Pathogenesis of enzootic nasal tumor virus

by

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Enzootic nasal tumor virus (ENTV) is a betaretrovirus of sheep (ENTV-1) and goats (ENTV-2) associated with neoplastic transformation of epithelial cells of the ethmoid turbinate. Confirmation of the role of ENTV in the pathogenesis of enzootic nasal adenocarcinoma (ENA) has yet to be resolved due to the lack of an infectious molecular clone and inability of the virus to propagate in cell culture. Very little is known about the prevalence of ENA, particularly in North America, and only one full length sequence was available for each of ENTV-1 and ENTV-2 at the initiation of this study. Serological screening has not been used for diagnostic purposes since reports have noted a lack of antibodies to the virus capsid in animals with ENA.

In this thesis, I hypothesized that ENTV-1 is the causative agent of ENA and that envelope mediated entry is a determining factor in the tissue tropism of ENA. Ten full-length ENTV-1 provirus sequences were determined from naturally occurring ENA tumors of sheep from North America (ENTV-1NA). Overall, there was an unusually high degree of amino acid conservation among the isolates compared to other RNA viruses suggesting that ENTV-1 is under stabilizing selection and the bias towards synonymous mutations we observed in the viral
coding sequences support this hypothesis. Induction of ENA in a healthy lamb was shown after inoculation with filtered tumor homogenate, thereby demonstrating the transmissible nature of ENA. Detection of ENTV-1 antigens and virions with betaretrovirion morphology in tissue homogenates from the experimentally induced ENA supports ENTV-1 as the causative agent of ENA. A molecular clone of ENTV-1 was generated but virus generated from the molecular clone was defective in protease processing unless complemented with JSRV Gag-Pro-Pol polyprotein and was unable to induce tumors \textit{in vivo}.

A highly specific and sensitive RT-PCR assay for detection of ENTV-1 genomic RNA in sheep nasal swab samples was developed based on the previously determined sequences. Antibodies reactive with the ENTV-1 envelope and capsid antigens were detected in sheep affected with ENA as well as in sheep kept in proximity to diseased animals, but results of serology based diagnostic tests were inconsistent.

Truncation of the cytoplasmic tail of the ENTV-1 envelope protein was shown to dramatically increase transduction of pseudotyped virions from two separate retroviral vector systems. \textit{In vivo} transduction with lentivirus vectors pseudotyped with the truncated ENTV-1 envelope protein demonstrated that the envelope protein is not the restricting viral factor for determining the tissue tropism of ENA.

In summary, I have shown that ENA is transmissible in sheep and that ENTV-1 is the causative agent of ENA but that induction of ENA is not the most common outcome of infection with ENTV-1.
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Declaration of Work Performed

All work reported in this thesis was performed by myself under the supervision of Dr. Sarah Wootton and the guidance of my advisory committee; Dr. Dorothee Bienzle, Dr. Geoff Wood and Dr. Peter Krell, with the following exceptions:

Dr. Nicolle Linnerth-Petrik performed all histology and immunohistochemistry in Chapters 2 and 3. Aimee Laporte assisted with the PCR, cloning and sequencing involved in Chapter 2. Kevin Stinson assisted with the ENTV-1 antigen specific ELISAs and was crucial in the development and optimization of the ENTV-1 RT-PCR diagnostic assay in Chapter 5. Darrick Yu and Dr. Jondavid de Jong assisted with in vivo infections and, along with Betty-Anne McBey, assisted with blood sampling for Chapter 3 and 4. Dr. Robert Foster performed the necropsies reported in Chapters 2 and 3 and Dr. Nicolle Petrik, Darrick Yu, Betty-Anne McBey and Kevin Stinson assisted with the post-mortem tissue sampling in Chapters 4, 5 and 6. Kevin Stinson, Lisa Santry, Maegan Melillo and Dr. Paula Menzies assisted with on-farm sample collection in Chapter 5.

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Chapter One:

Introduction

Retroviral Virion Properties

Taxonomy

Jaagsiekte sheep retrovirus (JSRV) and enzootic nasal tumor virus (ENTV) are highly related viruses of the genus *Betaretrovirus* in the family *Retroviridae* (1). The *Retroviridae* family represents a diverse group of single-stranded, positive-sense RNA viruses with reverse transcriptase activity. The virion structure, genome organization and replication cycle of all retroviruses are quite similar, yet the species infected and the diseases they cause are vastly diverse (1). The *Retroviridae* family is divided into seven genera and split between two subfamilies based on the method of virion egress, pathogenesis, virion morphology and host range (Table 1-1) (1, 2). The *Orthoretrovirinae* subfamily includes the *Alpharetrovirus, Betaretrovirus, Deltaretrovirus, Epsilonretrovirus, Gammaretrovirus* and *Lentivirus* genera. The *Spumaretrovirus* is the only genus of the *Spumaretrovirinae* subfamily (1). Although there is significant similarity in virion structure between the different species of the *Retroviridae* family, negatively-stained electron microscopy (EM) images reveal certain aspects of retroviral virion morphology that can be exploited to differentiate between viral species (1, 2). Based on virion morphology, all retroviruses can be classified into at least one of four morphological categories: A-type, B-type, C-type and D-type (2). A-type particles are non-enveloped, found exclusively in the cytoplasm and it is believed that these immature capsid structures are products of translation from non-infectious endogenous retrovirus-like elements (2). B-type particles are enveloped extracellular particles characterized by the presentation of prominent envelope spikes and an eccentric core (2). C-type particles are enveloped extracellular particles characterized by barely
visible spikes and a concentric core (2, 3). D-type particles are enveloped extracellular particles, slightly larger than that of B- and C-type particles and characterized by moderately prominent spikes as well as a cylindrical concentric core (4). A distinguishing feature of D-type particles is that they are assembled in the cytoplasm whereas B- and C-type particles are assembled at the plasma membrane (5, 6). Interestingly, JSRV and ENTV display characteristics of both D- and B-type viruses (7) such that assembly occurs in the cytoplasm and the capsid is spherical with an eccentric location in relation to the viral envelope (7).

<table>
<thead>
<tr>
<th>Subfamily/Genus</th>
<th>Morphology</th>
<th>Pathology</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthoretrovirinae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpharetrovirus</td>
<td>C-type, spherical concentric core</td>
<td>avian malignancies</td>
<td>Avian leukosis virus</td>
</tr>
<tr>
<td>Betaretrovirus</td>
<td>B- and/or D-type, spherical or cylindrical, eccentric or concentric core</td>
<td>carcinoma, lymphomas</td>
<td>Jaagsiekte sheep retrovirus, Enzootic nasal tumor virus</td>
</tr>
<tr>
<td>Deltaretrovirus</td>
<td>C-type, spherical, concentric core</td>
<td>malignancies</td>
<td>Bovine leukemia virus</td>
</tr>
<tr>
<td>Epsilonretrovirus</td>
<td>C-type, spherical, concentric core</td>
<td>solid tumors of fish</td>
<td>Walleye dermal sarcoma virus</td>
</tr>
<tr>
<td>Gammaretrovirus</td>
<td>C-type, spherical, concentric core</td>
<td>malignancies</td>
<td>Murine leukemia virus</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>cylindrical or conical concentric core</td>
<td>immunodeficiency and neurological disease</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>Spumavirinae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spumavirus</td>
<td>spherical, uncondensed concentric core</td>
<td>none apparent</td>
<td>Chimpanzee foamy virus</td>
</tr>
</tbody>
</table>

Table 1-1. Classification of the Retroviridae family.
The subfamilies and genera of the Retroviridae family are grouped based on classifications of virion morphology, pathology, and host. An example of a viral species for each classification is listed [modified from (7)].
**Genome Organization**

The single stranded positive sense genomic RNA of ENTV and JSRV has a structure analogous to that of cellular mRNA, with 5’ and 3’ untranslated regions (UTR), a 7-methylguanosine cap and a polyadenylated 3’end (1, 2, 7). All retroviral genomes share a basic canonical structure, R-U5-\textit{Gag-Pro-Pol-Env}-U3-R, with three to four open reading frames (ORF) and flanking untranslated regions and terminal repeats (LTR) on either end (2, 7). Some viruses encode accessory proteins that are not required for replication but may act to enhance the replication rate or infectivity of the virus. Due to the increased complexity imparted on the genome by the extra coding capacity, such viruses are termed complex viruses whereas viruses with a classical genome structure are termed simple retroviruses (2).

Each ORF of the genome codes for two or more functional protein subunits. The \textit{gag} (group antigen) gene encodes the structural proteins that make up the capsid (CA) and matrix (MA) layer or shell, as well as the nucleocapsid (NC) protein, which interacts directly with the genomic RNA (2, 7). The \textit{pro} gene encodes the viral protease (PR) responsible for proteolytic processing of viral proteins (2, 7). The \textit{pol} gene encodes reverse transcriptase (RT) and integrase (IN), the replicative enzymes required for reverse transcription and integration of the genome (2, 7). The \textit{env} gene encodes both SU (surface) and TM (transmembrane) subunits of the envelope glycoprotein (2, 7). Ribosomal frameshift or readthrough (termination) suppression, mediated by a pseudoknot (8), is required for translation of the pro and pol proteins as a fusion protein with Gag (Gag-Pro or Gag-Pro-Pol) (9), whereas the env protein is translated from a singly spliced transcript. Proteolytic processing of Gag, Pro and Pol polyproteins by the viral protease and processing of the env protein by host proteases, cleaves the viral proteins into their functional
Figure 1-1. Schematic of JSRV Genome Organization.

The complete JSRV provirus is shown annotated with all ORFs. JSRV is a simple retrovirus, encoding Gag, Pol, Pro and Env and flanked by the long terminal repeats (LTRs). An additional open reading frame X (Orf-X) is present within the Pol region with unknown function. Genomic mRNA and a singly spliced transcript are shown with the recognized protein products of each transcript. Loci of ribosomal frameshift to produce fusion Gag-Pro and Gag-Pro-Pol polyproteins are indicated by large black arrowheads. Purple arrowheads indicate viral protease cleavage sites. Orange arrow indicates host protease cleavage site [modified from (10)].

Although JSRV and ENTV-1 are classified as simple retroviruses, the JSRV genome has been shown to support expression of a small accessory protein named Rej, as the regulator of JSRV expression (11, 12). Rej is derived from the signal peptide of the envelope protein and is expressed either from a doubly spliced transcript (13), or as a part of the envelope protein with subsequent separation from the full-length protein by means of signal peptide peptidase cleavage (11, 12). Rej binds to JSRV mRNAs through interaction with a stem-loop-stem structure, termed the Rej response element (RejRE), in the area of the transcript that correlates with the coding sequence of the envelope protein carboxy terminus (11). This interaction results in a dramatic increase in JSRV gag protein expression in vitro (12) but has not been tested in vivo. The JSRV genome also carries an alternate reading frame within the pol gene, which encodes the putative Orf-x protein (Figure 1-1) (14). The function of Orf-x in the context of JSRV infection,
replication and pathogenesis is unknown. This same ORF is disrupted by two stop codons in the
ENTV-1 genome (15) and mutagenesis studies eliminating full-length Orf-x expression in JSRV
have shown no effect on viral replication or pathogenesis (16). Although a 3.2 kb spliced JSRV
RNA that could support expression of the Orf-x protein has been detected in ovine pulmonary
adenocarcinoma (OPA) tumors and transiently transfected 293T cells (17), non-optimized codon
usage of the putative coding sequence implies a low level of expression of the protein (15, 18,
19). Indeed, efforts to detect any Orf-x peptide expression during infection have proven
impossible due to the lack of a proper antiserum (16).

Retroviral long terminal repeats (LTRs) are generated as a by-product of reverse
transcription as both the 5’ UTR (U5 region) and the 3’UTR (U3 region) are duplicated onto the
opposing terminus of the genome. Therefore, the LTRs are present in their complete form only in
the provirus. Each LTR is composed of three DNA elements, the previously discussed U3 and
U5 regions separated by a short repeat (R) region in the intervening space (Figure 1-2) (2, 7).
The U3 region contains both the retroviral promoter/enhancer elements as well as the poly (A)
tail signal sequence (20, 21). Therefore, in the context of viral expression, the transcriptional
activity of a particular U3 region depends on which end of the genome it is located (22); the 5’
U3 region is responsible for initiating transcription through the promoter and enhancer activities,
whereas the 3’ U3 region mediates termination of transcription through the poly (A) signal
sequence.
Figure 1-2. Structure of the JSRV proviral LTR.

The linear proviral genome is flanked by identical long terminal repeating (LTR) sequences on both ends. The LTR contains three internal DNA elements, the U3, U5 and R regions. The U3 region contains transcription factor binding sites (promoter/enhancer) and transcription termination signal (poly (A) signal).

**Virion Structure**

Retroviral virions are enveloped with an internal capsid core. Virions measure between 80 and 100 nm in diameter and are roughly spherical (1). The virion surface is populated by evenly dispersed and distinctive glycoprotein spike projections. Glycoprotein surface projections are characteristically 8 nm long and are composed of trimerized glycosylated viral envelope proteins (1, 2).

Retroviral capsids are spherical with icosahedral symmetry, except members of the *Lentivirus* genus, whose capsids have a rod or truncated cone morphology (1). The capsid is typically located concentrically within the virion envelope. Species of the *Betaretrovirus* genera stray from this standard morphology and have an eccentric capsid (Figure 1-3)(4). This can be used in EM analysis to distinguish betaretrovirus virions from virions of other retroviral genera.

The RNA genome is contained, in duplicate, within the capsid of retroviral virions along with the replicative proteins, integrase and reverse transcriptase, and a transfer RNA (tRNA) primer (2). Virions have a buoyant density in sucrose of 1.13-1.18 g cm$^{-3}$ (1).
Figure 1-3. Immature and mature conformation of a betaretrovirus virion

All viral proteins are depicted in predicted location in the virion. Virion organization and capsid conformation is altered by viral protease-mediated proteolytic cleavage following viral release, producing the mature form of the virus. The capsid is eccentric relative to the envelope, a feature common to all betaretroviruses [modified from (10)].

Retrovirus Replication Cycle

Viral Entry

Retroviral cell entry is achieved through a membrane fusion event mediated by the interaction of the viral envelope protein with one or more receptor proteins expressed on the surface of the host cell membrane. In some viruses, interaction of the viral envelope protein with the host receptor is sufficient to trigger fusion of the viral envelope and plasma membrane and subsequent release of the virion capsid into the cytoplasm (23, 24). In other viruses, such as JSRV and ENTV, fusion is a pH-dependent event, requiring receptor-mediated endocytosis of the viral particle and subsequent exposure to an acidic environment in an endosomal or lysosomal vesicle (25–28). Upon entry of the retrovirus into the cytoplasm of the target cell the
capsid begins to degrade, allowing for reorganization of the proteins in the virion to form the reverse transcription complex (RTC) and initiate reverse transcription (29).

Reverse Transcription

The RTC is a composite nucleoprotein structure consisting of several different viral and host proteins. The protein composition of the RTC varies among different virus genera and species with the presence of the IN protein being an absolute requirement for successful integration. The HIV RTC has been reported to contain the matrix protein, viral protein R (Vpr), a multifunctional auxiliary viral protein, reverse transcriptase, integrase, and select cellular proteins (29–31), whereas participation of the capsid protein in the RTC appears to be required in murine leukemia virus (MLV) replication and integration (32). The RTC mediates the conversion of the viral genome from the single-stranded RNA form into the double-stranded DNA form (33).

Reverse transcription (Figure 1-4) is initiated from a tRNA primer (31, 34), which anneals to the primer-binding site of the genome and primes negative-strand DNA synthesis from the RNA genome template (35). Occurring concurrently with reverse transcription, the template RNA strand of the resulting DNA/RNA duplex is degraded by the RNase H catalytic domain of the RT protein (36). This process culminates to generate a negative-sense single-stranded DNA intermediate of the 5’ R and U5 region. The DNA intermediate is transferred to the 3’ end of the genome through annealing of the R region sequence of the DNA intermediate to the complementary R region sequence at the 3’ terminus of the RNA template (37). Once transfer is complete, the DNA intermediate primes reverse transcription of the remainder of the genome. RNase H digestion of the template strand is not complete because the poly-purine tract (PPT), a 7-18 purine rich sequence just upstream of the U3 sequence, is relatively resistant to
degradation and can be utilized to prime positive strand DNA synthesis of the 3’ LTR (38). Finally, a second transfer of the positive sense DNA strand to the 5’ end of the genome, mediated by annealing to the PBS sequence, and completion of transcription of the genome produces a full-length double-stranded DNA genome with a complete LTR on each terminus (39).

Figure 1-4. Generation of LTRs during reverse transcription of retroviral genome

The single-stranded RNA genome of a retrovirus is converted into a double-stranded DNA copy through the process of reverse transcription. The reaction is mediated by reverse transcriptase, which catalyzes polymerization of DNA on the RNA template and concurrent digestion of the RNA strand. The genomic RNA is packaged in the virion with a cellular tRNA hybridized to the primer-binding site (PBS) near the 5’ end. Short repeat sequences (R) located on both termini of the retroviral RNA and a small poly purine tract (PPT), which is resistant to degradation by the reverse transcriptase, are integral for reverse transcription. The entire process yields a double-stranded DNA molecule that has a long terminal repeat (LTR) at each end [modified from (40)].
Integration

Upon completion of reverse transcription and generation of a single double-stranded DNA molecule, the remnants of the RTC structure become known as the pre-integration complex (PIC). The blunt-ends of the linear proviral molecule within the PIC are processed by the integrase protein (41) and the terminal two nucleotide bases of each 3’ end are removed. Termini processing liberates a 3’ hydroxyl group, which is required for integrase-mediated integration of the viral DNA into the host genome (33).

Retained within the PIC nucleoprotein complex, the processed provirus is transported into the nucleus. Betaretroviruses are unable to traverse intact nuclear membranes and seem to require nuclear membrane breakdown during infection to allow for integration and completion of the replication cycle (42). Consequently, betaretrovirus infection is limited to actively dividing cells. In contrast, lentiviruses are able to traverse the nuclear membrane and infect both non-dividing and dividing cells (42, 43).

Once inside the nucleus, the provirus integrates into the host genome at random loci, although some retroviruses appear to use certain sites for chromosomal integration at an increased frequency (44–46). The 3’ hydroxyl group liberated in termini processing achieves integration through integrase-mediated nucleophilic attack of phosphodiester bonds in the chromosomal DNA (45). As a by-product of integration, a short sequence from the target site is duplicated, creating a flanking direct repeat of 4–6 bp (33). Integrated viral DNA is termed a provirus and, since integration is an irreversible process, and the provirus is transmitted as a Mendelian locus (47).
Assembly

Once integrated, the provirus utilizes the cellular machinery to translate viral proteins, replicate the viral genome and assemble progeny virions. Translation occurs from either the genomic mRNA or a singly spliced mRNA transcript as discussed previously. For most retroviruses, the structural protein products are targeted for transport to the plasma membrane via cellular transport mechanisms that are reliant on signals in the Gag protein (48). Association of retroviral Gag polyproteins with the genomic RNA brings the individual moieties into close proximity and causes dimerization of proteins at hydrophobic interfaces and assembly into an immature virion (49). The subcellular location of packaging differs between viral species but is largely dependent on the method of egress employed. Most retroviruses assemble at the membrane concurrent with budding (6, 50, 51). Assembly of foamy and betaretroviruses, however, occurs intracellularly at a perinuclear location in association with the microtubule organization center (MTOC) (52–54). The viral particle acquires an envelope as it buds from the plasma membrane releasing extracellular virus (5, 55).

Maturation

Regardless of the cellular location of assembly and pathway of egress, virion maturation occurs after or concurrent with budding of the virion (2). Maturation is initiated by release of the protease from the viral Gag-Pro-Pol polyprotein, via autocatalytic cleavage (56), followed by processing of the remainder of the polyprotein by the protease into the active subunits. Considerable structural changes occur during this process (57, 58), resulting in condensation of the smooth Gag shell, characteristic of immature particles (59), to the electron dense core visible in mature particles (Figure 1-3) (4). The trigger and mechanism of protease activation is still in contention but it appears that folding and dimerization of protease subunits is an important step
in this process (60, 61). A reduction-oxidation has also been implicated in retroviral protease activation (62). Regardless, premature activation and inactivation of the protease is detrimental to replication and infectivity (63, 64).

**Endogenous Retroviruses**

Integration of the viral genome into the host cell genome is a unique replication strategy of retroviral infection and replication. Once integrated the provirus remains in the host cell genome throughout the life of the cell and, as a consequence, is present at the same genomic loci in all progeny cells (44). Exogenous retrovirus infection and subsequent integration into a germline cell genome leads to the fixation of the provirus into the gene pool of the host species as an endogenous retrovirus (ERV)(65, 66). Mutations occurring after integration which inactivate viral replication and/or disable viral pathogenic activity stabilizes the proviral sequence in the host genome (67). ERVs are then transmitted vertically in Mendelian fashion (68), whereas the exogenous form of the virus is transmitted horizontally throughout the host population.

**Ovine endogenous betaretroviruses (OEBRVs)**

The sheep genome is colonized by at least 27 endogenous retroviruses at distinct genomic loci (69). Analysis of the nucleotide sequences of these sheep ERVs has revealed a high degree of homology with the currently circulating exogenous JSRV genome (70, 71). As a result, the sheep ERV sequences have been termed endogenous JSRV (enJSRV) sequences but they will be referred to more appropriately as ovine endogenous betaretroviruses (OEBRVs) for the remainder of this document because they also share homology with ENTV-1.
**OEBRV Genome Structure**

Comparison of OEBRV sequences to the exogenous JSRV and ENTV provirus sequences shows an increased degree of variability at three specific genomic loci, termed hypervariable regions 1-3 (70). Hypervariable region 1 and 2 (VR1 and VR2) are located approximately 60 residues apart in the matrix protein coding sequence of the \textit{gag} gene (70). Interestingly, VR1 contains a proline rich region, which is conserved between the exogenous sequences but is absent in most endogenous sequences (70). Hypervariable region 3 (VR3) spans the cytoplasmic tail sequence of the envelope gene of the provirus (Figure 1-5) (70). A single tyrosine residue at amino acid position 590 in JSRV envelope protein, which is important in pathogenesis (72), is lacking in all known OEBRV sequences (Figure 1-5) (70, 73). Therefore, it would appear that elimination of the tyrosine residue, and its associated oncogenicity, is required for stable transmission of the endogenized proviruses. Alternatively, the primordial exogenous virus from which the OEBRVs are derived was not oncogenic.
Figure 1-5. Schematic representations of JSRV Env structure and sequence alignment of Env cytoplasmic tail
(A) JSRV Env is type I transmembrane protein 615 amino acids in length. It is composed of two subunits, surface (SU) and transmembrane (TM). SP represents the signal peptide. M is the membrane-spanning domain. CT is the cytoplasmic tail. (B) Sequence alignment of the cytoplasmic tail of JSRV Env, ENTV Env and endogenous sheep retrovirus Env. Note that a YXXM motif is conserved among all transforming exogenous viruses, but absent from endogenous viruses. JSRV21 (AF105220) and JSRV7 (AF357971) are two JSRV strains isolated from the UK. ENTV was obtained from a sheep in the UK (Y16627). The enJSRV26 is an endogenous sheep retrovirus (EF680297). Dashes denote deletions [modified from (74)].

**OEBRV env Involvement in Placental Morphogenesis**

The nucleotide sequence of the U3 region is significantly different between exogenous JSRV and OEBRV sequences in comparison to the rest of the genome (70, 73). The sheep betaretroviral transcription regulatory elements (promoter and enhancer) are contained within the U3 region (20, 75). Cell culture transfection studies have found that the exogenous JSRV promoter is responsive to lung-specific transcription factors, such as HNF3α and HNF3β, while the OEBRV promoters elicit no response to these transcription factors (75). Instead, transcription from the promoter of the OEBRV sequences seems to be driven by progesterone stimulation through the progesterone receptor protein (76). As a result, OEBRV transcripts can be detected in abundance in the ovine uterine endometrial epithelial cells with transcript levels that peak in
correlation with that of progesterone levels in the peripheral blood (76). As a result, OEBRV mRNA production peaks in correlation with the establishment and development of the placenta after successful fertilization. *In situ* hybridization analysis has localized OEBRV RNA production to the luminal and glandular epithelium of the uterine wall as well as the binucleate cells of the conceptus trophoderm in the developing placenta (76). Binucleate cells of the conceptus play an essential role in the development of the placenta, as it is these cells that fuse with the maternal endometrial epithelium to initiate placental formation (77).

Immunohistochemical analysis with capsid and envelope protein-specific antibodies confirmed that protein expression correlates with the viral RNA profile observed in the binucleate and endometrial cells (76). Due to the apparent cell-specific restriction of viral RNA expression, it is believed that the OEBRV envelope protein is playing a role in the development of the placenta. In further support of this hypothesis, a recent study found that morpholino-based siRNA knockdown of the envelope protein expression *in utero* blocks placenta formation and causes conceptus decline (78). Endogenous envelope proteins are actively involved in placental formation and, therefore, required for normal conceptus development in several other mammalian species, including humans (79–82).

**OEBRV-Mediated Immune Tolerance**

Serum analysis of individual sheep presenting with OPA or ENA, caused by JSRV and ENTV, respectively, have shown a lack of JSRV or ENTV-specific antibodies (83). It is not known whether JSRV infection induces immunosuppression in the host but it appears that infected sheep are simply unable to generate a significant immune response against the virus, which is indicative of immune tolerance toward the virus (84). Expression of proteins in the cortico-medullary junction of the thymus may be responsible for this phenomenon of immune
tolerance because it is in this region of the thymus that self-tolerance of the immune cells develops (85, 86). As the endogenous viral proteins are expressed in the thymus they are processed and presented to the immature immune cells in major histocompatibility complex (MHC) molecules (87). Immune cells that recognize the peptides presented in the thymic MHC molecules with high affinity are deleted, resulting in the development of immune tolerance. Since the exogenous JSRV and OEBRV protein sequences are highly homologous (70), the induction of immune tolerance towards endogenous proteins results in a lack of immune response to exogenous JSRV infection.

**Pathogenesis of JSRV and ENTV**

**Disease**

JSRV is an acutely transforming oncogenic betaretrovirus of sheep and goats. JSRV is the causative agent of a neoplastic transformation of secretory epithelial cells of the distal lung, or OPA (88). Tumor cells have morphological and functional features of alveolar type II cells (ATII) and to a lesser extent of Clara cells, such as secretion of proteins. Typically, OPA lesions are observed 2 to 3 weeks post-inoculation of concentrated JSRV particles into newborn lambs, although the incubation period in naturally infected animals may be 2 to 4 years (89, 90). Therefore, under natural conditions, the majority of infected animals do not develop clinical abnormalities during the course of their commercial lifespan (89). The clinical features of OPA include lung dysfunction, laboured breathing and secretion of copious amounts of virus-containing fluids.

