter plate wells), followed by addition of a substrate (ie. biotin), leads to a colored product which can be spectrophotometrically analyzed much as is done during ELISA. There are a number of methods being developed that vary little from the above description of PSSPD. Another method that appears to have great potential is termed branched PCR (Holland *et al*, 1991). This method involves the addition of a fluorogenic oligonucleotide probe which anneals to one of the strands of the product of PCR amplification (ie. nested primer that is tagged). A polymerase that has 5' to 3' endonucleolytic activity is used and as PCR proceeds, the fluorometric tag (ie. 6-carboxy-fluorescein) is cleaved off of the annealed probe to produce a photometric reaction that can be detected spectrophotometrically. This method requires the use of a modified thermocycler which can also detect light.

These methods are not yet widely used but once they become established, ALV monitoring using PCR will be much more amenable to large scale screening and hopefully decrease the likelihood of false positive results due to cross contamination during nested amplification.

Concluding remarks.

With all the research that has been done on ALV and the closely related sarcoma viruses, results from this project indicate that the story is not complete. Much of the earlier research

assumed much on the ALV life cycle and did not take ALV latency into consideration. It is also believed that the stability of the ALV genome was highly overestimated. From the observations and results obtained here, it appears that defective or altered proviral integrations occur at a high frequency, producing ALV quasispecies in an infected individual (Steinhauer and Holland, 1987). These possibilities must be addressed prior to proclaiming eradication of ALV from any flock. The primers that have been designed will aide in following the course of an ALV infection both in the laboratory and in infected flocks. If used properly, it is believed that PCR detection of ALV infection will have a higher degree of reliability than the more traditional monitoring methods. Testing entire populations and culling birds which carry subgroup-specific PCR products will be the next step in determining whether or not PCR detection will help in eradicating ALV from a given flock.

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