ENTV is a betaretrovirus and is the suspected causative agent of ENA in sheep and goats. ENA is characterized by oncogenic transformation of nasal turbinate epithelium causing obstruction of the nasal passage and production of abnormal levels of nasal exudate(91). ENTV
is consistently found in the tumor tissue and nasal exudate fluid of ENA. The infectious etiology of ENA has been demonstrated through transmission of the disease after inoculation of a healthy goat with nasal exudate derived from ENA tumors (92). The viral species infecting goats is designated as ENTV-2 (93) and is slightly different at the nucleotide level from ENTV-1, which infects sheep (15). Nevertheless, both viruses have the same pathological effect in their respective hosts. JSRV and ENTV species utilize Hyaluronidase 2 (Hyal2), a glycophosphatidylinositol (GPI)-anchored membrane protein, as a receptor for viral entry (94–96). Hyal2 is a member of a large family of hyaluronoglucosaminidases, which catalyze the degradation of hyaluronan in the extracellular matrix (97, 98). The hyaluronidase activity of Hyal2 is very low in comparison to the enzymatic activity of the other hyaluronoglucosaminidase proteins (97). Also, Hyal2 seems to be ubiquitously expressed (97). Hyal2 was identified as the receptor for JSRV and ENTV through hamster cell line-based whole human genome radiation hybrid mapping (94). It was found that hybrid cells containing the p21.3 region of human chromosome 3, which contains the Hyal2 gene, were susceptible to JSRV and ENTV envelope-mediated infection (94). Meanwhile, all hybrids lacking this region remained resistant to infection. This result was confirmed when expression of human or ovine Hyal2 orthologs rendered cells susceptible to infection that were previously resistant (99).

**Mechanism of Oncogenesis**

As a direct consequence of proviral integration during retroviral replication, all retroviruses have an innate propensity to induce genetic instability. Most commonly, retroviral-mediated oncogenesis occurs through either insertional mutagenesis or host oncogene capture (2, 7). Insertional mutagenesis is characterized by insertion of the provirus into a locus of the host chromosome that causes inactivation of a tumor suppressor gene or constitutive activation of a
proto-oncogene (100, 101). The locus of integration is essentially random but common integration sites causing cellular oncogene upregulation have been identified for some retroviruses (102). The incubation period (time between initial infection and tumor development) is usually extended in cases of insertional mutagenesis due to the time it takes for a tumor-inducing integration event to take place as well as the time required for additional genetic changes to complete cell transformation (2). Oncogene capture, on the other hand, is a multi-step process, initiated by virus integration within a proto-oncogene. This event is then followed by either a deletion of the 3’LTR or inefficient polyadenylation of the viral genome leading to the production of transcripts containing both viral and cellular RNA. This hybrid RNA is then packaged into virions along with wild-type RNA and infectious virus particles produced. In cells infected with the hybrid virus, illegitimate recombination during reverse transcription may take place allowing for production of integration-competent proviruses containing host-derived oncogenes (103). This oncogenic mechanism is usually characterized by a short incubation period due to the elevated expression of one or more copies of a modified oncogene under the control of a retroviral promoter. Although insertional activation and virus capture of host oncogenes represent the classical and most commonly observed mechanisms of retroviral oncogenesis, some retroviral species employ alternative mechanisms to induce transformation (2).

The mechanism of JSRV oncogenesis does not involve viral transduction of an oncogene or insertional mutagenesis because the JSRV genome contains no known oncogene and no common integration sites have been identified (104). Also, multiple proviral insertions into a single cell genome is unlikely to be the cause of tumorigenesis because it has been shown that the level of Hyal2, the cellular receptor for JSRV, displayed on the cell surface is down-
regulated in conjunction with JSRV envelope expression (105). The mechanism of Hyal2 down-regulation is not completely understood but it is thought to be caused by interaction or complex formation with the JSRV envelope protein.

The presence of a putative accessory protein (Orf-x) raised the possibility that Orf-x was a novel oncogene and was responsible for JSRV-mediated transformation, but truncation of the Orf-x reading frame through mutagenic insertion of a stop codon had no effect on virus replication or oncogenesis in vitro (16). Instead, it was discovered that the viral envelope protein alone is sufficient to induce transformation in cell culture. The transforming property of the JSRV and ENTV envelope (Jenv and Eenv respectively) protein has been demonstrated in vitro in select rodent, canine, avian and human cell lines (105–108). Transformed foci can be observed in most cell cultures two to three weeks after Jenv or Eenv expression is induced (108). Immunoblotting analysis of transformed cells has shown that there is an increased level of phosphorylation of MAP kinase and Akt signalling pathway members in transformed cells (Figure 1–6), implicating the envelope protein as an activator of these pathways (109). Activation of these pathways promotes proliferation and cell survival, necessary steps in the progression towards a transformed state (110). Furthermore, it seems that activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is required for cell transformation and maintenance of the transformed phenotype, as inhibition of PI3K with LY294002, a PI3K-specific inhibitor, impedes transformation by Jenv and reverses the phenotype of Jenv transformed 208F cells (rat fibroblasts) (111). Since PI3K is one of the signal initiating proteins of the Akt pathway, this implies that signal transduction through the PI3K protein is important for Akt pathway activation in Jenv transformation (111).
Verification of the transforming potential of Jenv and Eenv in an *in vivo* model was performed using adeno-associated virus (AAV) expression vectors. The env coding sequence was placed under the control of a Rous sarcoma virus (RSV) promoter in an AAV-vector and the resulting virus was administered to immunodeficient or immunocompetent C57BL/6 mice through intranasal inoculation (112). Necropsy revealed that although there was efficient vector delivery in all groups, tumour development was observed only in immunodeficient mice inoculated with AAV-Jenv or AAV-Eenv (112). The tumours were at bronchio-alveolar locations and expressed markers of alveolar type II cells, similar to what is found in OPA cases (112). Since there was no tumour formation in inoculated immunocompetent mice, it appears that Jenv and Eenv mediated tumourigenesis can be controlled by the normal immune system of these mice (112). This implies that the immunotolerance of sheep to exogenous viral proteins may be an important factor in JSRV oncogenesis.

The $Y_{590}xxM_{593}$ motif (where $x$ is any amino acid) located in the cytoplasmic tail of Jenv represents a putative docking site for the p85 subunit of PI3K (113, 114). Phosphorylation of the tyrosine residue 590 (Y590) is required for the SH2 domain of the p85 protein to recognize and bind to YxxM, but no phosphorylation of the Y590 residue has been detected (74). Also, interaction between Jenv and p85 has not yet been observed. Mutation of this motif significantly diminished the transforming activity of Jenv *in vitro* (111) and abolished tumor formation *in vivo* (16). Furthermore, it seems that the YxxM motif of Jenv plays no role in Env transformation of the avian fibroblast cell line DF-1 (107). Therefore, activation of the PI3K/Akt signalling pathway in Jenv transformed cells is not induced through a direct interaction between Jenv and PI3K and the requirement of the YxxM motif for transformation is cell line specific (106).
An alternative mechanism to explain JSRV-mediated oncogenesis has been hypothesized based on the discovery that Hyal2 displays tumor suppressor activities in BEAS2 cells, a human bronchial epithelium cell line immortalized with the SV40 large T antigen (TAg) (105). In BEAS2 cells, Hyal2 acts as a negative regulator of the receptor tyrosine kinase RON by maintaining RON in an inactive complex. Studies in BEAS2 cells demonstrated that Jenv activates the MAP kinase pathway through downregulation and sequestration of Hyal2 in the endoplasmic reticulum. This lack of Hyal2 at the cell surface causes a release of RON inhibition and allows RON to constitutively activate the MAP kinase pathway through the phosphorylation activities in its tyrosine kinase domain (105). Aberrant MAPK signals allow the affected cells to proliferate independent of growth factors, avoid induced cell death and therefore complete transformation (Figure 1-6) (105). Although these results are promising and seem to indicate a novel mechanism of retroviral oncogenesis, the mechanism seems to be restricted to BEAS2 cells. For example, the mouse NIH 3T3 cell line is susceptible to Jenv mediated transformation despite the fact that mouse Hyal2 is incapable of interacting with Jenv nor do these cells express RON (108). Moreover, constitutive expression of RON in rat 208F, NIH 3T3 and Madin-Darby Canine kidney epithelial cells, all of which lack expression of a RON ortholog, resulted in only low levels of transforming activity (106, 108). Therefore, the mechanism observed in the BEAS2 is not universal for Jenv-mediated oncogenesis. Also BEAS2 cells are notoriously difficult to transfect (Dr. Sarah Wootton, personal communication) and efforts to replicate these results have failed. Nevertheless, the fact that the Hyal2 coding region of chromosome 21 is often deleted in human lung tumors lends credence to the fact that Hyal2 may be functioning as a tumor suppressor (115).
Three major pathways have been uncovered in different cell lines transformed by JSRV Env, including the Akt pathway that is dependent or independent of PI3K, the Hyal2-RON pathway that activates both PI3K/Akt and Ras-MEK-ERK pathways, which includes p38. Except for the RON-Hyal2 pathway, how JSRV Env engages other signalling networks is not known. Note that MAPK/p38 activation plays an inhibitory role in cell transformation [modified from (74)].

**JSRV Infectious Molecular Clone**

All attempts to propagate JSRV viruses in a cell culture system have failed to produce high titers of progeny viruses. Lack of an appropriate cell culture system that adequately recapitulates the alveolar type II cellular environment may be the problem. When grown in culture, primary alveolar type II cells are unable to retain progenitor cell morphology and rapidly differentiate into type I cells (116). Growth of JSRV virus in differentiated type I cell cultures
and all other cell culture systems is extremely stunted, presumably because the transcription factors utilized by the virus for replication are unavailable (75). As a result, efficient JSRV propagation cannot be achieved using cell culture and an infectious molecular clone of the virus is required for virus production.

Two separate research groups have each generated an infectious molecular clone of JSRV from a natural case of OPA (117, 118). Led by Dr. Massimo Palmarini, a research group generated a clone derived from naturally occurring OPA tumor tissue from the United Kingdom. Utilizing a commercially available kit, a phage library was produced from a digest of genomic DNA extracted from a tumor sample (117). Once obtained, the JSRV clone was subcloned into pBluescript to create pJSRV21. An *in vivo* transfection, involving intratracheal inoculation of pJSRV21 complexed with cationic lipids, was performed on newborn lambs. Inoculated lambs did not develop tumors but virus sequences could be detected in the lungs and at sites peripheral to inoculation, although at extremely low levels (117). This result indicated that pJSRV21 contained an infectious provirus but was insufficient to recapitulate the disease. It was assumed that inoculation with high titer virus was required to cause disease therefore the pJSRV21 clone was adapted for high titer virus production in HEK 293T cells by replacement of the 5' U3 region with a CMV promoter (pCMV2JS21) (117). Cells were transiently transfected with pCMV2JS21 DNA and supernatant was collected, purified and concentrated to produce virus particles. Newborn lambs were inoculated with concentrated virus as described earlier. At four months post-inoculation, 1 of the 4 inoculated lambs showed clinical signs of OPA. OPA tumors were found in 2 of the 4 lambs after necropsy (117). Histologically, tumors stained positive for JSRV capsid protein, while surrounding tissue stained negative (117). DNA was extracted from the induced tumours and was screened for an exogenous JSRV sequence using the same process as
the phage library (117). All negative control samples tested negative for exogenous JSRV sequences and samples from both lambs showing lung lesions tested positive for exogenous JSRV sequences. Positive histological staining and DNA screening indicates presence of the virus genome in the samples as well as transcription and translation of viral genes (117). Collectively, this evidence fulfills Koch’s postulates and has established JSRV as the causative agent of OPA.

The second molecular clone of JSRV (pCMV-JSRV\textsubscript{JS7}) was isolated from a phage library derived from a continuous sheep lung tumor cell line (JS7). The JS7 cell line was established from a natural case of OPA and had epithelial morphology with distinct markers/features of ATII cells (118). As the clone name implies and as was the case with the previous clone, the 5’ U3 region was replaced with a CMV promoter. Expression constructs for the \textit{gag}-\textit{pro-pol} genes (pCMV-J:\textit{gag-pol}) and the \textit{env} gene (pCMV-J:\textit{env}) were created and transiently transfected into 293T cells in conjunction with pCMV-JSRV\textsubscript{JS7} to enhance virus production (118). An \textit{in vivo} infection trial was performed whereby newborn lambs were inoculated with virus concentrate derived from 293T transfected cells (118). Of the 4 lambs inoculated with virus, only one developed OPA lesions. Histological analysis verified the presence of viral proteins at the site of lesion (118).

\textbf{ENTV-1 Sequencing}

A research group in the United Kingdom assembled a full-length genomic sequence of ENTV-1. In order to do this, viral particles from nasal exudate of a sheep with naturally occurring ENA were purified using isopycnic centrifugation on 20 to 55\% (wt/wt) sucrose gradients (119). Genomic RNA was extracted from the purified virus particles and used as a template for cDNA synthesis by random-primed or oligo (dT)-primed reverse transcription. A
cDNA phage library was created from the reverse transcript pool. Selection of recombinant clones containing ENTV-1 sequences was performed initially with JSRV \textit{gag}, \textit{env} and U3 specific probes but was verified later with ENTV-1 specific probes (119). Positive clones were sequenced and a full-length sequence was constructed from overlapping sequences(119). The resulting full-length sequence had the accession number Y16627(119). Prior to the completion of this thesis, this sequence constitutes the only genetic information available for ENTV-1.

\textbf{Koch`s postulates and demonstration of disease etiology}

In the 19\textsuperscript{th} century, a German physician by the name of Dr. Koch established a list of four criteria that need to be fulfilled in order to conclusively demonstrate a causal relationship between infection with an organism and a disease. These criteria are known as Koch`s postulates and are listed below (120).

1. The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organism.
2. The microorganism must be isolated from a diseased organism and grown in pure culture.
3. The cultured microorganism should cause disease when introduced into a healthy organism.
4. The microorganism must be recovered from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

Since their inception, Koch`s postulates have been used widely to determine the etiology of many different kinds of diseases but it has become clear that Koch`s postulates cannot be fulfilled in all cases. This is particularly true in diseases caused by organisms that cannot be propagated by conventional laboratory methods, as that is a pre-requisite step in Koch`s postulates. As well, the increased use of PCR and other molecular biology techniques to detect
organisms and show associations with disease has demonstrated a need to reconsider Koch’s postulates. As such, several different revisions of Koch’s original postulates have been published, by Inglis (121), Fredricks and Relman (122) or Falkow (123) for example, but no single set of revised postulates has been accepted by the scientific community as of yet.

**Retroviral vectors in gene therapy**

Knowledge about molecular mechanisms of viral replication and other viral processes has allowed researchers to modify viruses for directed purposes, both in the lab and in the clinic. For instance, the ability of retroviruses to introduce their genetic information into the chromosome of infected cells and cause stable expression of the encoded protein (a process termed retroviral transduction) has been adapted for use in the treatment of certain genetic diseases (124, 125). Recombinant DNA techniques can be employed to create retroviral vectors through replacement of the viral genes required for viral replication with a desired transgene (126, 127). The resulting vector is replication deficient, meaning that it is unable to replicate without supplementation with the missing viral proteins. This process is exploited in retroviral gene therapy whereby a recipient cell is transduced with a vector containing a normal and/or functional gene to correct for a defective, disease-causing allele. Such vectors are advantageous because all progeny of the infected cell will contain the integrated vector DNA and maintain expression of the transduced gene. Retroviral vectors are currently widely used to transduce cell culture systems as well as in clinical trials for some common monogenic diseases, such as cystic fibrosis (CF) (128). Lentivirus-based vectors are predominantly used for this application because of their ability to transduce both dividing and non-dividing cells (43).
**Envelope Pseudotyping**

Since the viral envelope is derived from the plasma membrane, it often contains an assortment of cellular transmembrane proteins in addition to the viral envelope. Indeed, some host transmembrane proteins of the plasma membrane that are expressed at sufficient levels during infection will be packaged into the envelope of progeny virions (129). In the same manner, viral envelope proteins can be incorporated into retroviral virions comprised of heterologous structural proteins during virus production in cell culture in a process termed pseudotyping. This allows alteration of cell tropism (130) and is useful for targeting retroviral vectors to specific cell types (131, 132). Also, pseudotyping represents a practical method for discerning the cell type targeted (or tropism) by an envelope protein in the absence of the other viral proteins and any associated restricting effect on tropism they may have.

**Objectives**

The purpose of this thesis was to investigate the connection between ENTV-1 infection, the sheep immune response and development of ENA in order to better understand the pathogenesis of ENTV infection. I hypothesized that ENTV-1 is the causative agent of ENA in sheep and the entry tropism of the envelope protein is the determining factor for the tissue tropism of ENA. Furthermore, sheep can develop an immune response against ENTV-1 and this is a factor in determining the outcome of infection.

Thus, studies commenced with the aims to 1) determine the full-length proviral sequence of ENTV-1 from ENA samples from North America, 2) to construct a molecular clone of ENTV-1 to generate recombinant virus in cell culture for use in *in vivo* infection studies and thereby establish ENTV-1 as the causative agent of ENA by fulfilling Koch’s postulates, 3) create a
diagnostic test for detecting ENTV-1 infection before euthanasia and 4) investigate the role of envelope-mediated transduction in determining the tissue-specific tumor formation by ENTV-1 and JSRV.
Chapter Two:

Full-length genome sequence analysis of enzootic nasal tumor virus reveals an unusually high degree of genetic stability

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Abstract

Enzootic nasal tumor virus (ENTV) is a betaretrovirus of sheep (ENTV-1) and goats (ENTV-2) associated with neoplastic transformation of epithelial cells of the ethmoid turbinate. Very little is known about the prevalence of this disease, particularly in North America, and only one full length sequence is available for each of ENTV-1 and ENTV-2. In order to understand the molecular evolution of ENTV-1, the full-length genome sequences of ten ENTV-1 proviruses derived from clinical samples of ENA isolated from conventionally reared sheep in Canada and the United States were determined. The North American ENTV-1 (ENTV-1_{NA}) genomes shared greater than 96% sequence identity with the European ENTV-1 sequence (ENTV-1_{EU}). Most of the amino acid differences were found in Orf-x, which in the corresponding ENTV-1_{EU} genome is truncated by 44 amino acid codons by the presence of an additional stop codon. Apart from Orf-x, the long terminal repeat (LTR) is where the majority of differences between ENTV-1_{NA} and ENTV-1_{EU} reside. Overall, there was an unusually high degree of amino acid conservation among the isolates suggesting that ENTV-1 is under stabilizing selection and $\kappa_a/\kappa_s$ ratios calculated for each of the viral genes support this hypothesis. The unusually high degree of genetic stability of the ENTV-1 genome enabled us to develop a hemi-nested PCR assay for detection of ENTV-1 in clinical samples. Additionally, multiple nasal tumor cell clones were established and while most had lost the provirus by passage 5; one polyclonal line retained the provirus and attempts are being made to culture these cells. These tumor cells, the first of their kind, may provide a system for studying ENTV-1 \textit{in vitro}. This work represents an important step in the study of ENTV and sets the foundation for the construction of an infectious molecular clone of ENTV-1.
Introduction

Enzootic nasal adenocarcinoma (ENA) and ovine pulmonary adenocarcinoma (OPA) are contagious neoplasms of sheep and goats affecting the epithelial cells of the nose and distal lung, respectively (133, 134). Jaagsiekte sheep retrovirus (JSRV), an ovine betaretrovirus, is the causative agent of OPA (117). A similar betaretrovirus etiology has long been assumed for ENA due to the consistent detection of enzootic nasal tumor virus (ENTV) in nasal tumors and exudate of animals with ENA (92, 135, 136). Two distinct viruses are implicated in ENA, one in sheep (ENTV-1) (119) and one in goats (ENTV-2) (93) and successful transmission of ENA from goat to goat using clarified or concentrated nasal fluid with immunological evidence of the presence of ENTV-2 is described (92).

The ovine genome is colonized by more than 27 endogenous betaretroviral sequences (OEBRVs) (69). OEBRVs share a high degree of sequence homology with JSRV and ENTV. Past studies noted significant expression of OEBRV proteins in many tissues including the thymus, Peyer’s patches, spleen, bone marrow, peripheral lymph nodes, leukocytes, lung, kidney and uterine endometrial epithelium of the fetal lamb (70, 137). Due to the similarity of OEBRV sequences with that of exogenous JSRV and ENTV it is believed that expression of OEBRV proteins in the fetal thymus causes an immune tolerance to exogenous virus via a mechanism of clonal deletion of self-reactive T cells (87, 138). Moreover, no evidence for circulating antibodies has been reported for either ENA or OPA when using antigen from natural sources (83, 139, 140). The prevalence and geographical distribution of ENA is unclear as serological screening for the virus is not possible and reports of ENA in the USA and Canada are limited.

To date, only one full-length genome sequence of ENTV-1 has been determined (accession number NC_007015, hereafter referred to as ENTV-1_EU) (119). This sequence was derived from
a cDNA phage library of genomic RNA extracted from the purified nasal exudate of a spontaneous case of ENA in Europe. Currently, there is little epidemiological and no sequence data pertaining to ENTV-1 or -2 in North America despite the fact that intranasal tumors occur in sheep throughout Canada and the United States (141–145). There are no known cell culture systems that support the growth of ENTV-1 (or JSRV), nor is there an infectious molecular clone of ENTV-1. For this reason, there is very little known about the pathogenesis or the prevalence of ENA. The purpose of the present study was to elucidate the full-length genome sequence of ENTV-1 from naturally occurring cases of ENA in Canada and the United States in order to examine the genetic heterogeneity of ENTV-1 in naturally infected sheep and to expand the limited sequence information that is currently available. To this end, ten tumor samples from spontaneous cases of ENA in Canada and the United States were obtained and the complete nucleotide sequence of the ENTV-1 provirus determined. Phylogenetic analysis revealed that these North American isolates are genetically stable and closely related to ENTV-1 from Europe. Despite this marked sequence conservation, four distinct regions of variability were identified.

**Materials and Methods**

**Clinical samples**

The Animal Care Committee at the University of Guelph approved all animal use and related procedures. Nine sheep exhibiting clinical signs of ENA and one sheep with asymptomatic ENA were obtained from five geographically distinct flocks in Ontario, Canada and from one flock in Minnesota, USA (Table 2-1). Nasal fluid, serum and tissue samples (nasal tumor and adjacent unaffected nasal turbinate, trachea, lung, heart, spleen, liver and kidney) were collected and flash frozen in liquid nitrogen or preserved in RNAlater (Qiagen). Tissue from two apparently healthy
6-month old sheep reared at the Ponsonby Animal Research Facility, University of Guelph were used as negative controls.

Table 2-1. Source of ENA samples

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<th>Farm of Origin</th>
<th>Age (years)</th>
<th>Breed</th>
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<td>GU292314</td>
<td>19-Jun-09</td>
<td>USA</td>
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<td>Border Leichestor</td>
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</table>

NA – not applicable, ND – not determined

Cell isolation and culture of ovine nasal tumor (ONT) cells

Ovine nasal tumor cells were derived from three different sheep tumors (sheep # 2, 3 and 4, Table 2-1). One cm³ pieces of nasal tumor were excised, briefly dipped in 70% ethanol and then in sterile PBS containing penicillin and streptomycin (P/S) and finally submersed in DMEM-10% FBS-P/S until ready to culture. Tumor tissue was placed on a petri dish in the laminar flow hood and minced with rounded scissors until a homogeneous liquid-like consistency was achieved. Once the tumor was sufficiently minced, cells were drawn through a series of syringes starting with 16 gauge and ending with 20 gauge. Large pieces of tumor that were difficult to mince were removed and treated with trypsin for 1 hour at 37°C. Cells were washed two times in PBS before seeding onto 10 cm cell culture dishes in keratinocyte serum free medium (KSFM;
Gibco BRL) supplemented with epidermal growth factor, bovine pituitary extract and antibiotics. The medium was replaced the next day and every four days thereafter. Cells were passaged when they reached confluence. Fibroblast contamination was rare to non-existent when tumor cells were cultured in KSFM. Once cells with a uniform epithelial-like appearance were established, single-cell colonies were ring-cloned and cultured in a 1:1 mixture of KSFM and 3T3-conditioned medium. The epithelial origin of the cloned cells was confirmed by uniform immunofluorescence staining for pancytokeratin and lack of vimentin expression in western blots (data not shown).

**Immunohistochemistry and amplification of ENTV env from paraffin-embedded tissue**

Tissue was fixed for 48 hours in neutral buffered formalin and routinely embedded in paraffin. Immunohistochemical (IHC) detection of the ENTV envelope protein was conducted as described previously using a monoclonal antibody developed against the JSRV envelope protein that cross-reacts with the ENTV-1 envelope protein (146). Immunohistochemical detection of surfactant protein C (SP-C) (1:200; Santa Cruz Biotechnology, sc-13979) and pro-SP-C (1:2000; Chemicon, AB3786) was performed on 4-mm sections of formalin-fixed paraffin-embedded tissues as described previously (112). Genomic DNA was extracted from serial sections of paraffin-embedded lung tissue (sheep # 3) as follows. Sections of paraffin-embedded tissue were first dewaxed in xylene for 30 minutes followed by rehydration for 30 minutes in 100% ethanol, 95% ethanol and 70% ethanol. Slides were then washed with PBS for 15 minutes before tissue was removed from the slide and placed in 200 ul of PBS. Genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen) in accordance to the manufacturer’s protocol.

PCR was conducted using the 5 PRIME MasterMix (5 PRIME) plus 100 ng of genomic DNA and the following primer pairs: ENTV-Env Fwd: 5\(^{-}\)TTT TTA GTT CCC TGC CTC AC-3\(^{'}\) and
ENTV-Env Rv: 5'-CAA AAA ACA TCT GAG CCC TAT AA-3'; JSRV-Env Fwd: 5'-ATA CGG GAA CGG ATC TGG ACC-3' and JSRV-Env Rv: 5'-CAA CAT GAA TGG ATA CGG CAC GC-3' for amplification of the ENTV and JSRV envelope sequences, respectively. Cycling conditions were as follows: 94°C for 2 min followed by 30 cycles of 94°C for 30s, 55°C for 30s and 65°C for 1 min. A final extension of 7 min at 65°C concluded the program.

**RNA Extraction and RT-PCR of Surfactant Protein Genes**

RNA was extracted from JS7 cells, an OPA-derived lung tumor cell line (a kind gift from Dr. Mark Ackermann, Iowa State University), and ONT cells using Trizol according to the manufacturer’s instructions. 5 mg of RQ1 DNase (Promega)-treated total RNA was reverse transcribed for 1 hour at 50°C using Superscript III and 40 pmol of one of the following reverse primers: SP-A Rv: 5'-TCA GAA CTC ACA GAT GGC CAG TC G G-3'; SP-B Rv: 5'-TCA GAA GTG GGG GCT GTG GAT ACA CTG G; SP-C Rv: 5'-CCA AGT CTC GAG GCT CTC ACT AGA; and SP-D Rv: 5'-CTC CAC ACA GTT CTC TGA GCC GC C for a total for four different cDNA reactions. After reverse transcription, samples were treated with RNaseH for 30 min at 37°C. PCR was conducted using 2 µl of cDNA template and 5 PRIME MasterMix (5 PRIME) with 40 pmol of the same reverse primers described above paired with the corresponding forward primer: SP-A Fwd: 5'-ATG CTG CTG TGC TCT TTG ACC C; SP-B Fwd: 5'-TCT GCA TGT TTG TGA CCA CTC AGG; SP-C Fwd: 5'-ATG GAT GTG GGC AGC AAA GAG G; and SP-D Fwd: 5'-CAG GAT GCC CAG CAG GTC TGC. Cycling conditions were as follows: 94°C for 2 min followed by 30 cycles of 94°C for 30s, 55°C for 30s and 65°C for 1 min. A final extension of 7 min at 65°C concluded the program.
Isolation of genomic DNA and amplification of ENTV proviral DNA

Genomic DNA was extracted from 25 mg of homogenized nasal tumor tissue using sodium dodecyl sulphate (SDS) lysis, proteinase K digestion and phenol:chloroform:isoamyl alcohol (25:24:1 v/v, Invitrogen) extraction as described previously (147). Genomic DNA was extracted from cultured tumor cells using the QIAamp DNA mini kit (Qiagen) in accordance with the manufacturer’s protocol.

Table 2-2. Primers used to amplify exogenous ENTV-1NA

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<tr>
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<tr>
<td>IIIF</td>
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<td>IIIIR</td>
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<td>7776-7795</td>
</tr>
</tbody>
</table>

*aProviral genome coordinates refer to the base location starting from the beginning of the 5’ LTR.

Hemi-nested PCR

500 ng of genomic DNA isolated from ONT cells was subjected to amplification using exogenous ENTV-1 specific primers designed to span the U3 (U3F1 5’-GGG CTC AGA TGT TTT TTG GTT ATG CA-3’) to the gag (GAGR 5’-AGG GAC GCG AAT GTA GG-3’). DNA template integrity was assessed in a separate PCR reaction using GAPDH primers (148). 5PRIME MasterMix was used and the PCR reaction and cycling conditions were the same as described above for both rounds of PCR. For the nested PCR amplification, 2 ml of the first round reaction was used as template with an internal forward primer in the U3 (U3F2 5’-ATG ATC TTA AGT CAC CTA AGT TGC C-3’) and the original GAGR reverse primer.
Amplification of ENTV-1 proviral DNA

Provirus was amplified from tumor-derived genomic DNA using ENTV-1-specific primers (Table 2-2). Primers were designed against regions of dissimilarity identified by ClustalW alignment of ENTV-1_EU and enJS56A1 sequences (NCBI accession numbers NC_007015 and AY326472, respectively). PCR was performed on 100-500 ng of genomic DNA using PfuUltra II Fusion HS (Stratagene) DNA polymerase. PCR conditions were as follows: 1× PfuUltraII reaction buffer (providing 2 mM Mg²⁺), 10 mM each dNTP, 40 pmol each primer and 1U PfuUltraII Fusion HS DNA polymerase in a total volume of 50 µL. Cycling conditions were as follows: 95°C for 2 min followed by 30 cycles of 95°C for 20s, 56°C for 20s and 72°C for 40s (except for primer pairs II and III, which had extension times of 50s and 80s, respectively). A final extension of 3 min at 72°C concluded the program. PCR products were purified with either a PureLink quick gel extraction kit or PureLink PCR purification kit (Invitrogen). Purified PCR products were cloned into the pGEM-T Easy vector (Promega) following the manufacturer’s instructions and purified plasmid DNA sequenced with an ABI Prism DNA Sequencer. Internal primers were designed based on the initial sequence data for ENTV-1NA4 and sequencing of all subsequent amplicons was performed on gel purified PCR products.

Sequence and phylogenetic analysis

Full-length genomes were assembled using Lasergene v8.0 software (DNASTAR, Inc) and Vector NTI Contig Express (Invitrogen). Compiled sequences were analysed and sequence similarity examined using ClustalW (http://align.genome.jp/) (149) and the SIMPLOT v.3.5.1 software package (150). The K_a/K_s-Calculator program was employed to calculate K_a/K_s ratios (151). MEGA4 was utilized for phylogenetic analysis (152) and MatInspector was used for transcription factor binding site analysis (153). The program Knotinframe
(http://bibiserv.techfak.uni-bielefeld.de/cgi-bin/knotinframe) was used to predict ribosomal frameshift/slippery sequences (154).

**Nucleotide sequence accession numbers**

All of the sequences described in this manuscript are available in the GenBank Nucleotide Sequence Database under the accession numbers found in Table 1.

**Results**

**Clinical findings and gross pathology**

Nine domestic sheep from Ontario, Canada and one from Minnesota, USA were recruited for this study. The sheep, including Dorset, Suffolk and Leicester breeds, (Table 2-1), were females between 1.5 to 7 years of age presenting with signs of weight loss, chronic nasal discharge and difficulty breathing. Several sheep with nasal tumors were from the same farm (e.g. four from farm #2) but collected at different time points, thereby making it possible to assess the level of ENTV-1 nucleotide divergence within a given farm. In all cases, the sheep were examined at necropsy and confirmed to have nasal adenocarcinoma. The typical postmortem finding was a mass in the ethmoidal area of the nasal cavity, usually bilateral, ranging from 2 to 10 cm in diameter. In general, the cranial part of the mass appeared white and gelatinous (Figure 2-1A), while the caudal part of the mass tended to be more nodular with brownish-red areas of hemorrhage (Figure 2-1B). The caudal part of the mass often destroyed the ethmoid turbinate area, but never traversed the cribiform plate to the brain (Figure 2-1C). No lesions were observed in any other organ with the exception of the trachea, which was occasionally filled with white froth. One case, a 3-year-old Suffolk ewe (sheep #1), was found dead and submitted to the Animal Health Laboratory, University of Guelph for scrapie surveillance. There was no
history of nasal discharge or respiratory stridor, but a multi-nodular, well circumscribed, mostly pearly white, space-occupying mass was found within the nasal cavity (sagittal section Figure 2-1D) as an incidental finding.

![Figure 2-1. Gross photograph of ENA affected sheep](image)

Macroscopic findings in sheep naturally infected with ENTV. Tumor masses are demarcated by dotted lines. (A) Unilateral mass in the cranial nasal cavity with a soft, gelatinous consistency. (B) Bilateral tumor in the posterior (caudal) part of the nasal cavity containing brownish-red areas of hemorrhage. (C) The caudal aspect of a nasal tumor replacing the ethmoid turbinate area, but not traversing the cribiform plate to the brain. (D) Sagittal section of the nasal cavity of a sheep (sheep # 1) with asymptomatic ENA.

**Histopathology**

The most common and typical appearance of the nasal masses was a gradation of phenotypic differentiation cranial to caudal, with the more differentiated regions being cranial and the more invasive and anaplastic being caudal. There was a distinct transition from normal nasal epithelium to a hyperplastic, papillary and then more glandular appearance. In the mass, the cells were arranged in acinar, tubular or papillary arrangements (Figure 2-2A). Individual cells
were well differentiated and regular in size, being cuboidal to columnar in shape with some distinct cilia on their apical surface. The nuclei were round to oval with a central nucleolus and a two-fold variation in nuclear size. Mitoses were rare. In all cases, IHC with an envelope-specific monoclonal antibody (Mab) (146) was most intense on the apical cell membrane (Figure 2-2B). All isotypes and tissue controls were negative (data not shown). In one case (sheep #1), the nasal mass had a more gelatinous appearance nearer to the tip of the nostril, but was confirmed to be neoplastic in nature and not an inflammatory polyp. Nevertheless, this section of the tumor did not stain positive for ENTV-1 Env by IHC (data not shown), nor was it possible to amplify proviral DNA from this section of the tumor.

ENTV Env protein-positive cells were found in the lungs of an ENTV-1 infected (sheep #3) from farm #2 (Figure 2-2C and D). Foci of Env positive cells could be detected in multiple lung sections, with two to three clusters of positive cells per section. SP-C staining could not discriminate between lung and nasal tumor as both expressed the unprocessed (Figure 2-2E) and the processed form (Figure 2-2F) of SP-C. PCR was conducted on genomic DNA isolated from lung tissue sections to determine whether the Env-positive cells were infected with JSRV or ENTV. Amplicons were only observed when ENTV Env-specific primers were used (Figure 2-2G) suggesting that the Env positive cells detected in IHC were not the result of JSRV co-infection (Figure 2-2H).
Figure 2-2. Photomicrograph of ENA affected sheep

(A) Hematoxylin and eosin stained section of nasal tumor from an ENA affected sheep. (B) Envelope protein-specific monoclonal antibody staining of a nasal tumor from the same ENA affected sheep. (C) and (D) Envelope positive cells in the lungs of an ENTV-1 infected sheep. Pro-SP-C (E) and SP-C (F) staining of nasal tumors from ENTV-1 infected sheep. Panels A and B, 20X, scale bar 200 mM; Figures C to F, 40X, scale bar 100 mM.

Amplification of genomic DNA isolated from serial sections of lung tissue (SL1, SL2 and SL3) shown in part C and D using ENTV Env specific (G) or JSRV Env specific (H) primers. ENA genomic DNA was used as positive control (ENA) for the ENTV Env specific primers and JS7 genomic DNA (JS7) as a positive control for the JSRV Env specific primers. Genomic DNA isolated from healthy sheep lung tissue (normal lung) was used as a negative control.
Culturing ovine nasal tumors

Primary cultures were established from neoplastic and normal tissues extracted aseptically from sheep nasal turbinates. The ONT cells had an epithelial, cobblestone-like appearance (Figure 2-3A) and stained positive for pan-cytokeratin (Figure 2-3B). Nasal epithelial cells derived from healthy nasal turbinates could be maintained for only three passages. Neoplastic cells were passaged four times before single colonies were isolated. Nine clonal and one polyclonal line derived from three different tumors were maintained for 10 passages at which point the three most robust clones were selected for long-term culturing and have survived more than 30 passages. Envelope protein expression was detected by western blot during early passages, but expression waned after three to four passages (data not shown). Hemi-nested PCR for exogenous ENTV-1 was conducted on nine passage five clonal lines and one polyclonal line. In all cases, the original tumor produced a PCR product of the expected size without the need for hemi-nested PCR (Figure 2-3C, top panel, +). For the polyclonal cells, a PCR product corresponding to ENTV-1 proviral DNA was detectable without the need for hemi-nested PCR (Figure 2-3C, top panel, poly) whereas proviral DNA was detectable only after hemi-nested PCR in four out of nine clones (C2, C5, C6 and C11) in at least two out of three replicates (Figure 2-3C, middle panel). When the clones that were selected for long-term maintenance were screened for the presence of the ENTV-1 provirus all were negative and none were able to form colonies in soft agar (data not shown).

Continuous ONT cells were further characterized for surfactant protein (SP) gene expression by RT-PCR. SP-A was detected in both ONT and JS7 cells (118, 155). SP-B and SP-D were also detectable in both cell lines but the RT-PCR product was more intense for the JS7 cells,
suggesting that SP-B and SP-D are expressed at slightly higher levels in OPA-derived cells. SP-C was not detected in either cell line (data not shown).

![Figure 2-3](image)

**Figure 2-3. Detection of ENTV-1 provirus in ovine nasal tumor (ONT) cells.**

(A) Phase contrast image of ovine nasal tumor derived cells displaying an epithelial phenotype. (B) Pancytokeratin staining of ONT cells confirming epithelial origin. (C) Amplification of genomic DNA isolated from ONT cells (C1-C15 and Poly), normal sheep lung (-) and ENA nasal tumor (+) using exogenous ENTV-1 specific first round primers [U3F1 and GAGR] (top panel) and exogenous ENTV-1 specific hemi-nested primers [U3F2 and GAGR] (middle panel) and GAPDH primers (bottom panel). M indicates molecular weight marker.

**Sequencing ENTV-1 provirus**

The complete ENTV-1 proviral genome was obtained from 10 nasal tumors using primers designed to selectively amplify exogenous ENTV-1 (Table 2-2; Figure 2-4A). Genomic DNA isolated from lungs of healthy sheep was used as a negative control (Figure 2-4B). A consensus sequence for the North American ENTV-1 genome was determined (ENTV-1<sub>NA</sub>) and compared to that of ENTV-1<sub>EU</sub>, ENTV-2 (accession no. NC_004994), JSRV (accession no. AF105220) and the OEBRV, enJS56A1 (accession no. AF153615) using Clustal W (149). Comparison of the
nucleotide and amino acid sequence of the LTR, Gag, Pro, Pol and Env regions revealed that ENTV-1\textsubscript{NA} is highly homologous to ENTV-1\textsubscript{EU}. For most viral genes, ENTV-1\textsubscript{NA} is greater than 95% identical at the nucleotide level and greater than 98% identical at the amino acid level to ENTV-1\textsubscript{EU} (Table 2-3). Using the K\textsubscript{s}/K\textsubscript{s} Calculator program (151), which is designed to calculate the ratio of non-synonymous (K\textsubscript{s}) to synonymous (K\textsubscript{s}) mutations (K\textsubscript{s}/K\textsubscript{s}) for protein-coding gene sequences, ratios of 0.035 (p= 7.5E-29), 0.001 (p= 9.3E-13), 0.054 (p= 1.2E-34) and 0.043 (p=1.9E-31) were calculated for the gag, pro, pol and env protein-coding gene sequences respectively, when comparing the ENTV-1\textsubscript{NA} consensus sequence to ENTV-1\textsubscript{EU}. These ratios are significantly lower than the value of 1 expected under conditions of selection neutrality (151).

Similar analysis of individual ENTV-1\textsubscript{NA} isolates to the consensus sequence could not be performed due to the limited number of amino acid differences.

**Figure 2-4. ENTV-1 sequencing scheme**

(A) The relative location of oligonucleotide primers used to amplify overlapping fragments (I, II and III) of the ENTV-1 genome and (B) a representative gel of the resulting PCR products. ENA genomic DNA was used as a template in the positive lane and genomic DNA isolated from healthy sheep lung tissue was used as a template in the negative lane.
Table 2-3. Comparison of percentage nucleotide and amino acid identity of the ENTV-1NA consensus sequence with ENTV-1EU, ENTV-2, JSRV and enJS56A1

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* accession number AF105220
* accession number AF153615

FL – full-length, Ntd – nucleotide identity, Protein – amino acid identity, SP – signal peptide, SU – surface subunit, TM – transmembrane subunit

**U3 alignment reveals variability in this region**

The LTR of North American ENTV-1 isolates ranged from 367 to 378 nucleotides in length and this variability was due to deletions in the U3 region of the LTR (Figure 2-5). Eight of the
nucleotide differences common to all of the North American isolates of ENTV-1 relative to ENTV-1\textsubscript{EU} were similar to that of ENTV-2 and all were within a variable region of the U3 between nucleotides 118 to 243. There were only nine unique nucleotide changes that were not found in either ENTV-1\textsubscript{EU} or ENTV-2, thus the core group of transcription factors required for initiating transcription from the promoter and enhancer elements in the U3 of these viruses does not differ significantly between the European and North American isolates. ClustalW alignment of the LTR revealed a twelve-nucleotide deletion in the U3 region of ENTV-1\textsubscript{NA6} and ENTV-1\textsubscript{NA7} relative to the remaining North American ENTV-1 sequences, ENTV-1\textsubscript{EU} and ENTV-2 (Figure 2-5). \textit{In silico} analysis of transcription factor binding sites showed that the deleted region, which is located within a tandem repeat, corresponds to a putative binding site for the growth factor independence 1 (Gfi1) transcription factor (156).

![Figure 2-5. LTR alignment](image)

Alignment of the LTR nucleotide sequence of all ten ENTV-1\textsubscript{NA} isolates, ENTV-1\textsubscript{EU} and ENTV-2. ENTV-1\textsubscript{EU} was used as the reference sequence (top line) and only differences in the nucleotide sequence are illustrated. Dots indicate identical nucleotides and dashes indicate deletions. The R region is underlined and the U3 and U5 regions are indicated by elbow bend arrows. The deletion of a putative Gfi1 binding site is highlighted in black. The tandem repeats are indicated by narrow black lines.
Gag alignment reveals a variable polyproline region in the matrix

The ENTV-1\textsubscript{NA} consensus sequence and ENTV-1\textsubscript{EU} Gag are 99% identical at the amino acid level. The few differences are localized to two regions that have previously been described as variable regions 1 and 2 (VR1 and VR2) (70, 157). VR1 and VR2 fall within the matrix (MA) and p12 domains of the Gag polypeptide, respectively (Figure 6). The ENTV-1, ENTV-2, and JSRV MA contains a stretch of four, six and seven consecutive proline residues, respectively (Figure 2-6, inset). ClustalW alignment of the MA from ENTV-1\textsubscript{NA} isolates revealed an in-frame trinucleotide insertion resulting in an additional proline in the VR1 polyproline tract in four of the ten isolates sequenced. The proline insertion was present in all but one of the sequences (ENTV-1NA2) derived from farm #2, and was also present in the sequence from farm #1 (ENTV-1NA1). Two of the amino acid changes in VR2 were located immediately upstream of the two late domain motifs, PSAP and PPAY, which remain intact in all isolates (Figure 2-6).
Figure 2-6. Gag alignment

ClustalW alignment of the predicted amino acid sequence of the amino terminal portion of the Gag polyprotein from ten ENTV-1NA isolates, ENTV-1EU, and ENTV-2. ENTV-1EU was used as the reference sequence (top line) and only changes in the amino acid sequence are illustrated. Dots indicate identical amino acids and dashes indicate deletions. Elbow bend arrows indicate the predicted cleavage sites between the MA, p18, p12, and CA peptides. VR1 and VR2 regions are underlined and inserted proline residues are highlighted in black. The inset image is a more detailed alignment of the proline-rich VR1 region including JSRV (accession number AF105220) and the OEBRV, enJS56A1 (accession number AF153615).
**Pro**

The *pro* open reading frame encodes a bifunctional protease/dUTPase of 326 amino acids (119). As with other retroviruses, Pro is expressed as a polyprotein with Gag by a mechanism involving ribosomal frameshifting - the exact site of which and relative efficiency have yet to be determined. The frameshift/slippery sequence prediction program Knotinframe (http://bibiserv.techfak.uni-bielefeld.de/cgi-bin/knotinframe) (154) identified three potential slippery sequences within the overlap between the *gag* and *pro* ORFs; however the 1954GGGAAAC1960 motif was the most favoured. This putative frameshift signal closely resembles the *gag/pro* frameshift of MMTV and is likely the start of the *pro* coding sequence (8, 93). Overall, the Pro region of ENTV-1*NA* was highly homologous to that of ENTV-1*EU* and showed very little nucleotide or amino acid variability.

**Pol**

The Pol protein is synthesized as a polyprotein with Gag-Pro and is cleaved by Pro to produce reverse transcriptase (RT) and integrase (IN) after virus assembly. The frameshift/slippery sequence prediction program Knotinframe failed to predict a slippery sequence within the overlap between the *pro* and *pol* ORFs. Instead, a conserved 2844GGGG2847 motif immediately downstream of the beginning of the *pol* ORF has been suggested to function as a frameshift signal (93). As with *pro*, the *pol* genes of ENTV-1*NA* isolates are highly homologous to that of the European isolate with 98% amino acid identity. Nearly every amino acid difference was the result of a synonymous substitution.
Orf-x from ENTV-1_{NA} has a longer coding sequence than ENTV-1_{EU} and may encode a functional protein

Although JSRV and ENTV-2 are classified as simple retroviruses they both contain an additional open reading frame, termed orf-x, entirely inside the pol gene (14). For JSRV, a spliced transcript that would support translation of a 179 amino acid Orf-x protein has been identified in tumor cells and transfected cell cultures; however, efforts to detect expression of the Orf-x protein have not been successful (17). ENTV-2 Orf-x shares the same initiating methionine as that of JSRV but is truncated by a stop codon at amino acid 166. ENTV-1_{EU} Orf-x, on the other hand, is truncated by two stop codons at residues 86 and 130 and is initiated from a methionine 13 amino acids downstream from the initiating methionine of the JSRV Orf-x (Figure 2-7).

ClustalW alignment of the ten ENTV-1_{NA} Orf-x amino acid sequences revealed that ENTV-1_{NA} Orf-x initiates at the same methionine as JSRV Orf-x but that it truncates at amino acid 130, rather than 86 as is the case for ENTV-1_{EU} Orf-x (Figure 2-7). Several amino acid differences were detected within Orf-x when individual ENTV-1_{NA} sequences were compared to each other. The amino acid sequence divergence among the ENTV-1_{NA} isolates was much greater in Orf-x than in any other viral protein. Despite this sequence divergence, the location of the initiating methionine and the lack of a stop codon at residue 86 was completely conserved in all of the sequences analysed.
Figure 2-7. Orf-x alignment

ClustalW alignment of the predicted amino acid sequence of Orf-x from ten ENTV-1NA isolates, ENTV-1EU and ENTV-2. The Orf-x sequence of JSRV was used as the reference sequence (top line) and only differences in the amino acid sequence are illustrated. Dots indicate identical amino acids and dashes indicate deletions. Amino acid differences between the ENTV-1NA sequences and ENTV-1EU are shaded in black while amino acid differences among the ENTV-1NA isolates are shaded in grey. The light grey box highlights amino acids upstream of the first methionine in ENTV-1EU Orf-x, which is denoted by the arrowhead.

Env protein is highly homologous despite geographical differences

There were only nine amino acid differences between the Env proteins of ENTV-1NA and ENTV-1EU, three of which were located in the surface (SU) domain, after the signal peptide sequence (Figure 2-8). The only amino acid differences in the cytoplasmic tail (CT) were limited to the carboxy terminus, in a region deemed to be dispensable for transformation by JSRV Env (158). One of the amino acid changes in the CT was conserved in two partially sequenced ENTV isolates from Africa (159); (accession numbers AY271312 and AY271313). The three tyrosine residues found in the ENTV-1EU CT, which are known to be essential for Env-mediated transformation (111), were conserved among all ENTV-1NA isolates.
Figure 2-8. Envelope protein alignment

Alignment of the envelope protein amino acid sequence from ten ENTV-1NA isolates, ENTV-1EU and ENTV-2. ENTV-1EU was used as the reference sequence (top line) and only differences in the amino acid sequence are illustrated. Dots indicate identical amino acids and dashes indicate deletions. Elbow bend arrows indicate the predicted cleavage sites between the SP, SU and TM domains. As predicted for JSRV by Caporale et al (166), the putative nuclear localization signal (NLS) and nuclear export signal (NES) motifs are outlined with boxes and the putative ARM domain is underlined. The cytoplasmic tail (CT) is underlined and tyrosine residues are denoted by ↓.

Phylogenetic analysis of ovine betaretroviruses

Two unrooted bootstrapped neighbor-joining trees were generated; one based on full-length genome sequences (Figure 2-9A) and the other based on sequences comprising the end of env into the U3, so as to include partial sequence information of ENTV-1 isolates from Africa and the United Kingdom (Figure 2-9B). The phylogenetic analysis showed a distinct separation of JSRV and ENTV-2 from the ENTV-1NA isolates and a clustering of ENTV-1NA isolates with ENTV-1EU (Figure 2-9A). Viruses originating from farm #2 (ENTV-1NA2, ENTV-1NA3,
ENTV-1NA4 and ENTV-1NA5) and from farm #3 (ENTV-1NA6 and ENTV-1NA7) formed two separate groups within the ENTV-1_{NA} cluster. While the virus obtained from the tumor of an asymptomatic animal (ENTV-1NA1) clusters with the rest of the ENTV-1_{NA} isolates, it had an earlier evolutionary divergence from the group. Phylogenetic analysis of env/U3 sequences (Figure 2-9B) had ENTV-1 strains from Europe and Africa clustered together, separate from the ENTV-1_{NA} isolates. However, unlike JSRV, which has been postulated to form two distinct lineages (Type I and Type II) (73), ENTV-1 isolates are not divergent enough to be classified as two groups.

Similarity plot analysis (Figure 2-10) demonstrates that ENTV-1_{NA} is most similar to ENTV-1_{EU}, with the largest region of sequence divergence occurring in the U3 (~90% similarity). The JSRV and enJS56A1 sequences show less similarity to ENTV-1_{NA} than to ENTV-1_{EU} along the entire length of the genome, with four distinct regions of decreased homology (VR1-4). The fourth region of reduced sequence identity, not previously identified, was located within the U3 and represents the area of the genome with the most dramatic sequence divergence. In correlation with the identity values observed in Table 2-3, the ENTV-2 sequence shows the least similarity to ENTV-1_{NA}. Interestingly, there was an increase in nucleotide sequence similarity among all ovine betaretroviruses within an area of the pol gene that coincides with the location of the orf-x open reading frame.
Figure 2-9. Phylogenetic analysis of ovine and caprine betaretroviruses

Phylogenetic analysis of the evolutionary relationship between the (A) full-length genome sequence and the (B) env cytoplasmic tail/U3 region of the 3’ LTR of ten ENTV-1NA isolates, ENTV-1EU, ENTV-2, and three JSRV isolates. For the env cytoplasmic tail/U3 alignment, four additional sequences from sheep affected by ENA in Africa [ENTV-1ov10, ENTV-1ov8; accession numbers AY271312 and AY271313, respectively] and Spain [ENTV-ITNO35, ENTV-ITNO29; accession numbers AY196359 and AY196358] were included. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. All positions containing gaps and missing data were eliminated from the dataset. Phylogenetic analyses were conducted using MEGA4.
Figure 2-10. Similarity plot analysis of full-length ovine and caprine betaretroviral genomes.

Similarity plot analysis of the full-length genome (from R to R) sequences of ovine and caprine betaretroviruses, with proviral structures depicted on top. For analysis, the SIMPLOT v.3.5.1 software package Simplot 2.5 (http://sray.med.som.jhmi.edu/RaySoft) was used with a 200-bp window size in 20 step increments. Nucleotide position is represented on the horizontal axis and the similarity values with respect to the ENTV-1NA consensus sequence are represented on the vertical axis. Colours represent the corresponding proviral sequences.

Discussion

Here, we describe the genetic composition of ENTV-1 from clinically affected sheep in Canada and the United States. Understanding the genetic heterogeneity of ENTV-1 is important both as a tool for epidemiological studies and as means to clarify the origin and future evolution of ENTV-1. Phylogenetic analysis of ENTV-1 from naturally affected sheep and ultimately, construction of an infectious molecular clone of ENTV will make it possible to identify infected animals and to study the pathogenesis of ENTV in an experimental setting.

This is the first report demonstrating the presence of ovine betaretroviral Env proteins in the lungs of ENTV infected sheep as we found Env-positive cells in the lungs of one ENTV-1-
infected sheep. The Env-positive cells were found in several locations within the lungs and their nuclei were similar to alveolar type II cells seen nearby. We tried to verify their origin by staining the cells with a SP-C-specific antibody but both the nasal tumor and the lung parenchyma expressed SP-C. These ENTV-positive cells in the lungs could be aspirated nasal tumor cells, but their number and depth in the lung makes this less likely. PCR analysis on lung-derived tissue confirmed the presence of ENTV-1 viral sequence and that the cells were not infected or co-infected with JSRV (160).

Three different clonal nasal tumor-derived cell lines were established and all survived more than 30 passages. Regrettably, none of these clones contained the ENTV-1 provirus as verified by hemi-nested PCR. Since we could not detect Env protein expression by western blot (data not shown), it is unlikely that only the env gene was retained explaining the continuous nature of these cells. While only three clonal lines were selected for long-term growth, multiple clones were established and screened for the presence of integrated provirus. At passage five, a subset of clones were found to retain the provirus. These results are similar to previous attempts to isolate neoplastic cells from OPA lesions where JSRV-infected alveolar type II cells had a decreased lifespan compared to a subpopulation of neoplastic JSRV-negative cells (116). Efforts to establish OPA-derived cell lines resulted in the loss of integrated JSRV by passage five (116). Only one group has successfully derived a cell line with integrated provirus from JSRV-induced lung tumors and this required initial removal of tumor macrophages (118, 155, 161).

Nasal tumor cells were found to express the same SP genes as normal human nasal epithelial (NHNE) cells (SP-A, SP-B and SP-D) (162), suggesting that they have retained some of their normal phenotype and thus might be useful for propagating ENTV-1. Efforts are being made to
culture provirus-containing cells by early removal of macrophages and growth in three-dimensional culture conditions.

To characterize the strains of ENTV-1 in North America, we generated a series of exogenous specific primers that permitted amplification of the ENTV-1 proviral genome from ten clinical samples of ENA and subsequent phylogenetic analysis of this poorly characterized virus. To the best of our knowledge, this is the most comprehensive phylogenetic analysis of any member of the betaretrovirus genus, and especially ENTV-1. ENTV-1 sequences from different sheep reared on the same farm were nearly identical, confirming the genetic stability noted previously by Ortín et al. (93). The ENTV-1\textsubscript{EU} sequence, which was deciphered in 1999, is 96% identical to the ENTV-1\textsubscript{NA} consensus sequence suggesting that the ENTV-1 genome is relatively stable.

Similarly, three full-length exogenous JSRV genomes isolated over a 13 year period from 1992 to 2005 [NC\textsubscript{001494} (163), AF105220 (117), AF357971 (118)] share between 93% to 99% sequence identity and isolates of mouse mammary tumor virus (MMTV) (JYG-MMTV and BR6-MMTV) share over 96% sequence identity (164). This genomic stability is in contrast to the variability observed in the natural quasispecies of maedi visna virus (MVV) and caprine arthritis encephalitis virus (CAEV) which vary from 84% in \textit{gag} and \textit{pol} to 78% in \textit{env} and 65% in LTR (165). Taken together, it would appear that betaretrovirus genomes are more stable than other members of the retrovirus family, possibly with the exception of human T-lymphotropic virus type I (HTLV-1) (166) and feline leukemia virus (FeLV) (167).

The high conservation rate of ENTV-1 could be attributed to the fidelity of the reverse transcriptase enzyme, which is known to have a major influence on the mutation rate (168). It is possible that due to OEBRV protein expression during development (76, 78), the ovine betaretrovirus reverse transcriptase has evolved to be inherently less error prone in order to
prevent the inadvertent induction of an autoimmune response against OEBRV proteins.
Alternatively, since ENTV infection causes cell proliferation, the limited sequence diversity observed among isolates may be achieved through genome replication by the higher fidelity host DNA polymerase during clonal proliferation, as is thought to be the case for HTLV-1 (169).
Additionally, the host immune system may eliminate viral variants that differ significantly from OEBRV thus explaining the low prevalence of ENA in an affected flock (1-15%). Caporale et al reported that induction of pulmonary adenocarcinoma is not the most common outcome of naturally occurring JSRV infection and that the incidence of infection in an OPA-affected flock is likely much higher than previously thought (89). A recent study using computed tomography to monitor tumor development in neonatal lambs infected with JSRV found that regression of tumors was more common than progression. CD3+ T cell infiltration and low levels of serum-neutralizing activity were detected and were more prevalent in sheep with spontaneous regression of JSRV-induced OPA (170). While no evidence of circulating antibodies is described in animals affected by either ENA or OPA using antigens from natural sources (83, 92, 139, 171), Summers et al were able to induce JSRV-specific antibodies and T-cell responses in animals immunized with recombinant JSRV capsid protein and inactivated whole virus, respectively (172). Sheep could therefore mount a humoral and cell-mediated immune response against JSRV and ENTV. Identifying the mechanism of purifying selection causing the genomic stability observed for ENTV-1 requires an experimental infection model.
The limited sequence diversity observed in the ENTV genome seems to occur at discrete loci or variable regions in the Gag polyprotein, the envelope cytoplasmic tail and the U3 region of the LTR (Figure 1-10). The part of the genome that differed the most between European and North American ENTV-1 isolates was the LTR, the only untranslated region of the genome. Two of the
isolates from the same farm (ENTV-1NA6 and ENTV-1NA7) had a 12 nucleotide deletion in the hypervariable region of the U3, just upstream of a region where ENTV-2 incurred a 10 base pair insertion (Figure 2-5). This suggests that the LTR is susceptible to mutation. Within this hypervariable region there are two tandem repeats. These are more pronounced in JSRV, but are also present in ENTV. Small deletions and duplications in retroviral genomes are thought to be caused by template slippage or mispriming at direct repeats during reverse transcription (173), and this may explain why the LTR, in particular, is more susceptible to deletions and insertions. The insertion found within the gag gene also occurred in a stretch of proline repeats; therefore, almost every instance of insertion or deletion was in repeat regions of the genome. Whether this tendency for insertion or deletion within tandem repeat regions of the ENTV LTR plays a role in pathogenesis has yet to be determined. There is evidence that altering the number of tandem repeats may regulate gene expression in cells. In *Saccharomyces cerevisiae*, tandem repeats are often located in nucleosome-free regions of promoters and variations in repeat length resulted in changes in expression and local nucleosome positioning (174). Additionally, many of the promoter tandem repeats were A/T-rich, as are tandem repeats in the LTR of ENTV and JSRV, and this may facilitate DNA melting (174). Therefore, tandem repeats may be variable elements in ovine betaretroviral LTRs that facilitate evolutionary fine-tuning of gene expression by affecting local chromatin structure. Deletion of this tandem repeat from ENTV-1NA6 and ENTV-1NA7 may have removed a Gfi1 binding site (consensus DNA recognition sequence taAATCac(t/a)gca) (156). The putative Gfi1 binding site in ENTV-1 (aaAATCagtaat) shares 81% identity with the consensus sequence according to Zweidler-McKay (1996) (156). Gfi1 is a ubiquitously expressed zinc-finger proto-oncogene known to be required for development and differentiation of erythroid and megakaryocytic cell lineages (175). Gfi1 functions as a
transcriptional repressor that promotes silencing of its target gene promoters by recruiting histone methyltransferases to cause histone methylation and heterochromatin formation (176). Gfi1 binding sites are common in the promoter region of genes highly expressed in the lung and nasal mucosa (177). Electrophoretic mobility shift assays will be necessary to determine whether Gfi1 binds to the ENTV-1 LTR. The two sheep from which the LTR deletion mutant was isolated were significantly younger than the other animals at 1.5 years of age, so this virus might be more virulent.

There was very little amino acid diversity in the Gag polyprotein except in the two domains defined as variable (VR1 and VR2) (157), suggesting that this part of the genome may be able to withstand change. The trinucleotide insertion resulting in the expansion of a polyproline tract within VR1 was found in isolates from different farms so there may be some pressure for expansion in this region. This stretch of prolines is absent from OEBRV sequences as well as from other members of the betaretrovirus genus (70, 157). Mutation of this polyproline motif in JSRV had no effect on virus particle release nor did insertion of this proline motif into OEBRV rescue virus particle release (157). The significance of this stretch of proline residues is not known. ENTV has a shorter polyproline motif than JSRV (4 versus 7); however, nearly half of the ENTV-1NA isolates encoded an additional proline thus expanding this repeat from four to five prolines. The proline insertion was present in all but one of the sequences derived from farm #2 (ENTV-1NA2) but was also present in the ENTV-1NA1 sequence (sheep #1) from a separate farm (farm #1). This insertion event seems to be a recent occurrence and may have arisen simultaneously in two separate geographical locations.

Orf-x is the most genetically diverse protein coding sequence of ENTV-1. The high amino acid sequence conservation observed in all other areas of the genome compared to orf-x suggests that
orf-x may not encode a functional protein, as amino acid variability is unlikely to be localized to one particular open reading frame. While it is currently not known whether an orf-x transcript is produced in ENTV-1 infected cells, truncation of orf-x in JSRV did not alter the pathogenesis of the virus compared to wild type JSRV in experimental infection (16). Despite the apparent dispensable nature of orf-x, it is conserved in all exogenous JSRV isolates and an orf-x transcript is produced in OPA-derived lung tumors (17). It remains possible that the subgenomic RNA itself may serve some as of yet unidentified biological function.

When the highly conserved ENTV-1\textsubscript{EU} and ENTV-1\textsubscript{NA} Env sequences were compared to the JSRV Env protein, a minor sequence divergence in the signal peptide (SP) domain was noted. Previous analysis of the JSRV Env SP using the PSORTII NLS prediction software (178), identified a pat7 nuclear localization signal (NLS) motif \((2\text{PKRRAGF}_8)\) at the amino terminus (13). The amino acid sequence at the equivalent location in ENTV-1\textsubscript{EU} and ENTV-1\textsubscript{NA}, PKHRAGS, however, does not encode a pat7 NLS, which is defined as a proline followed by a stretch of four amino acids where three out of four must be basic residues. This sequence deviation in ENTV-1 Env also reduces the number of arginines from five to four in the putative arginine-rich RNA binding domain that was identified in the JSRV Env protein (13). Despite the apparent lack of an NLS, the putative nuclear export signal identified near the carboxyl terminus of the JSRV SP (13) is intact in ENTV-1. Nuclear localization of the JSRV SP facilitates nuclear export of unspliced viral mRNA (13). Whether the absence of an NLS in the ENTV-1 Env SP has any effect on nuclear export of unspliced viral mRNA remains to be determined.
Conclusions

This study represents the first description of full-length ENTV-1 sequences from North American sheep flocks and is the most comprehensive genomic analysis of any member of the betaretrovirus genus to date. ENTV-1 isolates were found to be highly conserved with less than 2% amino acid differences in the coding regions and less than 5% nucleotide differences in the non-coding regions between ENTV-1 isolated more than a decade apart from sheep living on different continents. Our results demonstrate that the genome of ENTV-1 is extremely stable and does not appear to be evolving except at discrete loci of variability (VR1-3). An additional variable region, termed VR4, was identified in the U3 region of the ovine betaretroviral LTR. Future efforts will involve application of our hemi-nested PCR protocol for preclinical diagnosis of ENTV-1 from nasal swabs and construction of an infectious molecular clone of ENTV-1 for subsequent pathogenesis studies.
Chapter Three:

Experimental transmission of enzootic nasal adenocarcinoma in sheep

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Abstract

Enzootic nasal adenocarcinoma (ENA) is a contagious neoplasm of the secretory epithelial cells of the nasal mucosa of sheep and goats. It is associated with the betaretrovirus, enzootic nasal tumor virus (ENTV), but a causative relationship has yet to be demonstrated. In this study, 14-day-old lambs were experimentally infected via nebulization with cell-free tumor filtrates derived from naturally occurring cases of ENA. At 12 weeks post-infection (wpi), one of the five infected lambs developed clinical signs, including continuous nasal discharge and open mouth breathing, and was euthanized. Necropsy revealed the presence of a large bilateral tumor occupying the nasal cavity. At 45 wpi, when the study was terminated, none of the remaining infected sheep showed evidence of tumors either by computed tomography or post-mortem examination. ENTV-1 proviral DNA was detected in the nose, lung, spleen, liver and kidney of the animal with experimentally induced ENA, however there was no evidence of viral protein expression in tissues other than the nose. Density gradient analysis of virus particles purified from the experimentally induced nasal tumor revealed a peak reverse transcriptase (RT) activity at a buoyant density of 1.22g/mL which was higher than the 1.18g/mL density of peak RT activity of virus purified from naturally induced ENA. While the 1.22g/mL fraction contained primarily immature unprocessed virus particles, mature virus particles with a similar morphology to naturally occurring ENA could be identified by electron microscopy. Full-length sequence analysis of the ENTV-1 genome from the experimentally induced tumor revealed very few nucleotide changes relative to the original inoculum with only one conservative amino acid change. Taken together, these results demonstrate that ENTV-1 is associated with transmissible ENA in sheep and that under experimental conditions, lethal tumors are capable of developing in as little as 12 wpi demonstrating the acutely oncogenic nature of this ovine betaretrovirus.
Introduction

Enzootic nasal adenocarcinoma (ENA) is a neoplasm of the secretory epithelial cells of the nose of sheep and goats (133). ENA tumors can arise unilaterally or bilaterally, originating from the ethmoid turbinate and often expanding to occlude the nasal cavity. No metastasis has been reported in ENA cases, but disruption of the nasal septum structure as well as erosion of the cribiform plate has been reported (142, 179). Due to the cell type transformed and the space-occupying nature of the tumor, the clinical signs of ENA include production of copious nasal exudate, open mouth breathing, dyspnea and facial asymmetry (133). A neoplasm of the secretory epithelial cells of the distal lung of sheep, called ovine pulmonary adenocarcinoma (OPA), is known to be caused by jaagsiekte sheep retrovirus (JSRV) (117) and a similar etiology is suspected for ENA (135, 136). ENA is associated with the betaretrovirus, enzootic nasal tumor virus (ENTV), which is genetically very similar to JSRV (119). ENTV is divided into two distinct sub-species, one infecting sheep (ENTV-1) (119) and the other infecting goats (ENTV-2) (93). Studies involving ENTV are hindered by the fact that there is no cell culture system for propagating the virus. Although a causal relationship between ENTV-1 infection and the development of ENA in sheep has not been proven, reverse transcriptase activity, ENTV-1 specific nucleotide fragments and antigens that cross react with antibodies against JSRV proteins are consistently found in nasal exudate as well as nasal tumor tissue (135, 179–181). ENA has been shown to be infectious in goats through intrasinus or intranasal inoculation of newborn goat kids with clarified nasal exudates pooled from three ENA affected goats (92). In 1953, Cohrs reported transmission of ENA in sheep using cell and bacteria-free tumor filtrate (182), but more recently, similar experiments attempting transmission of ENA in sheep were unsuccessful (Dr. James DeMartini, personal communication).
In this study, we tested the hypothesis that ENA can be induced in healthy 14-day-old lambs after exposure to nebulized cell-free tumor homogenate derived from sheep with ENA. We show that while the rate of tumor induction was low, clinical signs could be detected as early as 12 wpi. The clinical signs, histopathology, and tissue distribution of ENTV-1 provirus in experimentally and naturally infected animals were similar, thereby validating the experimental infection method used in this study and providing further support for the hypothesis that ENTV-1 is the causative agent of ENA in sheep.

**Materials and methods**

**Animals, inoculum, and sample collection**

The Animal Care Committee at the University of Guelph approved all animal use and related procedures. Five lambs born to dams from a research flock at the University of Guelph with no previous history of ENA were infected at 14 days of age with two mL of ENA inoculum. Two lambs were mock infected with vehicle alone and were housed with the experimentally infected animals. All lambs were examined by a veterinarian and were clinically healthy prior to infection.

The ENA inoculum, which was comprised of cell-free tumor homogenate, was prepared as follows. Equal amounts of tumor tissue (~1 g/tumor) from ten different ENA samples from North America (ENTV-1NA1 to 10; (179)) were combined and homogenized using a Warring blender. The homogenized tumor cell suspension was diluted in phosphate buffered saline (PBS) (10% w/v) and clarified at 18 000 × g for 40 min before concentration by ultracentrifugation in a SW31Ti rotor (Beckman Coulter Canada, Mississauga, Ontario, Canada) at 60,000 × g for 2 h at 4 °C. The resulting pellet was resuspended in 12 mL of PBS and passed through a 0.45 µm filter.
To ensure efficient delivery of the ENA inoculum to the entire respiratory tract, nebulization was employed. A mask possessing a rubber seal to optimize a tight fit around the nose was fabricated and fitted with an inhaler connector and a one way “T” valve. Conventional 6 mL misty-Neb nebulizer cups (Wilder Medical, Kitchener, ON, Canada) were employed to deliver the inoculum. Nebulization was performed using a PM14 compressor (Precision Medical Inc., Northampton, PA, USA).

Blood mononuclear cells (BMCs) were collected from infected and control lambs prior to inoculation and every two wpi and stored at −80 °C.

Animals were euthanized after the onset of clinical signs or, in the absence of clinical signs, at 45 wpi. At necropsy, the nose was serially cut transversely at ~2 cm intervals and samples of normal conchae and lesions were collected. Tissue samples collected at necropsy included, trachea, lung, submandibular lymph node, liver, spleen, and kidney. These samples were divided in half and stored at −80 °C for subsequent isolation of nucleic acids and protein or fixed in 10% neutral buffered formalin for 24 h prior to embedding in paraffin wax and sectioning.

**Computed Tomography (CT)**

Computed tomography of the head was performed using a GE Bright Speed 16-slice helical CT scanner. The scan parameters were 120kvp, 200mA, 1.25mm slice thickness, and 0.75 pitch. For the CT scan, lambs were anesthetized using routine veterinary methods for this species. Images were interpreted by a board certified veterinary radiologist (HC).

**Histopathology and immunohistochemistry**

Formalin-fixed tissues were trimmed, embedded in paraffin, sectioned at 5 µm and processed to obtain haematoxylin-eosin stained sections. The avidin-biotin-peroxidase complex (ABC)
method was used on paraffin-embedded tissue sections for immunohistochemical (IHC) demonstration of ENTV envelope protein expression as described previously (179). Neoplastic tissue was stained with antibodies to pankeratin (Cell Signaling, clone C11), CK7 (DAKO, clone OVTL 12/30) and vimentin (DAKO, clone V9).

**Heminested PCR (hnPCR)**

Genomic DNA was extracted from PBMCs and homogenized tissue samples using the Qiagen DNeasy blood and tissue kit (Qiagen) according to the manufacturer’s instructions. An exogenous ENTV-1 specific hnPCR assay was used to screen for integrated ENTV-1 provirus as described previously (179). Specificity of the PCR products was confirmed by sequencing. Genomic DNA extracted from the lung tissue of a healthy sheep served as a negative control.

**Buoyant density analysis**

Approximately 3 g of tumor tissue was homogenized as described above. Pelleted virus was resuspended in 1 mL of PBS, placed on a linear 20 to 60% (wt/wt) discontinuous sucrose gradient and centrifuged at 100,000 × g for 16 h at 4 °C in a SW41 rotor (Beckman Coulter Canada). 500 µL fractions were collected and their density determined using a refractometer (Fisher Scientific). Reverse transcriptase (RT) activity of each fraction was determined using the EnzChek RT assay kit (Invitrogen) according to manufacturer’s instructions.

**Western blot analysis**

Western blot analysis was conducted as described previously (183) using monoclonal antibodies specific for the envelope protein of ovine betaretroviruses (i.e. JSRV, ENTV and OEBRV) (146) and capsid (184) (kindly provided by Dr. Hung Fan, University of California, Irvine) proteins.

**Electron microscopy**
Buoyant density fractions containing the greatest RT activity were combined, diluted to 30 mL with PBS and placed on a 5 mL 20% sucrose cushion. Virus was pelleted by ultracentrifugation at 18,000 × g for 2 h at 4 °C and resuspended in 300 µL of HEPES buffer. Virus was then placed on a carbon grid, negatively stained with uranyl acetate and examined using a LEO 912ab transmission electron microscope at the Electron Microscopy Unit, University of Guelph.

Sequence analysis

Exogenous ENTV-1 specific primers (179) were used to amplify three overlapping fragments, covering the full-length ENTV-1 genome, from a genomic DNA extract of the experimentally induced tumor. The PCR products were sequenced directly and the full genome sequence designated, ENTV-1OVC, was analyzed using the MEGA5 software package.

Nucleotide sequence accession number

The nucleotide sequence of ENTV-1OVC was deposited in GenBank. Accession number: GenBank:KC189895.

Results

Tumor induction in a lamb after inoculation with ENA homogenate

In this study, we tested the hypothesis that ENA could be induced in 14-day-old lambs using a cell-free preparation of tumor homogenate derived from naturally occurring cases of ENA in sheep. ENTV-1 was detected in the ENA inoculum by RT activity (Figure 3-1A) and by immunoblot analysis with antibodies specific for ovine betaretroviral envelope and capsid proteins (Figure 3-1B). These results confirmed the presence of ENTV-1 antigen and reverse transcriptase activity in the inoculum. ENA inoculum was administered to five 14-day-old lambs (two mL each) using a nebulizer. Clinical signs were observed in one of the five infected lambs
at 12 wpi and included persistent nasal discharge, stridor, nostril flaring, head shaking, sneezing and open mouth breathing. The lamb was euthanized and a postmortem computed tomography (CT) scan was performed (Figure 3-2A). This showed a bilateral soft tissue density mass with poorly defined margins and a few pinpoint areas of mineralization. The mass was associated with adjacent sinusitis and fluid accumulation within the air spaces and sinuses. The mass was causing destruction of the bony cartilages of the nasal turbinates and occupied approximately 50% of the overall nasal cavity, and approximately 95% of the nasal cavity at the site where it was largest (Figure 3-2A).

Postmortem findings confirmed the results of the CT scan. The nose of the lamb was sectioned rostral to caudal, exposing a mass within both nasal cavities (Figure 3-2C). The tumor was approximately five cm in diameter and had a heterogeneous texture and colour, ranging from hard and pink to soft and red. It had several distinct nodules protruding from the surface. The lungs were palpated and inspected for tumors but no lesions were detected. The experiment was terminated at 45 weeks post-infection at which point the remaining four animals were anaesthetized and subjected to CT scan followed by euthanasia and necropsy. No signs of nasal tumor induction were observed in any of the four asymptomatic sheep either by CT (Figure 3-2B) or necropsy (Figure 3-2D).
Figure 3-1. Reverse transcriptase activity assay and immunoblot analysis of ENA inoculum.

The reverse transcriptase activity of the ENA inoculum was quantified using a fluorogenic based assay (A) and compared to a mock inoculum comprised of concentrated filtered supernatant from a sheep skin fibroblast cell line. (B) Immunoblot analysis of cell lysate from normal sheep nasal tissue (lane 1), ENA inoculum (lane 2) and cell lysate from a naturally occurring ENA tumor (lane 3) with envelope surface subunit (Env SU) and capsid (CA) specific antibodies. M indicates molecular weight marker.
Figure 3-2. Computed tomography and gross pathological analysis of sheep experimentally infected with ENA inoculum.

Three dimensional reconstructed computed tomography scan of sheep experimentally infected with ENA inoculum with (A) and without (B) nasal tumors. Serial sections of the nasal cavity of sheep experimentally infected with ENA inoculum with (C) and without (D) nasal tumors.
Histopathological identification of two tumor morphologies in Expt ENA

Several different regions of the experimentally induced ENA (Expt ENA) tumor were sectioned and separate adenosquamous and fibropapillomatosis components were found. The adenosquamous region (Figure 3-3A and B) had an outer layer of normally differentiating stratified squamous and keratinising epithelium which connected with underlying glandular epithelial cells that made up most of this component. The neoplastic glandular cells had twofold variation in nuclear size and five mitoses in 10 high-power fields.

The second region had a papillary appearance (Figure 3-3C and D) with an outer layer of normally differentiating, stratified squamous epithelium with long projections that extend into the underlying abundant fibrous tissue that made up most of this mass.

Tumor tissue from a case of Natural ENA (Figure 3-3E and F) had similar features to the adenosquamous region of Expt ENA, but lacked features characteristic of the fibropapillomatosis region.
Figure 3-3. Histopathology of nasal tumors from sheep experimentally infected with ENA inoculum and naturally acquired ENA.

Representative images of hematoxylin and eosin stained nasal tumors from sheep experimentally infected with ENA inoculum (A-D) and naturally acquired ENA (E, F). Representative images showing the adenosquamous (A, B) and fibropapilloma (C, D) component of the experimentally induced nasal tumor.
Immunohistochemical detection of ENTV envelope protein and cytokeratin 7 (CK7) in the adenosquamous region of Expt ENA

Immunohistochemical staining with a monoclonal antibody specific for the ENTV Env protein of ovine betaretroviruses (146) revealed a complete lack of staining of the normal respiratory mucosa (Figure 3-4A and B) but intense apical staining of epithelial cells comprising the Expt ENA tumor (Figure 3-4C and D). Within the tumor, there was strong surface and cytoplasmic staining of all epithelial cells displaying a glandular phenotype, but not of epithelial cells with a stratified squamous differentiation pattern. No specific staining of any of the stratified squamous epithelial cells or of the stroma of the fibropapillomatosis regions was observed. Staining of tumor tissue from a case of Natural ENA showed a very similar pattern (Figure 3-4E and F).

Staining with an anti-cytokeratin 7 antibody was strongly positive for all epithelial cells of the normal nasal mucosa including glands and luminal epithelial cells (Figure 3-5A and B). All cells with a glandular phenotype in the adenosquamous portion of the tumor had strong cytoplasmic staining (Figure 3-5C and D), but none of the cells in the fibropapillomatosis region had positive staining (Figure 3-5E and F). Since CK7 and envelope expression were observed in the adenosquamous region of the tumor but were lacking in the fibropapillomatosis region, this suggests that the adenosquamous region was derived from the tubuloglandular epithelial cells of the nose but that the fibropapillomatosis region was not. The fibropapillomatosis region was likely derived from stromal cell expansion induced to support the growth of the envelope-positive adenosquamous tumor region. Immunohistochemical staining for high molecular weight cytokeratin was absent from both the adenosquamous and the fibropapillomatosis regions of the tumor (data not shown).
Figure 3-4. Immunohistochemical staining for ENTV-1 envelope protein expression in nasal tumors from sheep experimentally infected with ENA inoculum and naturally acquired ENA.

Representative images showing a lack of envelope protein expression in normal sheep nasal epithelium (A, B) but robust expression on the apical surface of all cells within the adenosquamous portion of the experimentally induced (C, D) and naturally acquired (E, F) ENA.
Figure 3-5. Immunohistochemical staining for cytokeratin 7 (CK7) in experimentally induced nasal tumors and normal nasal mucosa.

Representative images showing low molecular weight CK7 staining in normal sheep nasal mucosa (A, B) as well as in the adenosquamous portion of the experimentally induced nasal tumor (C, D) and absence of staining in the fibropapilloma portion of the experimentally induced nasal tumor (E, F).
Tissue distribution of ENTV-1 proviral DNA

Genomic DNA was extracted from the nose, trachea, lung, mandibular lymph node, spleen, liver and kidney of experimentally infected sheep and analyzed for the presence of ENTV-1 proviral DNA using ENTV-1 specific hemi-nested PCR primers (179). All samples from mock-infected sheep were negative for virus (data not shown). ENTV-1 proviral DNA was detected in the nose of the sheep with experimentally induced ENA after the first round of PCR (Figure 3-6A). In a second round of hemi-nested PCR, proviral DNA was detected in the lung, spleen, kidney and liver, but not in the lymph node or the trachea of the sheep with the experimentally induced ENA (Figure 3-6B). Interestingly, when DNA extracted from the PBMCs of the experimentally infected sheep that developed a nasal tumor were analyzed using the hnPCR assay, ENTV-1 provirus could not be detected at any time, including two weeks after infection and two weeks prior to euthanasia (data not shown). For the experimentally infected sheep that did not develop ENA, ENTV-1 provirus was absent from all tissues examined (Figure 3-6C).

Tissues positive for ENTV-1 provirus were examined for viral protein expression by western blotting and immunohistochemical staining using an Env-specific monoclonal antibody. Using these methods, ENTV Env protein could only be detected in the nasal tumor tissue of the sheep with ENA (data not shown).
Figure 3-6. Tissue distribution of ENTV-1 provirus in sheep experimentally infected with ENA inoculum.

PCR (A) and hemi-nested PCR (B and C) amplification of exogenous ENTV-1 proviral DNA from genomic DNA extracted from various tissues of sheep experimentally infected with ENA inoculum. Distribution of ENTV-1 provirus in an experimentally infected sheep that developed a nasal tumor (A and B) and in a sheep that did not (C). Genomic DNA isolated from healthy sheep lung (-) and naturally acquired ENA (+) were used as a negative and positive controls, respectively. M indicates molecular weight marker.

Ovine betaretroviral virions detected in Expt ENA homogenate by buoyant density analysis and electron microscopy

Virus purified from the ENA inoculum and the Expt ENA tumor were subjected to buoyant density analysis on a discontinuous 20 to 60% sucrose density gradient. A total of 10 fractions of 500 µL were collected and analyzed for reverse transcriptase activity (Figure 3-7A), density (Figure 3-7A), and capsid protein content (Figure 3-7B). A peak of RT activity was observed in the ENA inoculum in fraction 6, which corresponded to a density of approximately 1.18 g/mL (Figure 3-7A). The adjacent fraction 7 had a density of 1.22 g/mL and also contained RT activity. By western blot, a 27 kDa protein corresponding to the capsid protein was detected in both fractions 6 and 7 (Figure 3-7B). Capsid protein was also detected in fraction 5 and 8. The Expt ENA sample showed an increase in RT activity in fraction 6 with a density of 1.18 g/mL; however the majority of RT activity in this sample was found in fraction 7, which corresponded
to a density of 1.22 g/mL (Figure 3-7A). Immunoblot analysis of Expt ENA fractions 6 to 8 revealed the presence of multiple capsid protein products of approximately 85, 78, 62, 57, 52, 40 and 27kDa (Figure 3-7B).

On electron micrographic analysis, the fractions from the buoyant density gradient with the highest RT activity (fraction 6 and 7) had virus particles of similar size and morphology in both the ENA inoculum (Figure 3-7C, left) and Expt ENA (Figure 3-7C, right) samples. Particles were spherical with a diameter of 80 to 120 nm. Located eccentrically was a spherical electron dense core. The particle surface was rough in appearance with the smooth membrane interrupted at even intervals by structured projections with an apparent trimeric symmetry. There was an abundance of cellular debris on the electron micrograph. No other viral particles were seen.
Figure 3-7. Buoyant-density and transmission electron micrograph analysis of ENA tumor homogenate.

(A) 20 to 80% (wt/wt) sucrose gradient isopycnic centrifugation of the original ENA inoculum and a preparation of cell free tumor homogenate from the experimentally induced nasal tumor. The RT activity (solid lines) and density (dashed line) of each of the ten fractions is shown. (B) Immunoblot analysis for ENTV-1 capsid protein in fractions one through ten. ENA inoculum was loaded as a positive control for capsid (lane C). (C) Transmission electron micrograph analysis of fractions six and seven of the ENA inoculum (left) and cell free tumor homogenate from the experimentally induced nasal tumor (Expt ENA; right) showing virus particles with typical betaretrovirus morphology.
**ENTV-1 sequences in the experimentally induced tumor are derived from ENTV-1NA9**

Overlapping fragments comprising the complete ENTV-1 provirus were amplified from the genomic DNA of Expt ENA tumor tissue and the full-length genome, designated ENTV-1OVC, sequenced. Tissues were taken from separate distinct areas of the nasal tumor and combined for DNA extraction and subsequent amplification. PCR products were sequenced in both directions and as reported previously (179), no variation or evidence of quasispecies was detected. Phylogenetic analysis showed ENTV-1OVC shared a node with ENTV-1NA9 and was thus most closely related to ENTV-1NA9 (Figure 3-8A). The ClustalW alignment of ENTV-1OVC differed at every nucleotide position in which ENTV-1NA9 differed from the consensus sequence of all ENTV-1 genome sequences available on GenBank. Wherever ENTV-1NA9 and ENTV-1OVC diverged from the consensus sequence, they shared 100% nucleotide identity (data not shown). ENTV-1OVC differed from ENTV-1NA9 at six nucleotide positions and these differences were not found in any ovine betaretrovirus sequences, exogenous or endogenous, currently available on GenBank. The six nucleotide differences were distributed across the genome (Figure 3-8B) with one occurring in the U5, three in the *gag* gene and two in the *pol* gene. None of the substitutions were located in the four hypervariable regions previously identified in the ENTV-1 genome (179). All of the nucleotide substitutions represented transition mutations. Only one of the nucleotide differences resulted in a non-synonymous mutation, causing an alanine to valine mutation at amino acid 335 of the *gag* gene.
Figure 3-8. Phylogenetic analysis of ENTV-1OVC.

(A) Phylogenetic relationship analysis of the full-length ENTV-1 genome sequence from the experimentally induced nasal tumor (ENTV-1OVC) and all other full-length ENTV-1 genome sequences available on GenBank as well as genome sequences from the genetically related ENTV-2 and JSRV. Accession numbers are shown in brackets. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Phylogenetic analyses were conducted using MEGA5 and all positions containing gaps and missing data were eliminated from the dataset. (B) A schematic showing the location of nucleotide differences between ENTV-1OVC and the closely related ENTV-1NA9. Small arrows indicated nucleotide changes and large arrows demarcate regions of variability previously identified.
Discussion

Although ENA is known to be a transmissible tumor in goats (92), studies on the transmissibility of ENA in sheep are lacking. While a longstanding correlation between ENTV-1 and ENA suggests that this ovine betaretrovirus is the causative agent of the nasal tumor (119, 135), Koch’s postulates had not been fulfilled. A nasal tumor with components identical to ENA was induced in an inoculated lamb, thus we provide convincing evidence that ENA is caused by ENTV-1. We used a cell-free ENA tumor homogenate to induce nasal tumors in healthy lambs and found ENTV-1 viral antigens, proviral DNA, and virus particles in the resultant tumor in one of five lambs.

The time to lethal tumor development in our experimental infection trial was surprisingly short. One of five inoculated animals developed clinical signs of ENA at 12 wpi. Normally the estimated time for the development of naturally acquired ENA ranges from one to three years or more. Moreover, in a similar study conducted in goats, the tumor latency was between 12 to 18 months (92).

Nebulization was chosen as the method for virus administration to efficiently distribute the inoculum throughout the upper and lower respiratory tract. ENTV-1 proviral DNA was detected in the nose, lung, spleen, liver and kidney of the animal with experimentally induced ENA suggesting a disseminated distribution. Although ENTV-1 provirus was detected in regions of the respiratory tract other than the nose, this did not correlate with viral protein expression. Our inability to detect protein expression in any of the tissues that were PCR-positive for the virus, other than the nose, suggests that either infection of these tissues is non-productive or that infection events in these tissues are so rare that only the use of extremely sensitive methods such as hnPCR are able to detect them. It is possible that the presence of proviral DNA in the spleen,
kidney and liver could be due to the migration of virus-infected macrophages or dendritic cells to these tissues (185). At no point during the infection trial was ENTV-1 proviral DNA detected in PBMCs. Ortin et al. were also unable to detect ENTV-1 in the PBMCs of ENA-affected animals (93). In contrast, JSRV is widely distributed both in T and B lymphocytes and mononuclear phagocytes of OPA-affected animals (185, 186). Pseudotyping and receptor binding experiments have shown that ENTV-1 and JSRV envelope proteins both bind to and utilize the same receptor, hyaluronidase 2 (Hyal2), for entry (96, 99, 187, 188), but the requirements for entry of these viruses have not been evaluated in the context of the native virion. The entry requirements for ENTV-1 and JSRV envelope pseudotyped MLV particles differ slightly as ENTV-1 envelope requires a lower pH than JSRV envelope to mediate entry in this context (28). It is possible that ENTV-1 requires an additional cofactor for entry that JSRV does not and that this cofactor is lacking in immune cells.

The tissue distribution pattern of ENTV-1 in the experimentally infected sheep with ENA was similar to that reported for naturally acquired ENA (93). This demonstrates that the infection method used in this study, while likely more efficient, closely recapitulates what is seen during natural transmission of the virus.

Tumor latency was much shorter in our experimentally infected sheep than has been reported in natural cases. This is likely a consequence of the amount of virus in the inoculum and the young age at which the lambs were infected, as a similar reduction in incubation period was observed for newborn lambs experimentally infected with JSRV (189). Betaretroviruses, such as ENTV-1, require active cell division to translocate their genomes into the nucleus where they integrate in order to complete their replication cycle (190). Fourteen day old lambs were used in this study since cells in the respiratory tract would be expected to have a higher rate of cell division than
adult sheep thereby maximizing infection efficiency. Additionally, the expression level of ovine hyaluronidase 2 (Hyal2), the receptor for ENTV-1, is high in the fetus and then markedly declines in the neonate (191).

The disease incidence was relatively low in our experimental infection trial as only one of five infected animals developed a nasal tumor and for the remaining four animals, ENTV-1 provirus was undetectable by hnPCR. Since our analysis confirmed the presence of ENTV-1 antigens, RT enzymatic activity, and intact virions in the ENA inoculum, it is unlikely that the absence of ENTV-1 proviral DNA in the four asymptomatic sheep was due to a lack of infectious virions in the inoculum. Possible explanations for the low tumor incidence include immune-mediated clearance of virus infected cells and differences in genetic susceptibility to the virus. Indeed, allelic and expression variability of restriction factors and infection co-factors have been shown to influence the replicative fitness of ovine retroviruses (192–196). As well, certain endogenous ovine betaretrovirus elements restrict JSRV release (54) and have been shown to have variable expression in respiratory tissues (197). Whether these endogenous elements have similar inhibitory effects on ENTV-1 is currently unknown.

Currently, the immunological response to ENTV-1 infection is poorly understood. ENA affected animals do not appear to develop circulating antibodies towards ENTV-1 (83). This has been attributed to the immune tolerance induced by expression of endogenous ovine betaretrovirus transcripts in the thymus and peripheral immune organs during ontogeny (87). Similar studies pertaining to JSRV and OPA have had limited success in detecting a humoral immune response (87). In sheep experimentally co-infected with JSRV and maedi-visna virus, CD3(+) T cell and JSRV-specific antibody responses were detected and these correlated with the spontaneous regression of JSRV-induced lung tumors (170). A cell-mediated response to ENTV-1 infection
has yet to be demonstrated and we did not see evidence of nasal inflammation or tumor-related leukocytes in our study. It is possible that the apparent lack of virus-specific immune response in ENA-affected sheep contributes to tumorigenesis in these animals while sheep that are able to mount a robust immune response against ENTV-1 are capable of clearing virus-infected cells before the onset of tumorigenesis.

There was no evidence of quasispecies in the proviral sequence extracted from the Expt ENA tumor (ENTV-1OVC), even though several different areas of the tumor were sampled and PCR products sequenced directly. We previously demonstrated that ENTV-1 genome sequences amplified from North American ENA samples are surprisingly stable (179). Since the ENA inoculum used in the experimental infection was generated from a combination of ten different North American ENA tumor samples, we were interested to determine which genome sequences would predominate in Expt ENA and the extent of genetic variation. The lack of variation in the ENTV-1OVC sequence suggests that the tumor likely originated from a single integration event. Phylogenetic analysis shows that ENTV-1OVC is most closely related to ENTV-1NA9 and inspection of the ClustalW alignment shows that ENTV-1OVC differs from ENTV-1NA9 at only six nucleotides positions. Recombination was ruled out as a factor for introducing these nucleotide changes because the nucleotide differences seen in ENTV-1OVC relative to ENTV-1NA9 are not shared by any of the other ten sequences represented in the virus inoculum. All six of the nucleotide changes represent transition mutations, which most likely occurred due to base mispairing during reverse transcription of the genome. It is well documented that retroviral reverse transcriptases have low fidelity (198, 199), thus it is likely that the RT of ENTV-1 is similarly error prone. Alternatively, the nucleotide differences observed in ENTV-1OVC existed previously in the ENTV-1NA9 tumor and this area of the tumor was not sampled during
sequencing. Only one of the ENTV-1OVC nucleotide substitutions corresponded to a
nonsynonymous change in the amino acid sequence at residue 335 in the Gag polyprotein. It is
unlikely that this mutation would affect the structure or function of the resulting protein since the
physiochemical properties of valine and alanine are conserved; therefore, it is not likely to confer
any advantage in terms of pathogenesis or viral replicative fitness.

Buoyant density analysis of the ENA inoculum fractions showed only one band in the capsid
immunoblot at 27 kDa, representing fully processed capsid. Conversely, buoyant density analysis
of Expt ENA showed multiple bands on the capsid immunoblot, the size of which correspond
well with the molecular weight of the various forms expected of unprocessed Gag and Gag-Pro
polyprotein as well as partially processed Gag products that contain the capsid subunit (63).
Therefore, it appears that mature as well as immature virions were liberated from the Expt ENA
tumor whereas only mature virions were found in the ENA inoculum. The peak RT activity of
Expt ENA occurred at a density of 1.22g/mL whereas the RT activity of ENA inoculum peaked
at 1.18g/mL, the buoyant density expected for a retrovirus. Studies of closely related
oncoretroviruses have noted that naked viral cores have a buoyant density of approximately 1.22
g/mL, which is distinct from the buoyant density of enveloped virions (1.15 to 1.18 g/mL) (117,
200). The immature nature of the virions and the increased buoyant density (1.22 versus 1.18
g/mL) indicate that although a proportion of the Expt ENA virions were enveloped and
processed, the majority of virus in the tumor was comprised of naked immature cores. It is
possible that the mechanical forces of homogenization sheared cells causing the release of
assembled but immature virus particles lacking an envelope membrane. This phenomenon was
not observed in the Natural ENA because the fast growth of Expt ENA and relative disorganized
structure likely impaired virion release and caused an accumulation of unenveloped virion cores
in the cytoplasm of tumor cells. Electron microscopic analysis supports this conclusion as mature enveloped virions were clearly visible in the ENA inoculum, but relatively rare in the virus isolated from in the Expt ENA tumor sample.

Taken together, the fact that the experimentally infected sheep that developed ENA was inoculated with filtered homogenate from ENTV-1 antigen and genome sequence-positive tumors strongly suggests that the nasal tumor observed in this animal was caused by ENTV-1, thereby further implicating ENTV-1 as the etiologic agent of transmissible nasal tumors in sheep.
Chapter Four:

Complementation of the defective protease activity of an ENTV-1 molecular clone with JSRV Gag does not induce tumors in vivo

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Abstract

Enzootic nasal tumor virus (ENTV-1) is a betaretrovirus of sheep that has been linked to enzootic nasal adenocarcinoma (ENA), a tumor of the nasal mucosa of sheep. We have performed transmission experiments that suggest that ENTV-1 is the causative agent of ENA, however this etiological relationship has not been conclusively proven due to an inability to propagate the virus. In this report, we constructed a molecular clone of ENTV-1 and used this clone to produce virus via transfection of HEK 293T cells. Analysis of the recombinant virus particles revealed a defect in the proteolytic processing of Gag, which is a phenotype that has been associated with a decrease in viral fitness and infectivity in other retroviral systems. Neither mutagenesis of amino acid variants within the protease gene nor exposure of recombinant virus to a reducing agent restored proteolytic processing. Co-expression of the heterologous Gag-Pro-Pol polyprotein from the highly related Jaagsiekte sheep retrovirus (JSRV) with the ENTV-1 molecular clone led to co-packaging of JSRV Gag and the production of mature fully processed virions. As it is possible that ENTV-1 Gag processing requires a sheep-specific factor that is absent from 293T cells or that human cells restrict ENTV-1 Gag processing, we investigated whether the complemented virus would be viable in vivo in the natural host. Infection of newborn lambs with the complemented virus failed to demonstrate provirus integration or viral protein expression suggesting that the defect in proteolytic processing is likely due to a characteristic of the ENTV-1 molecular clone rather than the producer cells. In summary, we have constructed the first molecular clone of ENTV-1 but the lack of proteolytic processing observed in the resulting virions needs to be addressed before Koch’s postulates can be fully tested.
Introduction

Protease-mediated processing of the Gag-Pro-Pol polyprotein is an essential step in the replication of retroviruses (56). The protease is activated concurrent with egress, or shortly thereafter, at which time it is released from the polyprotein via an autocatalytic reaction followed by proteolytic processing of the remainder of the polyprotein (201). The mechanism or trigger for activation of retroviral proteases is unclear. Strict regulation of the protease is required to prevent premature activation, which would inhibit virus assembly, budding and infectivity (202–205). This is especially important in betaretroviruses as the core assembles in an intracellular compartment before transport to the plasma membrane and release (201). Several studies have demonstrated that protein conformation/dimerization (60, 61, 203, 206) and oxidation (62, 207) play an integral role in protease activation, but activation of ovine betaretrovirus proteolytic processing has not been studied.

Inactive protease is detrimental to retroviral replication because in the absence of protease processing the virion will not convert to the mature metastable conformation required for the virus to become infectious. Indeed, there is a class of antiretroviral drugs designed to specifically inhibit the protease and consequently inhibit virus replication (64, 208).

Enzootic nasal tumor virus (ENTV)-1 is a betaretrovirus of sheep that is associated with enzootic nasal adenocarcinoma (ENA), a nasal tumor of sheep (209). Experiments showing transmission of ENA to a healthy lamb by inoculation with cell-free ENA tumor homogenate containing ENTV-1 antigens suggested that ENTV-1 is the causative agent of ENA, but did not fulfill Koch’s postulates (209). A factor limiting these studies is an inability to produced concentrated pure virus since the virus cannot be propagated in current cell culture systems. In the study presented here, we sought to resolve this issue by constructing a molecular clone from which
infectious ENTV-1 could be generated. Transfection of HEK 293T cells with the ENTV-1 molecular clone led to the production of virus particles but processing of the Gag polyprotein was not observed. The protease could not be activated by treatment with a reducing agent or by mutagenesis targeted to key amino acids. Complementation of the ENTV-1 molecular clone with the JSRV Gag-Pro-Pol polyprotein generated virus particles with fully processed Gag, but did not result in infection or disease development.

**Materials and methods**

**Cloning and vector construction**

The ENTV-1 genome was amplified from genomic DNA (previously designated as ENTV-1NA4, Genbank accession number FJ744146) isolated from an ENA sample in two overlapping fragments using PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies, Missisauga, Ontario, Canada) and the E5F/E5R and E3F/E3R primer pairs (Table 4-1) for the 5' fragment and the 3' fragment, respectively. PCR products were cloned using the TOPO TA cloning kit (Life Technologies, Burlington, Ontario, Canada). Subsequently, the cytomegalovirus (CMV) promoter was amplified from the pcDNA3.1 plasmid and fused to the 5' ENTV-1 genome fragment at the 5' border of the R region using overlap extension PCR (using primers CMVF, RCMVR, E5F and EGagR, Table 4-1). The CMV-RU5 fusion fragment was inserted into the 5' ENTV-1 fragment using XbaI and BsrGI restriction sites. The 5' fragment was excised using XbaI and Mmel and the 3' fragment was excised using NotI and Mmel and both were ligated into XbaI and NotI digested low copy number plasmid, pLG338/30 (provided by Dr. Ronald C. Montelaro through the AIDS Reagent Program, Bethesda, Maryland, USA) (210). Plasmids containing ENTV-1 genome fragments were prone to recombination in bacteria and thus were
incubated at a reduced temperature (30°C). The JSRV molecular clone, pCMVJSRV21 (117), and the JSRV Gag-Pro-Pol polyprotein expression vector, pCMVGPP-MX-4CTE (88), were kind gifts from Dr. Massimo Palmarini, University of Glasgow, Scotland.

Mutations of the ENTV-1 molecular clone (used in Figure 4-3) were created using a modified site-directed mutagenesis protocol as outlined by Wang et al. (211) using the primer pairs (EproS199P F/R, EproT202M F/R and EproT289I F/R) in Table 4-1 and the KOD Hot Start DNA Polymerase kit (VWR International, Mississauga, Ontario, Canada) according to the manufacturer’s instructions. Briefly, mutagenesis was performed with two separate amplification reactions containing either forward or reverse primers to amplify the molecular clone plasmid for three rounds. The two single-primer PCR products were combined in one tube and amplified for a further 14 rounds. Methylated parental plasmid DNA was digested with DpnI before transformation into competent E. coli cells.
Table 4-1. Envelope cloning and mutagenesis primers
Primers utilized in the generation and mutagenesis of the molecular clone and in screening for ENTV-1 transcripts derived from the molecular clone are listed along with the corresponding sequences on the right (5' to 3').

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</table>

RT-PCR

Total RNA was extracted from HEK 293T cells previously transfected with either pCMVENTV-1 or pCMVJSRV21 and cDNA was generated using the JEenvR primer (Table 4-1) and the SuperScript II Reverse Transcriptase kit (Life Technologies) according to manufacturer instructions. A PCR product representing spliced envelope transcripts with an expected size of 384 bp was amplified from the cDNA using Platinum PCR SuperMix (Life Technologies) and the JU5F/JenvR and EU5F/EenvR primer pairs (sequences shown in Table 4-1 and genome location shown in Figure 4-1A) to amplify JSRV and ENTV-1 mRNA transcripts, respectively.

Cell lines and virus production
HEK 293T, goat synovial membrane cell line immortalized with telomerase expression (GSM-T, a kind gift from Dr. Yahia Chebloune, Université Joseph Fourier), sheep skin fibroblasts (SSF) and ovine nasal tumor cell line (ONTC) (179) cells were grown in Dulbecco’s minimum essential medium supplemented with 10% fetal bovine serum in 10 cm tissue culture dishes at 37°C under 5% CO₂. To produce primary sheep lung and nasal epithelial cells, fetal lung and nasal tissue was diced with a scalpel and subsequently dispersed by passage through a 20-gauge needle. Non-adherent cells were removed 72 h after plating and the remaining adherent cells were treated with trypsin for three minutes to remove fibroblast cells and enrich for epithelial cells. The cells were propagated for three to four passages before transfection.

For virus production, HEK 293T cells grown on 10 cm tissue culture dishes were transfected using the calcium phosphate co-precipitation method and medium was replaced after 16 h with 5 ml of fresh medium. The virus-containing medium was harvested 48 h later, passed through a filter with 0.45 µm pores and pelleted by ultracentrifugation through a 20% sucrose cushion in a SW32 Ti rotor (Beckman Coulter Canada, Mississauga, Ontario, Canada) at 60,000×g for 2 h at 4°C. The viral pellet was resuspended in TNE buffer (117).

**Histopathology and immunohistochemistry**

Formalin-fixed tissues from sheep infected with JSRV-complemented ENTV-1 were trimmed, embedded in paraffin, sectioned at 5 µm and stained with haematoxylin-eosin. The avidin-biotin-peroxidase complex (ABC) method was used on paraffin-embedded tissue sections for immunohistochemical detection of ENTV envelope protein expression, as described previously (179).
Western blot analysis

Western blotting was conducted as described previously (183) using monoclonal antibodies specific for the envelope protein of ovine betaretroviruses (146) (i.e. JSRV, ENTV and enJSRV) and the capsid protein (12) (kindly provided by Dr. Hung Fan, University of California, Irvine).

Animals and in vivo infection and sample collection

The Animal Care Committee at the University of Guelph approved all animal use and related procedures, which also complied with the recommendations of the Canadian Council on Animal Care. Since a suitable cell culture system for infection and titration of ENTV-1 does not exist, the virus used in the following experiments was not titered but rather animals within the same group received equal amounts of virus as determined by Western blot assay of virion-associated capsid protein. Six lambs born to dams from a research flock at the University of Guelph with no previous history of ENA were infected at two days of age with 2 mL of nebulized virus as previously described (197). Two lambs were mock infected with vehicle alone and were housed with the experimentally infected animals. All lambs were examined by a veterinarian and were clinically healthy prior to infection. Animals infected with JSRV were housed in a biosafety level 2 animal isolation unit.

Animals were euthanized at the times post infection outlined in Table 4-2. At necropsy, the nose was serially cut transversely at ~2 cm intervals and samples of normal conchae and lesions were collected. Lung tissue samples were also harvested. Tissues were sectioned into halves and stored at −80°C for subsequent isolation of nucleic acids and protein or fixed in 10% neutral buffered formalin for 24 h prior to embedding in paraffin wax and sectioning.
Results

ENTV-1 molecular clone produces unprocessed immature virions

ENTV-1 cannot be propagated in conventional cell culture systems, therefore it was necessary to generate a molecular clone in order to produce virus for experimental purposes. The full-length ENTV-1 integrated genome was amplified from a DNA extract of ENA tumor tissue in two overlapping fragments and cloned into a plasmid with the CMV promoter fused to the 5' end of the R region (Figure 4-1A). The 5' U3 was replaced with the CMV promoter to allow high-level expression in human cells. The resulting plasmid (pCMVENTV-1) was transfected into HEK 293T cells and after 48 hours RNA was extracted and subjected to RT-PCR using a forward primer in the U5 region and a reverse primer in the signal peptide region of env (see black arrow heads in Figure 4-1A) producing a band at approximately 384 bp. This band represented spliced ENTV-1 envelope transcripts (as seen in Figure 1-1) and demonstrated that the molecular clone could support transcription of viral genes (Figure 4-1B). The JSRV molecular clone was analysed in a similar fashion and showed an identical result (Figure 4-1B). Recombinant ENTV-1 and JSRV particles produced in HEK 293T and concentrated by ultracentrifugation were subjected to SDS-PAGE separation and immunoblot analysis using a monoclonal antibody against the JSRV capsid, which cross-reacts with the ENTV-1 capsid. A protein of 26 kDa was detected in the lane containing JSRV lysate (Figure 4-2, lane 2) as well as in the lane containing bacterially-expressed ENTV-1 capsid (ECa) protein (Figure 4-2A, lane 4). However, this 26 kDa protein was absent in the ENTV-1 molecular clone (Figure 4-2, lane 3) and instead, a protein of approximately 78 kDa, which is the expected size of the unprocessed Gag polyprotein, was detected. Taken together, these results suggested that the ENTV-1 molecular clone was able to direct production of virus particles but that there was a defect in proteolytic processing.
The ENTV-1 genome was amplified from genomic DNA isolated from an ENA tumor in two overlapping fragments. The CMV promoter was fused to the 5' ENTV-1 genome at the 5' border of the R region in an intermediate fragment by overlap extension PCR and was introduced into the 5' genome fragment via the BsrGI restriction site. The fragments were ligated together at the MmeI restriction site and the ENTV-1 molecular clone was generated by ligation into the pLG338/30 plasmid. A schematic outlining this strategy and the location of the primers (arrowheads) used in the RT-PCR is shown (A). Singly spliced transcripts coding for the envelope protein were detected in RNA extracted from HEK 293 cells transfected with the JSRV or ENTV-1 molecular clone and the resulting PCR products are shown (B). M indicates the lanes containing 100bp DNA ladder (Life Technologies).
Figure 4-2. Lack of Gag-Pro-Pol polyprotein processing in virions derived from the ENTV-1 molecular clone.

Immunoblot analysis of capsid protein, using a monoclonal antibody specific to JSRV capsid, in the lysates of concentrated viruses derived from the indicated molecular clones after transfection of HEK 293T cells. M indicates the lane containing the molecular weight marker and lanes labelled as ECa contain bacterially expressed and purified ENTV-1 capsid protein. The black arrowheads indicates unprocessed Gag at 78 kDa and the red arrowheads indicate processed Capsid at 26 kDa.

Treatment with reducing agents was unable to restore ENTV-1 protease activity

A study of the simian betaretrovirus, Mason–Pfizer monkey virus (MPMV), demonstrated that treatment of unprocessed non-enveloped viral cores with the reducing agent dithiothreitol (DTT) resulted in processing, presumably by activation of the protease (62). In order to determine if the protease in the virions produced from the ENTV-1 molecular clone could be similarly activated, samples of purified and concentrated recombinant virus were exposed to 1mM DTT for 1 to 120 minutes (Figure 4-3A). Evidence of processed Gag was absent at every time point; thus, it appears that the protease of the ENTV-1 molecular clone was not activated by exposure to reducing conditions.
Mutagenesis of the ENTV-1 protease did not restore proteolytic processing

Given that the ENTV-1 molecular clone was generated from two large PCR products, we were curious whether mutations had occurred that could affect the protease processing activity. The ENTV-1 molecular clone, pCMVENTV-1, was sequenced in its entirety using primers described previously (179). Since virions derived from the JSRV molecular clone (pCMVJSRV21) contain fully processed Gag, the protease sequence of pCMVJSRV21 was compared to that of pCMVENTV-1, the ENTV-1 isolate (ENTV-1NA4) from which pCMVENTV-1 was derived and an additional ENTV-1 isolate (ENTV-1OVC) from an experimentally infected sheep using MEGA5 software. A proline to serine change at position 199 unique to the pCMVENTV-1 protease was identified (highlighted in Figure 4-3C). This amino acid difference was located in the peptidase domain, 18 amino acids downstream from the catalytic aspartate residue, based upon sequence similarity and alignment with MPMV protease (212). This mutation was of interest because prolines may influence secondary structure due to their “kinked” conformation (213–215). Since the infectious status of the ENTV-1NA4 isolate has not been confirmed, the protease sequence of ENTV-1OVC was also included in this alignment. The ENTV-1OVC sequence was isolated from an experimentally induced tumor; therefore it represented a validated infectious ENTV-1 sequence. Two differences between pCMVENTV-1 and ENTV-1OVC were noted, one in the peptidase domain (T versus M at position 202, highlighted in Figure 4-3C) and the other in the G-patch domain (I versus T at position 289, highlighted in Figure 4-3C). It should be noted that there was one additional amino acid difference that followed this pattern at position 147 but since it was localized to the dUTPase region of the polyprotein it was not included as a candidate for mutagenesis. Site-directed mutagenesis was performed on pCMVENTV-1 to first change the serine at position 199 to a proline (pCMVENTV-1S199P).
Subsequent amino acid changes were introduced into pCMV-ENTV-1S199P to produce pCMV-ENTV-1S199P, T202M and pCMV-ENTV-1S199P, T289I, which resembled the ENTV-1OVC sequence. Virus was generated from the modified molecular clones and analyzed by immunoblot for capsid protein expression (Figure 4-3B). Despite mutating the protease of the ENTV-1 molecular clone to resemble that of known infectious viruses, there was no change in the processing defect of any of the mutant viruses as only unprocessed Gag could be detected (Figure 4-3B). These results suggested that either all three amino acid variants within the protease needed to be corrected or that amino acid variants outside of the protease contributed to the processing defect.
Figure 4-3. Lack of processing after treatment with DTT and mutagenesis

(A) Immunoblot analysis of capsid protein, using a monoclonal antibody specific to JSRV capsid, in the lysates of concentrated viruses derived from the indicated molecular clones after transfection of HEK 293T cells. (A) A capsid protein immunoblot of lysate from the ENTV-1 molecular clone derived virus after treatment with DTT for increasing lengths of time. (B) Capsid protein immunoblot analysis of lysates from concentrated viruses derived from the indicated molecular clones containing the designated amino acid changes in the protease. M indicates the lane containing the molecular weight marker and lanes labelled as ECA contain bacterially expressed and purified ENTV-1 capsid protein. The black arrowheads indicate unprocessed Gag at 78 kDa and the red arrowheads indicate processed Capsid at 26 kDa. (C) ClustalW alignment of the predicted amino acid sequence of the pro gene of JSRV compared to that of the ENTV-1 molecular clone, ENTV-1NA4 and ENTV-1OVC. Dots indicate identical amino acids. Amino acids mutated are red with yellow highlight.
The JSRV Gag-Pro-Pol polyprotein can complement and correct the processing defect of the ENTV-1 molecular clone

Our results showed that the JSRV molecular clone was able to produce virions comprised of fully processed Gag polyprotein while pCMVENTV-1 could not. This phenotypic difference is difficult to reconcile in light of the extremely high amino acid identity shared between the two viruses (179). The mechanism responsible for initiating protease processing and maturation is not well understood. In order to determine whether the ENTV-1 genome and envelope protein contribute to the processing defect, the JSRV Gag-Pro-Pol polyprotein alone (pCMVGPP-MX-4CTE) was supplied in trans to allow co-assembly with the ENTV-1 polyprotein and packaging of the ENTV-1 genome. HEK 293T cells were co-transfected with pCMVENTV-1 and pCMVGPP-MX-4CTE and virus purified from the supernatant was analyzed by Western blot using an antibody specific for the JSRV capsid (JCa). Virus from cells transfected with either the JSRV molecular clone (Figure 4-4A, lane 2) or the ENTV-1 molecular clone (Figure 4-4A, lane 3) showed banding patterns identical to those observed previously in Figure 4-2. A single protein migrating at a molecular weight of 26 kDa was observed in virus particles produced from cells co-transfected with pCMVENTV-1 and pCMVGPP-MX-4CTE (Figure 4-4A, lane 4) indicating that JSRV Gag-Pro-Pol can complement ENTV-1. Since Gag polyprotein expression levels from the pCMVGPP-MX-4CTE plasmid were much greater than from either of the two molecular clones, we performed an experiment to determine the dose dependence of this complementation. Dose dependency experiments were performed with JSRV Gag-Pro-Pol maintained at a consistent level and ENTV-1 progressively increased (Figure 4-4B) as well as the inverse (Figure 4-4C). When ENTV-1 was supplied in excess, a band was detected at both 26 kDa and 78 kDa (Figure 4-4B lane 7 and 8 and 4-4C lane 3). Proteins of approximately 62, 57,
52 and 40 kDa in a banding pattern reminiscent of partially processed Gag (63, 209) were also detected (Figure 4-4B, lane 8). Since the JSRV Gag-Pro-Pol expression construct lacks a packaging signal, the virions produced in these experiments were presumed to have assembled around the ENTV-1 genome, indicating that the genomic RNA derived from the ENTV-1 molecular clone is not responsible for the processing defect.
Figure 4-4. Dose dependent rescue of ENTV-1 polyprotein processing defect by complementation with JSRV Gag-Pro-Pol

(A) Capsid protein immunoblot analysis, using a monoclonal antibody specific to JSRV capsid, of concentrated virion lysate from HEK 293T cells co-transfected with the ENTV-1 molecular clone and the JSRV Gag-Pro-Pol expression vector (ENTV-1/J-GP) showing protease processing of the gag polyprotein (lane 4). Virus derived from transfection of the JSRV (lane 2) and ENTV (lanes 3) molecular clones. The dosage dependence of polyprotein processing was evaluated by co-transfection of consistent levels of JSRV Gag-Pro-Pol (0.5µg) with increasing amounts of ENTV-1 (1 to 10 µg) (B) as well as consistent levels of ENTV-1 (5 µg) and increasing amounts of JSRV Gag-Pro-Pol (0.5 to 5 µg) (C). M indicates the lane containing the molecular weight marker and lanes labelled as ECa contain bacterially expressed and purified ENTV-1 capsid protein. The black arrowheads indicates unprocessed Gag at 78 kDa and the red arrowheads indicate processed Capsid at 26 kDa.
In vivo infection of newborn lambs with JSRV-complemented ENTV-1 virions

Since neither JSRV nor ENTV-1 can infect cells in culture, an in vivo infection model was utilized to determine whether JSRV-complemented ENTV-1 virions were infectious. Six lambs from a flock with no history of ENA or OPA were inoculated at two days of age with JSRV complemented ENTV-1 virus via nebulization. Infected lambs were monitored weekly for clinical signs of lung or nasal tumor development since we did not know how JSRV complementation would affect disease tropism. An additional group of four lambs were infected at two weeks of age with recombinant JSRV generated from the JSRV molecular clone in an identical manner to serve as a positive control for virus production and disease development. Between one and three months post-infection, an increased respiration rate was observed in a subset of animals infected with JSRV-complemented ENTV-1, but overt signs of disease were never observed and ultimately the respiration rate returned to normal. One lamb was euthanized at one-month post infection due to unrelated complications and the remaining infected lambs were euthanized at the times indicated in Table 4-2.

Abnormal clinical signs were never observed in the animals infected with JSRV and when euthanized at nine months post-infection there was no evidence of tumors in the lungs or nose upon macroscopic inspection. Compared to lung tissue from an uninfected sheep (Figure 4-5A and B), the lung tissue from JSRV-infected animals showed clusters of leukocytes in association with vasculature and airways (Figure 4-5C and D). A single 5 mm diameter hard nodule was discovered during tactile inspection of the distal lung of one of the JSRV-infected animals and was sampled for microscopic examination. The nodule showed proliferation of cells with papillary and acinar organization typical of ovine pulmonary adenocarcinoma (216) filling the alveolar spaces (Figure 4-5E). Immunohistochemical staining for envelope protein in the tumor
demonstrated robust expression in neoplastic cells (Figure 4-5F) but absence of expression in adjacent non-proliferating cells.

**Figure 4-5 Histopathology and immunohistochemical staining for JSRV envelope protein**
Representative images of hematoxylin and eosin stained sheep lung tissue from normal sheep (A and B) and sheep experimentally infected with JSRV (C-E). Lung sections from infected sheep showed increased leukocyte clusters around airways and blood vessels (C and D). One animal had a nodule consistent with OPA (E). Immunohistochemical staining showed envelope protein expression in the OPA-affected lung (F).

At necropsy animals infected with JSRV-complemented ENTV-1 showed no evidence of nasal tumors (Figure 4-6A and B). The peripheral lung in some of the animals had multifocal lesions that were raised, round, well circumscribed and firm, ranging from approximately 2 to 10 mm in
diameter and were lighter in colour relative to healthy lung tissue (Figure 4-6C and D). Samples from these areas were collected and upon microscopic examination were found to have a reduced alveolar space (Figure 4-7A) as well as an increased number of clusters of leukocytes in association with airways and vasculature (Figure 4-7B). There was no evidence of neoplasia in the lung. All nasal tissues examined appeared normal by histology with no detectable neoplasia or hyperplasia.
Figure 4-6. Macroscopic findings
Images of serial sections of the nasal cavity (A and B) and whole lungs (C and D) of sheep experimentally infected with JSRV complemented ENTV-1 showing a lack of tumors in the nose and multifocal raised and lighter coloured lesions in the peripheral lung (black arrowheads).
Figure 4-7. Histopathology and immunohistochemical staining for ENTV-1 envelope protein expression.
Representative images of hematoxylin and eosin stained lung (E and B) and nasal (E and F) sections from sheep experimentally infected with JSRV complemented ENTV-1. Histology showed clusters of leukocytes in association with airways and vasculature but no aberration in the nasal structures. Immunohistochemical staining indicated lack of envelope protein expression in lung (C) and nasal (G) sections except in occasional single cells in the interstitium of the lung (Black arrowhead in panel D) and near the basement membrane in the nose (Black arrowhead in panel H).
(Figure 4-7E and F). Immunohistochemical staining did not reveal clusters of cells expressing ENTV-1 envelope in either the lung or the nose tissue samples (Figure 4-7C and G, respectively); however, in rare instances single cells expressing envelope were observed in both the lung and nose (Figure 4-7D and H, respectively). The envelope-positive cell in the lung was cuboidal with a large amount of cytoplasm and located at the septal junction (Figure 4-7D). The envelope-positive cell in the nose was a small rounded cell situated in the basal region of the pseudostratified columnar epithelium, near the basal lamina (Figure 4-7H).

<table>
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<tr>
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<th>Sex</th>
<th>Tumor</th>
<th>αEnv</th>
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Mpi - months post infection, αEnv - result of immunohistochemical staining with the ovine betaretrovirus envelope-specific antibody.

**Discussion**

ENA is relatively common in North America (142, 179) but despite this, little is known about the pathogenesis of the disease, including confirmation of ENTV-1 as the etiologic agent. Much of what is known about ENA and ENTV-1 is inferred from studies on OPA and JSRV. We endeavoured to address this problem and build upon our previous transmission experiments involving cell-free tumor homogenate by constructing a molecular clone of ENTV-1 from which virus could be generated for *in vivo* infection experiments. Failure of the molecular clone to
produce mature virions comprised of fully processed viral proteins presented a unique impediment to these experiments, but ultimately revealed important clues to the mechanism of pathogenesis and replication of ENTV-1.

The defect in the processing of ENTV-1 Gag that we observed could be due to a cell-specific requirement for activation of the protease that is present in sheep cells but absent in HEK 293T cells. Alternatively, human cells may express a restriction factor that inhibits maturation of ENTV-1 virions. To address this possibility, we attempted to produce recombinant ENTV-1 via transfection of primary and continuous sheep and goat cell lines, but the transfection efficiency was not sufficient enough to detect virus even after ultracentrifugation of virus-containing supernatant (data not shown). Indeed, we struggled to observe ENTV-1 in HEK 293T cells in situations where the transfection efficiency was suboptimal; suggesting that virus generation by this method is extremely inefficient. Since JSRV particles produced from HEK 293 cells do not show a defect in polyprotein processing and the sequence identity between JSRV and ENTV-1 is high, it is unlikely that the defect in processing of particles from the ENTV-1 is due to a defect specific to the producer cells.

Co-transfection of the JSRV Gag-Pro-Pol expression vector with the ENTV-1 molecular clone generated virions with fully processed Gag (Figure 3A). The antibody used in this study does not distinguish between JSRV and ENTV-1 capsid proteins so it was not possible to determine whether rescue of polyprotein processing was due to processing of ENTV-1 Gag by the JSRV protease or displacement of the ENTV-1 polyprotein by the JSRV polyprotein. The JSRV Gag-Pro-Pol expression vector contains additional elements (e.g. MPMV CTE and SV40 polyA) (88) that promote much higher expression of Gag-Pro-Pol than the molecular clone so direct comparisons are not possible. Expression level differences were taken into account in the dosage

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response co-transfection experiments (Figure 3B and C) in order to encourage heterologous Gag polyprotein co-packaging (ie. generation of virions with polyproteins from both JSRV and ENTV-1)(217). Incomplete rescue of processing, seen as partially processed Gag in lane 7 and 8 of Figure 3B, indicates that the JSRV protease is processing the ENTV-1 polyprotein in trans in these virions and that there is a limit to which the JSRV protease can perform this function. Therefore, since the ENTV-1 polyproteins can be processed by the JSRV protease the processing defect is due to lack of ENTV-1 protease activity.

Since mutagenesis of the pCMVENTV-1 protease subunit to match the ENTV-1NA4 sequence failed to rescue the processing defect, it is possible that the virions derived from the ENTV-1NA4 sequence may have also shared this phenotype. Although only fully processed Gag was observed in virions purified from the ENTV-1NA4 tumor or any of the other ENTV-1NA tumors (ENA inoculum sample analysed in Figure 3-7B in Chapter 3), a processing defect may be observed outside the context of transformed sheep cells. In addition, further mutation to make the molecular clone protease subunit match that of ENTV-1OVC was also unable to rescue processing. Since ENTV-1OVC was extracted from a tumor transmitted by cell-free tumor homogenate (209), it was assumed that the sequence represented a replication-competent, infectious ENTV-1. It should be noted that homogenates from the ENTV-1OVC tumor contained unprocessed and partially processed Gag proteins which were thought to represent naked cores released from the cytoplasm of tumor cells due to mechanical disruption (209). This tumor was aggressive, causing clinical signs three months after infection, so observations may represent an intermediate stage in neoplastic development. There are still some amino acid differences between the ENTV-1 sequences and the pCMVJSRV21 pro gene sequence that remain to be explored. As well, it has been shown in other retroviral systems that regions of the
Gag-Pro-Pol polyprotein outside of the protease subunit can affect the activation status of the protease (218). Unfortunately, more complicated mutagenesis still needs to be performed to address the involvement of other polyprotein subunits.

It is possible that the processing defect seen in virions derived from the ENTV-1 molecular clone may be reflective of the phenotype of at least some circulating exogenous ENTV-1 particles. If that were the case, these virions would not be infectious, which is a requirement to remain in circulation, unless supplemented with an active protease, perhaps from endogenous betaretrovirus sequences (219). Expression of the endogenous ovine betaretroviruses is localized primarily to tissues of the reproductive tract (76), but transcripts have been detected in the lungs (197). Human endogenous retrovirus (HERV) sequences are upregulated in neoplastic tissues due to genomic instability and epigenetic changes during transformation (220–222) and a similar phenomenon occurs in HIV-infected cells (223). Furthermore, HERV-K Gag can co-assemble and co-package with HIV-1 Gag (224). Therefore, it is possible that infection of sheep cells with ENTV-1 and subsequent transformation results in upregulation of endogenous betaretrovirus transcription, which in turn supplies polyproteins with active protease for co-packaging.

Assembly of retroviral Gag polyproteins into virion cores is performed in a relatively non-specific manner involving dimerization of proteins at hydrophobic interfaces once they have been brought into close proximity (49). This is typically by association with the genomic RNA but evidence for alternative mechanisms has also been observed (50). Therefore, structure of heterologous Gag proteins resulting in similar spatial positioning of the hydrophobic regions would allow for coassembly of heterologous Gag proteins into viral cores. Particles derived from co-assembled HERV-K (a member of the Betaretrovirus genus) and HIV Gag have been observed but were found to have decreased infectivity and release efficiency (224).
Furthermore, it has been shown that endogenous Gag can coassemble with JSRV Gag and, in the presence of a certain mutation (R21W), restrict the release of exogenous JSRV particles in a transdominant fashion (54, 225). The high degree of similarity shared between ENTV-1, JSRV and endogenous ovine betaretroviruses (179) means that since ENTV-1 can co-assemble with JSRV, it can likely also co-assemble with at least some of the endogenous ovine betaretrovirus Gag proteins.

Since we were able to detect a tumor in one of the animals infected with virus derived from the JSRV molecular clone, this would suggest that the virus production method we employed was capable of producing infectious virus. However, the OPA tumor we observed was small and barely detectable at necropsy and the incubation period between initial infection and the development of neoplasia was relatively long (9 months). Since we used nebulization to deliver the virus and we have no method for measuring infection efficiency, it is possible that poor infection efficiency led to a longer incubation period and ineffective disease development, as the remaining three infected lambs showed no signs of tumor development or virus replication. It has, however, been noted that tumor development is not the most common outcome of infection with the ovine betaretroviruses (89) and this is supported by the low disease incidence noted in other experimental infections (117, 118) and in the field (209). Recent evidence has demonstrated that sheep can develop an immune response against viral antigens in JSRV (140, 170, 226) and ENTV-1 (see Chapter 5) infections, which would contribute to the low disease occurrence discussed above. The reduced alveolar spaces and airway associated clusters of leukocyte infiltrates observed in lung tissues from the animals infected with JSRV-complemented ENTV-1 suggests that an immune response developed against the virus in this study. Serum samples from infected animals were tested for ENTV-1-specific antibodies and,
although an elevated level was detected, this was not significantly different from naïve controls (data not shown).

Although disseminated infection/virus replication and disease development was not observed in the animals infected with JSRV-complemented ENTV-1, viral protein expression in a small subset of cells was observed (black arrowhead in Figure 4-7D and H). The localization and morphology of the envelope-positive cells in the nose and lung were reminiscent of basal cells and type II alveolar cells, respectively. Both of these cell types are progenitors that are capable of differentiating into and replacing all of the cell types in the surrounding tissue structure (227). Interestingly, lung adenocarcinoma in JSRV-infected sheep originates from proliferating type II alveolar cells (228, 229). We have also observed transduction of basal cells and type II alveolar cells in sheep infected with lentiviral particles pseudotyped with JSRV and ENTV-1 envelope protein (manuscript in preparation, see Chapter 6).

In summary, we have constructed a molecular clone of ENTV-1 that is capable of directing the production of virus particles. However these particles are immature with defective Gag-polyprotein processing. JSRV Gag-Pro-Pol polyprotein can complement the ENTV-1 molecular clone and, although infection was observed, no lung or nose tumors were detected in sheep inoculated with JSRV-complemented ENTV-1. Since ENTV-1 and JSRV cannot be titered, it is impossible to determine whether JSRV complemented had low infectivity, a low yield or lacked oncogenicity. Further work involving the ENTV-1 molecular clone, especially concerning the mechanism of the protease defect, is required.
Chapter Five:

Development of an ante-mortem diagnostic test for enzootic nasal tumor virus (ENTV-1)

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Abstract

Enzootic nasal adenocarcinoma (ENA) is a contagious neoplasm of the nasal mucosa of sheep and goats and is associated with enzootic nasal tumor virus (ENTV). Since ENA is a common disease in North America and vaccination against ENTV-1 is unavailable, diagnostic tests that can identify infected animals and assist with eradication and disease surveillance efforts are greatly needed. In this study, we endeavoured to develop a novel, non-invasive diagnostic tool that could be used to not only validate clinical signs of ENA, but also to detect ENTV-1 infection prior to the onset of disease signs (i.e. preclinical diagnosis). Cytology, serology and RT-PCR-based diagnostic methods were investigated. Although the serology and cytology-based assays were able to detect ENTV-1 infection in some animals, they had insufficient sensitivity and specificity required for a diagnostic test. For this reason, serological or cytological detection of ENTV-1 infection were not considered viable ante-mortem diagnostic approaches. In contrast, RT-PCR on RNA samples extracted from nasal swabs reliably detected exogenous ENTV-1 sequences, did not amplify endogenous ovine betaretroviral sequences, demonstrated high concordance with the immunohistochemistry for envelope protein and had perfect sensitivity and specificity. This report describes the practical and highly specific RT-PCR technique for the detection of clinical and preclinical ENA that may prove beneficial in future control or eradication programs.
**Introduction**

Enzootic nasal adenocarcinoma (ENA) is an economically important contagious tumor of the nasal mucosa of sheep and goats (133). ENA has been recorded worldwide wherever sheep and goats are farmed with a prevalence of up to 10% in some areas (133). The true economic impact of ENA is not known because affected animals are culled before actual diagnosis, and suspected disease incidence is rarely reported. Adenocarcinomas of the nasal cavity in sheep have the highest reported prevalence in the United States, Canada, France, Germany, and Spain (91). Although the exact prevalence of ENA is unknown, it is not an uncommon disease in North America (179) and is very likely under-diagnosed. Clinical signs include seromucosal nasal discharge leading to a “washed nose” appearance, accompanied by snoring, coughing, wheezing and dyspnea. The duration of disease, from the appearance of clinical signs to the time of death, varies from three weeks to one year or more. ENA has been experimentally transmitted to goats using concentrated nasal fluid, proving the infectious nature of this disease (92). We recently conducted transmission studies in newborn lambs and demonstrated the transmission of ENA using cell-free tumor homogenate (209).

The retrovirus, enzootic nasal tumor virus (ENTV), has been implicated in the etiology of this lethal and contagious nasal tumor (119, 230). Using a mouse monoclonal antibody against the envelope glycoprotein (Env) of ovine betaretroviruses (146), of which ENTV is a member, we have been able to verify the presence of the virus in all nasal tumor samples evaluated to date (179). This monoclonal antibody is exquisitely specific in immunohistochemical staining making it an ideal tool for diagnostic purposes; however, acquisition of nasal biopsies is costly, therefore this tool has been restricted to post-mortem use.
Currently, diagnosis of ENA depends on ante-mortem clinical signs, and histological findings following post-mortem analysis. Serology has not been used for diagnostic purposes since there is a lack of antibody production to the virus capsid in animals with ENA (83). Possible causes were elimination of reactive B cells during negative selection due to expression of endogenous ovine betaretrovirus proteins in the thymus and lymphoid tissues (70, 71). Additionally, our inability to detect ENTV provirus in the blood mononuclear cells (BMCs) of either experimentally or naturally infected sheep at any point during the course of infection rules out the use of whole blood for diagnostic purpose (209). Computed tomography scanning can detect tumors in the nose (209) and lungs (170) of sheep, associated with ENTV and JSRV infection respectively, but this procedure is expensive, and is stressful for the animal as it requires transport and anaesthesia. Also, detection of a tumor in these anatomical locations does not conclusively implicate the involvement of ovine betaretroviruses. Therefore, the purpose of this report was to evaluate the utility of RT-PCR and immunohistochemical analysis of materials obtained from nasal swabs or nasal fluid for diagnosis of ENTV infection in sheep. With this information in hand, it was envisaged that a combination RT-PCR/cytospin assay could be implemented in a test-and-removal program aimed at eradicating ENTV from sheep flocks as was done for the highly related, Jaagsiekte sheep retrovirus (JSRV) in Iceland (231).

**Materials and methods**

**Sample collection**

The Animal Care Committee at the University of Guelph approved all animal use and related procedures. Two groups of sheep were sampled. The first group consisted of 15 Suffolk rams from a research flock in Quebec, Canada, of which some members had displayed clinical signs
of ENA, including nasal discharge and noisy breathing, for more than two years. Approximately 0.5 ml of nasal exudate was combined with 0.75 ml of Trizol LS RNA preservative (Life Technologies, Mississauga, Ontario, Canada) (2:3 ratio), was transported on ice and stored at -80°C. RNA was extracted according to the manufacturer’s instructions and stored at -80°C until cDNA synthesis. A second group was enrolled in the study that consisted of eighty horned Dorset sheep aged 6 months to 16 years belonging to a commercial flock with a long history of ENA. Both nostrils were sampled by gently rubbing the nasal turbinates with an 8-inch sterile cytobrush (Fisher Scientific, Whitby, Ontario, Canada). Two nasal swabs were obtained per animal. The first swab was transferred to a charged microscope slide (Superfrost Plus; Fisher Scientific) using standard procedures and transported to the laboratory immersed in PBS. Once in the lab, the slides were fixed in 4% paraformaldehyde for 10 min, and stored in PBS at 4°C until immunocytochemical staining. For the second swab, the tip of the cytobrush was then cut off and placed into a 1.5 ml microfuge tube containing 0.5 ml of RLT buffer (Qiagen, Toronto, Ontario, Canada). Specimens were transported to the laboratory on ice and frozen at -80 °C within 4 h of collection. Blood samples were obtained by venipuncture of the jugular vein using serum-separating vacutainer tubes (BD, Mississauga, Ontario, Canada). A set of animals showing clinical signs of ENA were euthanized, the nose sectioned and tissue samples were taken for formalin-fixation.

**Immunohistochemical and immunocytochemical staining**

Fixed tissues were embedded in paraffin, sectioned at 5 µm and stained with haematoxylin-eosin. The avidin-biotin-peroxidase complex (ABC) method was used on paraffin-embedded tissue sections for immunohistochemical demonstration of ENTV envelope protein as described previously (209). Immunocytochemical staining of nasal smears was conducted as above except
deparaffinization and hydration steps were excluded. After staining, nasal swab cytology slides were evaluated by three graders using the following grading scheme, ~ for no staining, * for low intensity, ** for moderate intensity and *** for high intensity staining.

**Enzyme-linked immunosorbent assay (ELISA)**

Serum antibodies reactive with ENTV-1 capsid and envelope proteins were detected by indirect ELISA. Recombinant antigens used in the ELISA were prepared as follows: Recombinant capsid protein from ENTV-1 (ECa) was expressed in *E. coli* using the pET28a System (Novagen, Mississauga, Ontario, Canada) and purified using a nickel column as described previously (209). ESU-IgG, a fusion protein comprised of the surface (SU) subunit of the ENTV-1 envelope protein fused to the Fc domain of human IgG₂ (99), was purified from the supernatant of 293T cells transfected with pCMV-ESU-IgG using a HiTrap protein-G sepharose column (GE Healthcare, Mississauga, Ontario, Canada) according to manufacturer instructions. Briefly, flat-bottomed 96-well plates (VWR International, Mississauga, Ontario, Canada) were coated with purified ECa (2 µg/ml) or ESU-IgG (2 µg/ml) and incubated at 4°C overnight. Plates were then washed with PBS-0.5% Tween 20 and blocked for 1 h at room temperature with PBS containing 5% non-fat dry milk. Serum samples heat inactivated at 56°C for 30 min were diluted in blocking buffer and added to the 96-well plates. Plates were then incubated at room temperature for 2 h. Following three washes in PBS-0.5% Tween 20, horseradish peroxidase (HRP) conjugated rabbit anti-sheep IgG (Life Technologies) (diluted 1:10,000 in blocking buffer) was added and plates were incubated for 1 h at room temperature. Plates were washed and the reaction was visualized by the addition of 100 µl of ABTS substrate [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); Mandel Scientific, Guelph, Ontario, Canada] for 10 min. Absorbance was measured at 405 nm using a microplate reader (Bio-tek Instruments, Inc.,
Rabbit polyclonal antibodies raised against bacterially-expressed ENTV-1 capsid protein (ECa) (Pacific Immunology, California, USA) and a mouse monoclonal antibody that cross-reacts with the ENTV envelope protein (146) were used as positive controls.

**Immunoblot analysis**

ECa and ESU-IgG proteins resolved on a 15% SDS-PAGE gel were transferred to polyvinyl difluoride (PVDF) membrane (Thermo Fisher Scientific) and blocked overnight at 4°C in PBS-Tween 20 containing 5% non-fat dry milk. The membrane was then cut into evenly sized strips and probed with individual sheep serum samples (diluted 1:50 in blocking buffer) at 4°C for 16 h. The strips were washed with PBS-0.5% Tween 20 and incubated with a 1:5,000 dilution of HRP conjugated rabbit anti-sheep IgG (Life Technologies) in blocking buffer for 1 h at room temperature. The membrane strips were developed using Western Lightning Plus ECL (PerkinElmer, Woodbridge, Ontario, Canada) and imaged with a ChemiDoc XRS (Bio Rad).

**Virus neutralization assay**

The ability of serum samples to neutralize ENTV-1 was assessed by neutralization assay. Briefly, NIH 3T3/LL2SN cells (95), which overexpress the receptor for ENTV-1, Hyal2, were seeded in 12 well plates at 1x10⁵ cells per well. After 16 h, sheep serum samples were heat-inactivated at 56°C for 30 min, mixed at 1:50 dilution with ~300 infectious ENTV-1 Env-pseudotyped murine leukemia virus (MLV) vectors expressing human placental alkaline phosphatase (AP) in a total volume of 50 µL, and incubated for 30 min at 37°C. The virus-serum mixtures were then added to NIH 3T3/ LL2SN cells with 500 µL medium containing 8 µg/ml polybrene for 4 h before replacing with 1 mL of fresh medium. Cells were fixed 48 hours later with 3.7% formaldehyde and stained for AP activity as described (92). The assay was performed
in quadruplicate and neutralization was defined as a 50% or more reduction in AP positive foci compared to PBS treated samples.

**RNA extraction and RT-PCR**

Total RNA was extracted from nasal exudate stored in Trizol LS according to the manufacturer’s instructions. Total RNA was extracted from the nasal swabs stored in RLT buffer using the RNeasy kit (Qiagen, Toronto, Ontario, Canada). cDNA libraries were synthesized using qScript™ Flex cDNA SuperMix (VWR International) and a combination of random and oligo dT primers. A fragment of the 5’end of the ENTV genome was amplified (Figure 5-1) using Platinum® PCR SuperMix (Life Technologies) and the ENTV-U5-F (5’-GAT GCT CCG TTC TCT CCT TAT A-3’) and GAG-R (209) primer pair. The efficiency of RNA extraction and cDNA generation was tested using forward (5’-TGT TC C AGT ATG ATT CCA CCC-3’) and reverse (5’-ATA AGT CCC TCC ACG ATG CC-3’) primers specific for ovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH)(148). Select RT-PCR products were sequenced to verify the identity of the amplicons.

**Results**

**Pilot study to evaluate the efficacy of a RT-PCR based assay to detect ENTV-1 in nasal exudates**

Efforts to develop an ante-mortem diagnostic test were initiated when we received 15 nasal exudate samples from a research flock of Suffolk rams in Quebec, Canada. Many of these rams had displayed clinical signs of ENA, including nasal discharge and noisy breathing, for more than two years. Samples were collected in Trizol LS (2:3 ratio of sample to Trizol LS) to preserve RNA. Based on sequence information that we recently obtained from ten North
American ENTV-1 isolates (179), primers were designed against the U5 and gag regions to specifically detect exogenous ENTV-1 (Figure 5-1A). RNA extracted from healthy sheep lung (a negative control) and naturally acquired ENA tissue (a positive control) were screened for the presence of ENTV-1 genomic RNA by RT-PCR. In this RT-PCR assay, an ENTV-1 positive sample produced a PCR product of approximately 592 bp (see positive control Figure 5-1B, lane 15). No amplification was detected in the negative control (Figure 5-1B, lane 17) confirming the specificity of these primers for exogenous ENTV-1. After confirming the ability of the RT-PCR assay to selectively amplify exogenous ENTV-1 by sequencing of the product from QB-12 (data not shown), RNA was extracted from all 15 nasal exudate samples and subjected to cDNA synthesis and RT-PCR for both GAPDH, as a control for RNA quality, and ENTV-1 (Table 5-1). The RT-PCR results for a subset of animals are shown in Figure 1B. Of the 15 samples submitted for RT-PCR analysis, 4 were positive for ENTV-1. PCR amplicons were cloned and sequenced to confirm that exogenous ENTV-1 was amplified (data not shown).
Figure 5-1. Detection of ENTV-1 genomic RNA in nasal swabs from sheep showing clinical signs of ENA
(A) A schematic of the ENTV-1 genome showing the location of primers used in the RT-PCR assay. (B) A representative image showing the results of RT-PCR amplification of exogenous ENTV-1 from RNA extracted from nasal swabs. RNA isolated from healthy sheep lung (Neg) and naturally acquired ENA (Pos) were used as a negative and positive controls, respectively. M indicates 1Kb Plus DNA ladder (Life Technologies).

Three RT-PCR-positive and two RT-PCR-negative rams from the putative ENA cluster in Quebec were euthanized and submitted for post mortem analysis. Upon gross inspection, the three RT-PCR positive rams had visible tumors while the nasal cavity of the two RT-PCR negative rams looked unremarkable (Table 5-1). Tissues harvested from the nasal cavities were subjected to histology and immunohistochemical staining with an ENTV-1 Env specific monoclonal antibody. ENTV-1 envelope expression and histopathological features consistent with ENA were detected in all three RT-PCR/tumor-positive cases (QB-1, QB-12 and QB-15). For one of the RT-PCR-negative cases (QB-6), a small ENTV-1 Env positive lesion was identified in the nose by immunohistochemistry. The other RT-PCR-negative sample appeared normal upon histological and immunohistochemical examination (QB-7). Results from the pilot study revealed what appeared to be a good correlation between RT-PCR positivity and a positive result by immunohistochemistry for envelope protein expression, which is the current gold
standard test. Lack of sample availability limited our ability to test the remaining cases by immunohistochemistry and therefore limited our ability to analyse the specificity and sensitivity of the RT-PCR test.

Table 5-1. RT-PCR and histology results from pilot study

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<td>QB-15</td>
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</tr>
</tbody>
</table>

(+) - positive, (-) - negative, (*) - IHC positive early neoplastic lesion, (ND) - not determined

Study to evaluate serology, cytology and RT-PCR based assays for ante-mortem detection of ENTV-1

Given the promising results of the pilot study we decided to launch a full-scale study involving the flock of 80 horned Dorset sheep from Ontario previously described. Additional control samples were obtained from ten sheep chosen at random from a research flock at the University of Guelph with no history of ENA and which has been closed for 25 years to all new animal additions. In this study, we expanded our sample repertoire to include nasal swabs, cytology samples and serum. Two nasal swabs were collected per animal. One swab was placed in RLT
buffer for subsequent RNA extraction and the other was applied to a charged microscope slide for cytological examination.

**Gross and histopathological detection of ENA showed good correlation with RT-PCR results**

Total RNA was extracted from nasal swabs and subjected to RT-PCR as described above. Based on the RT-PCR results (Table 5-2), seven ewes that were RT-PCR-negative for ENTV-1 infection, 14 ewes that were RT-PCR-positive for ENTV-1 infection, and one lamb (2013-1) of undetermined status but born to a mother who was RT-PCR-positive, were submitted for post-mortem analysis. As shown in Figure 5-2, tumors of various sizes, ranging from 0.5 cm to 8 cm, were detected upon dissection of the nasal cavity. All tumors stained positive for ENTV-1 Env expression by immunohistochemistry (Table 5-2 and Figure 5-3), whereas none of the sections from RT-PCR-negative animals did (Table 5-2). Importantly, all of the animals that were RT-PCR-positive for ENTV-1 had tumors whereas none of the animals that were RT-PCR-negative for ENTV-1 had tumors. No evidence of tumors or envelope-positive cells was detected in the lamb born to a mother who was RT-PCR-positive for ENTV-1 (Table 5-2). The specificity and sensitivity of the RT-PCR test was perfect [100% for both (Table 5-3)] when the immunohistochemical staining for envelope protein was used as the current gold standard test for calculation purposes. Taken together, these findings suggest that RT-PCR analysis of RNA extracted from nasal swabs represents a sensitive and specific method for detection of ENA.
Figure 5-2. Gross pathology of sheep nasal tumors

Representative images of tumors observed in the nasal cavity at necropsy (A-D). Examples of a large, soft unilateral tumor obstructing the entire nasal passage (A), a medium-sized, hard unilateral tumor (B) and a small soft tumor (C and D) are shown.
Figure 5-3. Histopathology and immunohistochemical staining for ENTV-1 envelope protein expression in nasal tumors

Representative images of hematoxylin and eosin stained nasal tissues from ENA positive (A and C) and ENA negative (E) sheep from a flock with endemic ENA. Immunohistochemical staining for ENTV-1 envelope protein in ENA tissue obtained from macroscopically evident tumors (B and D) and macroscopically normal nasal tissue with an early envelope positive lesion (F).
**Immunohistochemical staining of nasal smears for detection of ENTV-1**

To evaluate the use of nasal swab cytology as a method to diagnose ENA, slides were immunocytochemically stained for ENTV-1 envelope protein and blindly graded by three individuals. Slides were graded as (−) for no staining, (+) for low intensity, (**) for moderate intensity and (***) for high intensity staining (Figure 5-4 A, B, C and D, respectively). Isotype control staining was performed on a slide from a control animal and no staining was observed (data not shown). Although grading results were consistent for some samples (see sample 2011-50 in Table 5-2), overall there was poor intergrader agreement (Table 5-2). In addition, the quantity and quality of cells on the slide varied from sample to sample as well as between different regions of the same slide and, in a subset of cases, poor sample representation made grading difficult. Taken together, these results suggest that ENTV-1-infected cells can be identified by immunocytochemical staining of nasal smears, but that sample quality would need to be improved to allow appropriate and consistent interpretation. The specificity and sensitivity was relatively low compared to the RT-PCR test with 66.7% and 50% respectively (Table 5-3). These values were calculated from absolute positive/negative values derived from a consensus of the results from all three graders and any results with no clear consensus value not included (Table 5-2).
Figure 5-4. Nasal swab cytology detection of ENTV-1 envelope protein expression

Representative images showing nasal swab cytology slides stained with an anti-envelope monoclonal antibody and graded as ~ for no staining (A), * for low intensity (B), ** for moderate intensity (C) and *** for high intensity staining (D).
Detection of ENTV-1-reactive sheep serum by ELISA and western blot analysis

To investigate whether sheep mount an immune response to ENTV-1, serum samples were tested for reactivity against recombinant ENTV-1 capsid (ECa) and envelope surface subunit (ESU-IgG) proteins using an indirect ELISA (Figure 5A and 5B, respectively). Serum samples from the previously described closed research flock with no history of ENA (Figure 5-5, group i) were used to determine the background cut-off value, which was calculated as the mean of the naïve samples plus three times the standard deviation of those samples. Of the eight sheep that had no lesions of ENA by immunohistochemistry (Figure 5-5, group iii), three (37.5 %) tested positive by the ECa ELISA and five (62.5%) were positive by the ESU-IgG ELISA. In the group of animals confirmed to have ENA by post-mortem analysis (Figure 5-5, group ii), 3/13 (23.1%) were positive in the ECa ELISA and 5/13 (38.5%) were positive in the ESU-IgG ELISA. Only two animals (2006-37 and 2008-1) had serum that reacted positively using both the ESU-IgG and ECa ELISAs. Of the remaining 55 flock mates for which no post-mortem data were available (Figure 5-5, group iv), 8/55 and 15/55 serum samples were positive by ECa and ESU-IgG ELISA, respectively. Three of those serum samples (2008-1, 2011-36 and 2011-23) from group iv were positive on both ELISAs. Both ELISA-based tests show poor agreement with the results of the immunohistochemical staining for envelope protein as the resulting values for specificity and sensitivity of ESU-IgG ELISA was 62.5% and 37.5%, respectively (Table 5-3). The ECa ELISA showed similar specificity and sensitivity with 23.1% and 42.9%, respectively (Table 5-3). Thus, the ELISA tests would not be suitable for diagnostic purposes.
Figure 5-5. ELISA detection of antibodies reactive against ENTV-1 capsid and envelope proteins (A) Sheep serum samples tested in an indirect ELISA using purified ENTV-1 capsid (ECa) and (B) envelope (ESU-IgG) proteins as antigens. The dotted line represents the background cut off value. Group i, serum from control sheep with no history of ENA; group ii, serum from sheep confirmed to have ENA, group iii, serum from sheep confirmed to be free of ENA; and group iv, sheep of unknown ENA status. Chequered bars represent RT-PCR-positive sheep with unknown disease status.
Sera from animals submitted for post-mortem analysis were analyzed by strip-western blotting to investigate reactivity against denatured ENTV-1 antigens and its correlation with ELISA results. Recombinant ECa and ESU-IgG proteins were used as antigens and polyclonal rabbit ENTV-1 capsid antibody (209) and monoclonal anti-Env antibody (146) were used as positive controls, respectively (Figure 5-6, lane C). Bands were detected in most strips treated with serum samples, including some of the naïve sheep samples, at the appropriate molecular weight corresponding to capsid and ESU-IgG proteins (Figure 5-6). There appeared to be a higher correlation between ESU-IgG ELISA and western blot results compared to the correlation between ECa ELISA and western blot results (5/6 ESU-IgG ELISA positive samples were positive in western blot whereas only 2/10 ESU-IgG ELISA negative samples were positive on western blot). Conversely, ECa protein was detected in the majority of serum samples, including many of those from the control animals (see summary of ELISA results as shown in Figure 5-6). Nonetheless, these results demonstrate that sheep are able to mount an antibody-mediated immune response against ENTV-1

**Serum neutralization of ENTV-1 envelope-pseudotyped virions**

Heat-inactivated serum samples from animals submitted for post-mortem analysis, and thus with a known disease status, were evaluated for their ability to inhibit infection mediated by ENTV-1 envelope pseudotyped MLV particles. Neutralization was defined as a 50% or greater reduction in infection (as determined by foci of alkaline phosphatase expression) relative to untreated virus. Many of the serum samples were unable to neutralize infection according to the above criteria, however a subset were able to dramatically reduce virus transduction (Table 5-2). Interestingly, the sample that showed the most robust neutralization was from a 2-week-old lamb
(sample 2013-1) whose mother was ENA-positive. Eight serum samples remain to be tested before full analysis of the relationship between neutralizing antibodies, presence or absence of disease, and RT-PCR test results.

**Figure 5-6. Western blot analysis of sheep serum against ENTV-1 envelope and capsid proteins**

Serum samples from sheep with ENA (red) and from disease-free sheep that were housed with diseased animals (black) were tested for reactivity against ENTV-1 envelope (ESU-IgG) and capsid (ECa) proteins in a strip western blot. Naïve serum was obtained from a high health status research flock with no history of ENA. ELISA results for the various serum samples are shown above (for ESU-IgG) and below (ECa).
Table 5-2. Summary of sample analysis from sheep submitted for post-mortem examination from the Ontario flock consisting of Horned Dorset sheep.

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<td>*</td>
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<td>**</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>2010-43</td>
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<td>+</td>
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<td>*</td>
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<td>-</td>
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<tr>
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<tr>
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<td>+</td>
<td>**</td>
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<tr>
<td>2012-49</td>
<td>Lesion</td>
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<tr>
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<td>+</td>
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<td>P/S</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
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<td>NA</td>
</tr>
</tbody>
</table>

(+) - positive, (-) - negative, (*) - IHC positive early neoplastic lesion, (P/S) - poor sample, (~) - no staining.

(*) - low intensity staining, (**) - moderate intensity staining, (***) - high intensity staining. Grey background – positive by IHC for envelope as the gold standard.
Table 5-3. Specificity and sensitivity values for the diagnostic tests evaluated.

<table>
<thead>
<tr>
<th>Test</th>
<th>Specificity</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Result</td>
<td>95% CI</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>100%</td>
<td>62.9% - 100%</td>
</tr>
<tr>
<td>Cytology</td>
<td>66.7%</td>
<td>22.7% - 94.7%</td>
</tr>
<tr>
<td>ECA ELISA</td>
<td>62.5%</td>
<td>24.7% - 91.0%</td>
</tr>
<tr>
<td>ESU-IgG ELISA</td>
<td>37.5%</td>
<td>9.0% - 75.3%</td>
</tr>
</tbody>
</table>

CI – confidence interval

Discussion

ENA is a common disease among North American sheep flocks (179) and as producer and veterinarian awareness increases, so too does the need for an accurate ante-mortem diagnostic test. Since vaccination is not a viable approach, diagnostic tests that can identify infected animals will greatly assist with disease surveillance and eradication efforts. Based on our previous findings, PBMCs are not an appropriate source material for detecting ENTV-1 since at no point during the course of infection can ENTV-1 be detected in PBMCs by PCR. In this study, we endeavoured to develop a novel, non-invasive diagnostic tool that could be used to not only confirm that clinical signs of dyspnea were likely due to ENA, but also to detect ENTV-1 infection prior to the onset of disease (i.e. preclinical diagnosis). The latter would assist in surveillance programs and eradication efforts to quarantine and/or eliminate ENTV-1 infected animals from a flock, which would ultimately enhance the health and profitability of the sheep industry. Using clinical samples from a flock with a long history of ENA, we developed and tested serology, cytology, and RT-PCR based assays for utility as diagnostic tools for identification of ENTV-1 infected or ENA-affected sheep. A comparison of RT-PCR, ELISA and cytology results with post-mortem and histological findings showed poor correlation between the presence or absence of ENA tumors and results of cytology, ELISA and western
blot. However, based on small numbers of animals, RT-PCR on RNA extracted from nasal swabs proved to be highly sensitive and specific. Of the 14 sheep that tested positive by RT-PCR and were submitted for post-mortem analysis, all 14 had nasal tumors that were confirmed by IHC to be ENA. An additional eight RT-PCR negative sheep were confirmed to be free of disease. As an indication of the sensitivity of this assay, a sheep less than 8 months of age, which was positive by RT-PCR, was found to have a small neoplastic lesion comprised of approximately 50 ENTV-1 Env-positive cells (Figure 5-3).

The RT-PCR assay described in this study has many advantages. In addition to being highly specific for exogenous ENTV-1, it is non-invasive and sample collection is simple to perform. In fact, when we retested nasal swabs that were collected by the producer we found that the animals had the same RT-PCR status irrespective of who collected the sample (data not shown). Since the sampling materials are inexpensive, more than one sample per animal can be collected at one time so as to have a backup should the RNA extraction fail. By generating cDNA using oligo dT and random primers, RNA quality and presence of ENTV-1 can be assayed from the same cDNA; however, the RT-PCR assay was not amenable to multiplexing so separate PCR reactions were required to assess ENTV-1 and GAPDH. This assay could be useful in situations where the veterinarian or producer would like confirmation that clinical signs are in fact due to ENA and not some other condition such as nasal bots or chronic rhinitis. Indeed, one of the sheep in our study had experienced chronic rhinitis and nasal discharge for many months and appeared to be suffering from ENA. This animal was repeatedly negative for ENTV-1 by RT-PCR and upon necropsy it was discovered that the animal had an extensive bacterial infection but no evidence of ENA.
Sheep are thought to be immune tolerant to exogenous ENTV-1 infection. It is been hypothesized that immune cells reactive to endogenous ovine betaretroviruses, which are transcribed in the thymus and peripheral lymph nodes during ontogeny (87), undergo clonal deletion rendering sheep unable to mount an immune response against invading exogenous ovine betaretroviruses. A study by Ortin et al. compared serum from healthy sheep with serum from sheep affected with ENA for the presence of antibodies against ENTV-1 capsid fused to glutathione S-transferase (GST). Although some serum samples reacted positively to the antigen, the results were difficult to interpret due to cross-reactivity with the GST protein alone (83). For this reason, we chose to produce recombinant ENTV-1 capsid protein using a hexa-His tag bacterial expression system and to remove the His tag prior to using it as an antigen. The results presented here indicate that sheep can respond immunologically to exogenous ENTV-1. Antibodies reactive with both the capsid and the envelope protein of ENTV-1 were detected in the serum of sheep from a flock with a high incidence of ENA in both ELISA and western blot analysis. There was, however, no consistency within ENA affected and disease-free groups, such that animals in both groups had immunoreactive antibodies. Similar results were obtained with the virus neutralization assay in that the ability to neutralize a virus pseudotyped with the ENTV-1 Env protein did not correlate with the absence of presence of ENA. Despite the fact that serology cannot reliably be used to diagnose ENTV-1 infection, it is clear that some sheep are able to mount an immune response against the virus. We do not however, know whether disease-free sheep that mounted an immune response against ENTV-1 were at one point infected with the virus and cleared it. Nevertheless, it is possible that the immune system may be a factor in determining the outcome of ENTV-1 infection, particularly in animals where tolerance to ENTV-1 is broken and an immune response is developed. It is likely that the antibodies detected
in these experiments represent low affinity antibodies because high affinity antibody producing cells would be deleted in the thymus (86). Low affinity antibody-producing cells on the other hand could escape clonal deletion and be induced to expand when exposed to exogenous ENTV-1 antigens on antigen-presenting cells in the periphery.

Given that ENTV-1-infected sheep can harbor infectious tumors and exist in a flock unnoticed for a considerable amount of time, or be sold as healthy animals, there is an urgent need for a rapid and reliable ante-mortem diagnostic test for ENA for use in individual sheep. This report describes a practical and highly specific RT-PCR technique for the detection of clinical and preclinical ENA that may prove beneficial in future control or eradication programs.
Chapter Six:

Truncation of the ENTV-1 envelope protein cytoplasmic tail increases fusogenicity and transduction efficiency

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Manuscript in preparation for submission to the Journal of Virology
Abstract

Enzootic nasal tumor virus (ENTV) and Jaagsiekte sheep retrovirus (JSRV) are simple betaretroviruses of sheep and goats that share greater than 90% sequence identity and utilize the same cellular receptor for virus attachment, but cause neoplasms of the nose and lung, respectively. It is not known why ENTV-1 and JSRV cause such distinct neoplasms, but often the envelope protein is the viral element that dictates disease tropism. To address this question, JSRV and ENTV envelope (Jenv and Eenv)-pseudotyped virions were generated and transduction was evaluated both in vitro and in vivo. The cytoplasmic tail (CT) of retroviral envelope proteins is a key modulator of envelope-mediated fusion and pseudotyping efficiency, especially in the context of heterologous Gag proteins. Since Eenv does not pseudotype conventional vectors efficiently, progressive truncations of the Eenv CT were tested for increased pseudotyping efficiency in two distinct vector systems. Complete truncation of the Eenv cytoplasmic tail (at amino acid 575) increased transduction of pseudotyped murine leukemia virus (MLV) and human immunodeficiency virus (HIV) viral vectors by increasing fusogenicity without affecting sensitivity to inhibition by lysosomotropic agents, subcellular localization, or efficiency of inclusion into virions. Transduction of sheep cells was more efficient than of murine fibroblasts overexpressing the virus receptor, Hyal2. Transduction of lung and nose cells was observed in fetal lamb primary cells as well as lambs infected in vivo with Jenv and Eenv-pseudotyped lentiviruses, in which transduction of progenitor cell types was observed. These results suggest that envelope-mediated entry is not the primary determinant of disease tropism for ENTV-1 and JSRV.
**Introduction**

Enzootic nasal tumor virus (ENTV) and Jaagsiekte sheep retrovirus (JSRV) are simple betaretroviruses of small ruminants that have been shown to induce tumors in the nose (209) and lung (117), respectively. Despite sharing approximately 95% overall amino acid similarity (179), JSRV and ENTV have distinctly different tissue tropisms. While the long terminal repeat (LTR) of JSRV is preferentially active in type 2 alveolar cells (75), the LTR of both ENTV and JSRV has been shown to be active in the nose, trachea and lung epithelium in mice using an adeno associated virus (AAV) vector system (232) suggesting that the promoter is not the determining factor of disease tropism. The envelope protein of ENTV and JSRV is an oncogene and expression of the envelope protein *in vivo* has been shown to induce tumors, but in a non-tissue specific manner (146). The viral envelope glycoprotein mediates interaction with the cellular receptor and entry into target host cells. The cellular receptor for ENTV-1 and JSRV is hyaluronidase 2 (Hyal2) (94, 99, 187), a glycosyl phosphatidyl inositol (GPI)-anchored cell surface protein and a member of a group of proteins that degrade hyaluronan (98, 233), a glycosaminoglycan present in the extracellular matrix. The expression level and distribution of the cellular receptor can restrict viral tropism, but since Hyal2 is expressed on most tissues (97), including the entire respiratory tract, it is unlikely that the tropism of these viruses is limited by the expression profile of the receptor. There are other host-derived factors that can affect virus entry beyond the receptor, including restriction factors (193, 234, 235) and enzymatic processing events (236, 237), which can be reliant on interactions with the envelope protein. To determine whether the envelope proteins of JSRV and ENTV-1 are responsible for determining disease tropism, pseudotyping experiments can be conducted.
The cytoplasmic tail of the ENTV-1 envelope contains several motifs associated with subcellular sorting and localization. Tyrosine (Yxxθ, where θ represents a hydrophobic amino acid) and dileucine (Lθ) sorting motifs in the cytoplasmic tail of viral glycoproteins, including those of Moloney murine leukemia virus (MLV) and Mason-Pfizer monkey virus (MPMV), have been shown to influence subcellular localization and pseudotyping efficiency (238). The subcellular location of assembly and Gag-Envelope protein interactions differ among retroviruses (239). Betaretroviruses are unique among orthoretroviruses because their viral cores assemble at a perinuclear location (53) rather than at the plasma membrane like gamma and lentiviruses (51, 201). This difference can cause a decrease in pseudotyping efficiency due to incompatibility between the localization of envelope and virion core protein interactions required for assembly and release (240). Truncation of the cytoplasmic tail of numerous retroviral envelope proteins has been shown to circumvent inhibitory or non-productive envelope and heterologous core interactions and to rescue inefficient virus assembly (241–245). In contrast, truncation of the JSRV envelope protein cytoplasmic tail has been shown to increase fusogenicity at neutral pH but ultimately decrease transduction of pseudotyped MLV particles (246). It is not known whether truncation of the Eenv cytoplasmic tail will have the same effect or alternatively, enhance passive incorporation into budding virions.

In this report, we examine the effect of progressively truncating the cytoplasmic tail of Eenv on pseudotyping efficiency and transduction both in vitro and in vivo. Partial truncation resulting in removal of all tyrosine-based motifs did not significantly affect pseudotyping efficiency; however complete truncation of the cytoplasmic tail dramatically increased pseudotyping efficiency. Mutation of the dileucine motifs in the cytoplasmic tail of Eenv did not significantly alter subcellular localization, but transduction and fusion efficiency was increased to a level
equivalent to that of the fully truncated Eenv protein. *In vivo* transduction with pseudotyped lentiviruses did not show a significant difference in the transduction profile between Jenv and truncated Eenv pseudotypes suggesting that the envelope proteins of ovine betaretroviruses do not define disease tropism.

**Materials and methods**

**Cloning and mutagenesis**

The envelope gene of JSRV was obtained from Dr. Dusty Miller in an expression vector containing the LTR and a truncated intron from MoMLV upstream of the JSRV envelope gene (pSX2Jenv). The envelope gene of ENTV-1 was amplified from a plasmid containing a fragment of the ENTV-1NA4 genome (Genbank accession number: FJ744146) using primers to add *Dra*III (Eenv F) and a *Cla*I (Eenv R) (Table 6-1) restriction sites to the 5’ and 3’ termini, respectively and the KOD Hot Start DNA Polymerase kit (VWR International, Mississauga, Ontario, Canada). Both Eenv and Jenv were cloned into pCIneo for expression in human cells via amplification with Eenvsc F or Jenvsc F primers respectively, and the Envsc R primer (Table 6-1) followed by TA cloning into the pGEMTEasy system (VWR International) and subsequent subcloning into the *Eco*RI site of the plasmid pCIneo. Truncation and dileucine mutants in Figure 6-1 were created using a modified site-directed mutagenesis protocol as outlined by Wang *et al.* (211) using the corresponding primers shown in Table 6-1. Briefly, mutagenesis was performed with two separate amplification reactions containing either forward or reverse primer to amplify the parental envelope expression plasmid for three rounds. The two single-primer PCR products were combined in one tube and amplified for a further 14 rounds. Methylated parental plasmid DNA was digested with *Dpn*I before transformation into competent *E. coli*
cells. VSV-G pseudotyped viruses were created using the pCMV-VSV-G expression vector (Addgene plasmid 8454)(247).

Table 6-1. List of primer sequences utilized in cloning and mutagenesis

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’ to 3’</th>
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<tbody>
<tr>
<td>Eenv F</td>
<td>CACCCGTTGAAAGTTAATTCCAGCAGCCAG</td>
</tr>
<tr>
<td>Eenv R</td>
<td>CATCGATGGGACAGGGAGCTTAGGTACT</td>
</tr>
<tr>
<td>Eenvsc F</td>
<td>GAAACATGCCGAAGCACCCTGGATCCCCGAA</td>
</tr>
<tr>
<td>Jenvsc F</td>
<td>GAAACATGCCGAAGGCCCCGCCTGGATCCCCGAA</td>
</tr>
<tr>
<td>Eenvsc R</td>
<td>CAAAACCTAGAGCCTGGACACACTCAGTAC</td>
</tr>
<tr>
<td>Eenv1575*F</td>
<td>TTTTAGTTCCCTGCCTCACTCGAGATTTATAGAAAGATTCTACATCAAAATGAG</td>
</tr>
<tr>
<td>Eenv1575*R</td>
<td>CAATCTCTATTTGTAGGAATCTTTCTATAATTTGAGGAGGAACACT</td>
</tr>
<tr>
<td>EenvY590*F</td>
<td>CCTACAAATGAGTTGAGTTAATAATACATATGAAATAGAGGAGGAACCT</td>
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<tr>
<td>EenvY590*R</td>
<td>GGTATTGCAACATGTATCTCTATTTTATGATATTAATTCAACTCTTTGTAGG</td>
</tr>
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<tr>
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<tr>
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<td>TAAAAAAAGGAGGGAGTGGTCGGGGGACAGCCCCG</td>
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<td>Eenv I575SF</td>
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<td>Eenv I586SF</td>
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</tr>
<tr>
<td>Eenv I586SR</td>
<td>TATTTCATATGGCTTTATCTCTCTCTCTTTGGTAGG</td>
</tr>
</tbody>
</table>
Cell culture and virus production

Primary sheep lung and nasal epithelial cells were isolated from fetal lung and nasal tissue diced with a scalpel blade and subsequently dispersed by passage through a 20-gauge needle. Non-adherent cells were removed 72 h after plating and the remaining adherent cells were treated with trypsin for three minutes to remove fibroblast cells and enrich for epithelial cells. The cells were propagated for three to four passages before transfection. Primary cells as well as HEK 293, NIH 3T3 LGPS/LAPSN (248), NIH 3T3/LL2SN (188), HeLa Tat3 and HeLa-CD4-LTR-β-gal (MAGI) (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH; HeLa-CD4-LTR-β-gal from Dr. Michael Emerman (249)) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in 10-cm tissue culture dishes at 37°C under 5% CO₂. For lentivirus production, HEK 293 cells were transfected using the calcium phosphate co-precipitation method with pTYEFnLacZ, pHpdIN/A, pCEP4Tat, pCMVBgal and the appropriate pCIneo envelope expression vectors. Pseudotyped MLV particles were generated by transfection of NIH 3T3/LGPS-LAPSN cells with pSX2 based envelope expression vectors by the calcium phosphate co-precipitation method. In both cases, medium was replaced after 16 h with 5 ml of fresh medium. Supernatants were harvested 48 to 72 h post-transfection and passed through a 0.45-µm-pore-size filter. Virus samples were either used immediately for infection, or used from aliquots stored at -80°C.

Immunoblotting

Viruses in supernatants analysed in the envelope inclusion assay were pelleted by ultracentrifugation through a 20% sucrose cushion in a SW32 Ti rotor (Beckman Coulter Canada) at 60,000xg for 2 h at 4°C. The viral pellet was re-suspended in radioimmunoprecipitation assay (RIPA) buffer, resolved by sodium dodecyl sulphate-
polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyl difluoride (PVDF) membrane. Membranes were blocked with 5% skim milk in PBS-0.5% Tween20 and probed with antibodies against the JSRV envelope (146) and the HIV capsid (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Monoclonal Antibody to HIV-1 p24 (AG3.0) from Dr. Jonathan Allan (250)) or MLV capsid protein (a kind gift from Dr. Stephen Goff, Columbia University).

**Flow cytometry**

HEK 293 cells (2x10^6 cells/6-cm plate) were transiently transfected by the calcium phosphate precipitation method with envelope expression vectors. Media was changed 16 hours post-transfection and cells harvested 48 hours post-transfection using PBS/0.2% EDTA treatment and agitation with a pipette. Cells were maintained at 4˚C throughout the sample preparation procedure. Cells were washed with PBS supplemented with 2% bovine serum albumin (wash buffer) before staining with murine hyperimmune serum directed against the JSRV envelope (diluted 1:200 in wash buffer). Cells were washed and stained with an Alexa Fluor 488 conjugated goat anti-mouse IgG secondary antibody (Life Technologies, Burlington, Ontario, Canada). Cells were washed and stained with propidium iodide in order to exclude non-viable cells during analysis. Cells were analysed on a FACScan flow cytometer (Becton Dickinson Biosciences, San Jose, California). FlowJo cell analysis software (Tree Star Inc, San Carlos, California) was used to evaluate cell populations, which were gated based on cells stained with secondary antibody alone.

**Pseudotyping, infection and lysosomatropic agent inhibition**

NIH 3T3/LL2SN cells were seeded on 12-well plates (5x10^5 cells/well) and, in experiments involving lysosomatropic agents, were pre-treated with ammonium chloride and chloroquine for
two hours at the indicated concentrations and infections were performed in the presence of the drugs. No pre-treatment was used for bafilomycin A1. NIH 3T3/LL2SN cells were infected with equivalent amounts of pseudotyped MLV or HIV vector containing supernatant in the presence of 8 µg/mL polybrene (Sigma) for 16 h. Transduction was quantified 48 h post-infection by fixation of cells with 3.7% formaldehyde and staining for AP or nLacZ marker protein expression (99). Histograms were generated and statistical analysis was performed using GraphPad 6.0 for Windows (GraphPad Software, Inc, La Jolla, CA, USA) Two-way ANOVA was used to determine statistically significant differences among pseudotype transduction groups. P-values of 0.05 or less were considered to be statistically significant.

**Cell-cell fusion assay**

HEK 293 cells were seeded in 12-well plates at 1x10^4 cells/well and transfected with pCEP4Tat and envelope expression vectors by the calcium phosphate method. After 16 h, the media was replaced and Magi cells were added (1x10^4). After 24 hours, co-cultures were treated with citrate buffer of the indicated pH for 5 minutes. Treated cells were rinsed twice with PBS and incubated with fresh media for 16 h before fixation with 3.7% formaldehyde for 10 min and staining with Xgal staining solution at 37°C for 16 h. Nuclear β-galactosidase positive syncytia were counted manually. Syncytia containing two or fewer nuclei were excluded.

**Immunofluorescence assay**

HEK 293 and NIH 3T3/LGPS-LAPSN cells were seeded onto coverslips treated with FNC Coating mix (Anthena) at 1x10^4 cells/well in a 6-well dish. Envelope constructs were co-transfected with either pHIVGagGFP (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pGag-EGFP (Cat#11468) from Dr. Marilyn Resh)(251) or pMLVGagYFP (Addgene plasmid #1813) (252). Cells were fixed 48 h after transfection and
stained using murine hyperimmune serum directed against the JSRV envelope protein. Cover slips were with, mounted onto slides using Prolong Gold Antifade Reagent (Life technologies) which contains DAPI counterstain and analyzed with a Leica DM 6000B confocal microscope (Molecular and Cellular Imaging Facility, University of Guelph).

Results

Truncation of the cytoplasmic tail of Eenv enhances transduction efficiency of pseudotyped virions

Site-directed mutagenesis was used to truncate the cytoplasmic tail of the ENTV-1 envelope glycoprotein at the indicated amino acid positions (Figure 6-1A). NIH 3T3/LL2SN cells were transduced with MLV particles pseudotyped with the Eenv truncation mutants and the numbers of AP-positive foci were compared to that of the wildtype JSRV and ENTV-1 envelope proteins (Figure 6-1B). Results were reported as a percentage of the number of AP-positive foci in cells transduced with Jenv pseudotyped MLV virions, with Jenv set at 100%. As has been noted by other groups, Eenv pseudotyping efficiency was significantly lower than that of Jenv. Complete truncation of the Eenv cytoplasmic tail (represented by the Eenv I575* mutant) dramatically increased transduction efficiency, approaching that of Jenv. The remaining truncation mutants were not significantly different from wildtype Eenv although a slight increase was observed in the truncation mutant lacking the transforming tyrosine-based motif (EenvY590*). Since the Y590* truncation mutant did not significantly increase transduction efficiency, we reasoned that an element in the intervening sequence (amino acids 575 to 590) must be inhibiting transduction. Analysis of the CT sequence of Y590* revealed the presence of two dileucine motifs (LI residues shown in red in Figure 6-1A) that are conserved in Eenv but not found in Jenv.
Interestingly, this region in Jenv has been shown to form an amphipathic helix structure (158) but whether this is the case for Eenv has not been determined. The second hydrophobic residue of each of the dileucine motifs was mutagenized to serine (I575S and I586S, labelled as diL), in order to ablate the motif and any associated putative trafficking function. Dileucine mutations were introduced in the context of both the full-length cytoplasmic tail as well as the Y590* truncation with the aim of determining the role of the dileucine motifs with and without the tyrosine motif. The dileucine mutants were tested in both MLV and HIV vector systems. Transduction efficiency was unchanged in comparison to wildtype for the diL mutant, but a dramatic increase in transduction efficiency was observed in diLY590* mutant to a level equivalent to EenvI575* (Figure 6-1C). Single dileucine mutants were also generated but had no effect on transduction efficiency (data not shown).
Figure 6-1. Truncation of the Eenv cytoplasmic tail increases transduction efficiency

(A) Schematic of truncations and mutations introduced into the cytoplasmic tail (CT) of Eenv. The CT spans amino acid positions 572 to 617 of the envelope protein. Putative dileucine and tyrosine based motifs in the CT sequence are in red. Truncation mutant constructs were named by the amino acid at which the truncating stop codon was introduced. diL indicates constructs in which the two membrane proximal dileucine motifs have been abolished by mutation of the isoleucines at amino acid position 575 and 586 to serine. MSD - membrane spanning domain, SU - surface subunit, TM - transmembrane subunit, ED - ectodomain. (B) NIH 3T3 cells overexpressing human Hyal2 (NIH 3T3/LL2SN) were incubated with MLV virions pseudotyped with the indicated Eenv CT truncation mutant proteins and the number of transduced foci counted 48 hours later after staining for alkaline phosphatase expression. Foci counts are expressed as a percentage of Jenv pseudotyped virion transduction from five independent experiments. (C) Transduction values of an equivalent experiment using MLV and HIV virions pseudotyped with the indicated Eenv CT truncation and dileucine motif mutants. The 2way ANOVA was used for statistical analysis and bars indicate standard error of the mean. Bars marked with * are statistically significant from the corresponding wildtype Eenv (B and C).
Lysosomotropic agent and V-ATPase inhibitor treatment has identical effects on wildtype and CT truncated Eenv

pH-dependent viruses utilize endocytic pathways to transport virions into endosomal compartments where the acidic environment triggers a conformational change of the surface subunit and the ectodomain that drives membrane fusion (23). Fusion by Jenv and Eenv pseudotyped virions is inhibited by lysosomotropic agents/V-ATPase inhibitors and is increased at low pH suggesting that fusion takes place in the late endosome/early lysosomal compartments (27, 28). To investigate whether this requirement was maintained in the EenvI575* truncation mutant or if the fusion kinetics and/or subcellular compartment of entry was changed, a transduction assay was performed in the presence of lysosomotropic agent/V-ATPase inhibitors (Figure 6-2). A significant difference between untreated and all treatments was observed in cells transduced with Jenv pseudotyped virions, as was also observed by Bertrand et al (27).

Transduction by Eenv pseudotyped virions was significantly inhibited by NH₄Cl and bafilomycin A1 treatment, but even though fewer foci were observed in chloroquine-treated wells compared to untreated wells, the reduction was not significant (Figure 6-2). The same pattern was observed in EenvY590* and Eenv I575* transduced wells. Taken together, these results suggest that truncation of the cytoplasmic tail does not affect the pH-dependent entry of Eenv-pseudotyped virions.
Figure 6-2 Truncation of the Eenv cytoplasmic tail does not affect the pH-dependent entry of Eenv pseudotyped virions

For the lysosomotropic agent/V-ATPase inhibition experiments, cells were pretreated with NH₄Cl or chloroquine for 2 h at the indicated concentrations (no pretreatment was performed with bafilomycin A1) and exposed to MLV virions pseudotyped with the indicated envelope proteins in the presence of inhibitors. After 16 h the media was replaced and 48 h later cells were stained for AP activity. AP positive foci were counted and percent infection was calculated relative to results for vehicle (dimethylsulfoxide) treated cells. The 2way ANOVA was used for statistical analysis and bars indicate standard error of the mean. Bars marked with * are statistically significant from the untreated transduction with the same envelope protein.
Cytoplasmic tail modification does not affect surface expression of envelope or incorporation into virions

Since MLV and HIV particles assemble and bud from the plasma membrane, presentation of the envelope protein on the cell surface is an important property for inclusion into virions. The relative level of envelope protein cell surface expression in transiently transfected HEK 293 cells was quantified by flow cytometry using murine anti-envelope polyclonal antiserum. A prominent shift in fluorescence intensity was observed in all cell populations expressing wildtype and mutant ovine betaretroviral envelope proteins (Figure 6-3A) indicating that modification of the cytoplasmic tail had no effect on surface expression of Eenv.

Studies probing the pseudotyping efficiency of several other retroviral envelope glycoproteins have noted the presence of inhibitory signals in the cytoplasmic tail that diminish the efficiency of incorporation into heterologous viral particles (244). Inclusion of envelope proteins into particles in these situations is extremely low unless these signals are removed. Thus, differences in transduction efficiency between wildtype Eenv and cytoplasmic tail truncated Eenv proteins could be due to differences in envelope incorporation efficiency. To address this possibility, envelope inclusion into MLV and HIV-based vectors was investigated by analyzing envelope to capsid protein ratios of equal volumes of concentrated pseudotyped virus particles by Western blot. A single Western blot representative of three to four independent experiments is shown for MLV (Figure 6-3B) and HIV (Figure 6-3C) vector systems. The ratio of envelope to capsid protein was determined and is shown at the bottom of each series of western blots. Lower values signify virions that have a large number of envelope moieties per capsid moiety and have therefore inefficiently incorporated envelope. No significant increase in inclusion was observed
for Eenv I575* over that of the wildtype Eenv envelope. Interestingly, Jenv showed a decreased incorporation compared to all Eenv pseudotypes in MLV particles, but not in HIV particles. Taken together, these results indicate that truncation of the cytoplasmic tail of Eenv does not interfere with cell surface expression or virion incorporation.
Figure 6-3. Truncation of the Eenv cytoplasmic tail does not influence cell surface expression or envelope inclusion

(A) HEK 293 cells transiently transfected with expression vectors for the indicated ovine betaretrovirus envelope proteins were stained with mouse anti-envelope polyclonal primary antibody and Alexa Fluor 488-labelled anti-mouse secondary antibody and analyzed by flow cytometry. Solid coloured lines represent the fluorescence shifts of the corresponding ovine betaretrovirus envelope proteins and filled area represents cells incubated with secondary antibody alone. Representative immunoblots show envelope protein inclusion in HIV (B) and MLV(C) pseudotyped virions relative to capsid protein. Note that Jenv shows at a lower molecular weight compared to Eenv. Band intensities were determined by densitometry and the ratio of envelope to capsid protein density is shown below each lane.
Cytoplasmic tail modification of Eenv does not affect co-localization with Gag

To determine whether truncation of the cytoplasmic tail or mutation of the membrane proximal dileucine motifs changes co-localization of Eenv with HIV or MLV Gag, colocalization experiments were conducted. HIV Gag-GFP and MLV Gag-YFP fusion proteins were co-expressed with the various Eenv constructs in HEK 293 cells. Envelope proteins were immunolabelled with murine polyclonal anti-Env primary antibody followed by Texas Red-conjugated anti-mouse secondary antibody. Comparison of colocalization between the different ENTV-1 envelope proteins in the context of both HIV (Figure 6-4) and MLV (Figure 6-5) Gag did not demonstrate a substantial difference, although Jenv showed an increased colocalization with HIV Gag (Figure 6-4). Cells transfected with HIV Gag-GFP showed a more diffuse staining pattern than MLV Gag-YFP transfected cells, but distinct punctate areas of fluorescence were observed intracellularly and at the plasma membrane in both cases. The majority of GFP/YFP punctate structures were closely associated with a more diffuse cluster of fluorescence in the red channel, which was demonstrated as a yellow area with a red halo in the merged image. Interestingly, these co-localized foci were observed intracellularly as well as at the membrane. Results from these experiments suggest that modification of the ENTV-1 Env cytoplasmic tail does not significantly affect co-localization with either MLV or HIV Gag.
Figure 6-4. Co-localization of HIV Gag with ovine betaretrovirus envelope proteins

HEK 293 cells were co-transfected with the indicated pClneoEnv, Eenv or Eenv mutant construct and HIVgagGFP expression vector for forty-eight hours before fixation and permeabilization. Polyclonal mouse antibody raised against ovine betaretrovirus envelope proteins labelled with a Texas red-conjugated goat anti-mouse secondary antibody. Nuclei were stained with DAPI. Confocal microscopy images are shown.
Figure 6-5. Co-localization of MLV Gag with ovine betaretrovirus envelope proteins

HEK 293 cells were co-transfected with the indicated pClneoEnv, Eenv or Eenv mutant construct and MLVgagYFP expression vector for forty-eight hours before fixation and permeabilization. Polyclonal mouse antibody raised against ovine betaretrovirus envelope proteins labelled with a Texas red-conjugated goat anti-mouse secondary antibody. Nuclei were stained with DAPI. Confocal microscopy images are shown.
Fusogenicity of Eenv at neutral pH is increased by cytoplasmic tail truncation and
dileucine motif mutation

The ability of a viral envelope protein to mediate fusion is essential for infection to occur, thus
we investigated the fusogenicity of the Eenv truncation mutants in reference to the wildtype
envelopes at a neutral pH. VSV-G was included as a control and showed frequent fusion with
many syncytia containing ten or more nuclei (Figure 6-6A). Fusion foci in Jenv and wildtype
Eenv-transfected wells was an extremely rare event and contained only three to five nuclei
whereas up to seven to eight nuclei were observed in Eenv I575* and EenvdiLY590* fusion foci
(Figure 6-6A). Total fusion foci counts correlated well with the above observations. VSV-G
wells had the greatest number of fusion foci counts (~175 fusion foci/well, Figure 6-6B) while
wildtype Jenv and Eenv wells showed the lowest (~20-30 foci/well, Figure 6-6B). A significant
increase was observed in Eenv I575* and EenvdiLY590* fusion foci counts (~60 foci/well,
Figure 6-6B) compared to wildtype Jenv and Eenv. Therefore, truncation of the Eenv
cytoplasmic tail enhances fusogenicity.
Figure 6-6. Cell-cell fusion assay

(A) Representative images showing the number of βgalactosidase-stained nuclei in syncytia among cells transfected with VSV-G or EenvI575* expression vectors. 
(B) A bar graph showing the number of fusion foci/well observed after transfection with the indicated envelope expression vector. Bars represent standard error of the mean, * are statistically significant from wildtype Eenv.
Jenv and Eenv pseudotyped virions transduce sheep primary cells more efficiently than NIH 3T3/LL2SN cells

Primary cells were established from fetal nose and lung tissue as described above. Primary sheep cells and NIH 3T3/LL2SN cells were transduced with equal amounts of lentivirus pseudotyped with VSV-G, Jenv, Eenv or Eenv I575* and the resulting nLacZ positive nuclei quantified.

Transduction of primary sheep cells by VSV-G pseudotyped virions was significantly less than NIH 3T3/LL2SN cells (Figure 6-7). This pattern was reversed with Jenv and EenvI575*-pseudotyped virions where transduction of both primary nose and lung cell populations was significantly higher than NIH 3T3/LL2SN. Nuclear LacZ counts were nearly equivalent in Jenv and EenvI575*-transduced wells, but approximately 100 fold less in Eenv transduced NIH 3T3/LL2SN and primary nose and lung cells. Although a similar increasing trend was observed in reference to transduction of primary cells compared to NIH 3T3/LL2SN cells in Eenv-pseudotyped virions, total nuclear LacZ counts were not high enough to reach significance.

Taken together, these results indicate that truncating the cytoplasmic tail of Eenv increased transduction efficiency of pseudotyped virions in primary sheep lung and nose cells and that Jenv and truncated Eenv (Eenv I575*) had a similar tissue tropism in vitro.
Figure 6-7. Transduction of primary sheep lung and nasal epithelial cells with lentivirus vectors pseudotyped with ovine betaretrovirus envelope proteins

Primary cells derived from fetal lamb lung and nose were transduced with HIV-based vector particles pseudotyped with VSV-G, Jenv, Eenv and EenvI595*. Cells were stained for nuclear βgalactosidase activity and positive foci were counted and the results shown as a bar graph. Bars marked with * are statistically significant from NIH 3T3/LL2SN transduction levels with the indicated pseudotype.

In vivo transduction and tropism

Lentiviruses expressing nuclear LacZ were pseudotyped with Jenv or Eenv I575* as described above. Filtered virus was concentrated by ultracentrifugation and a small sample was subjected to SDS-PAGE separation and probed with anti-envelope or anti-capsid antibodies to confirm successful virus preparation (data not shown). The concentrated virus was titered on NIH 3T3/LL2SN cells and 2-day-old lambs were inoculated with 2 mL of virus (2x10^5 virions total) via nebulization. Lambs were euthanized at 1 month post-infection and tissues from the nose, lung and trachea harvested and stained for nLacZ expression. Transduced cells were identified
by dark blue staining of the nuclei when exposed to X-gal staining solution. Positive cells were observed in the nose and lung of animals transduced with Eenv I575* (Figure 6-8A and B, respectively) as well as Jenv (Figure 6-8C and D respectively) pseudotyped virus. Within the nose, the ciliated, pseudostratified columnar epithelial cells appeared to be unaffected whereas individual round cells with large nuclei and comparatively small cytoplasm at the basement membrane of the epithelial layer, likely basal cells, showed staining (black arrows in Figure 6-8A and C). Transduction of cells within the lamina propria was also observed in association with Bowman’s gland cell clusters. A fraction of the transduced cells had distinct cuboidal morphology (green arrows in Figure 6-8B and D) similar to cells of the Bowman’s glands but there were still other cells in close proximity to the glandular cell clusters that were also transduced. These cells ranged from endothelial to fibroblast in morphology. The staining observed in the lung was reminiscent of the location and morphology of type II alveolar cells (red arrows in Figure 6-8B and D). It appears that resident alveolar macrophages were also transduced (white arrows in Figure 6-8B and D). These results indicate that lentiviruses pseudotyped with Jenv and Eenv I575* can infect the upper and lower respiratory tract of newborn lambs, albeit with poor efficiency, and that both envelopes target the lung and nose equivalently.
Figure 6-8. *In vivo* infection of sheep with Jenv and EenvI575* pseudotyped lentiviral vectors expressing nLacZ.

Images of nose and lung tissues from lambs transduced with HIV-based vectors pseudotyped with EenvI575* (A and B respectively) or Jenv (C and D) glycoproteins. Slides were counterstained with nuclear fast red. Arrows indicate nuclear βgalactosidase positive cells of various morphologies (red=alveolar type II, white=alveolar macrophage, black=basal cell, green=Bowman gland cell).
Discussion

Assembly of both HIV and MLV particles occurs at the membrane whereas assembly of betaretroviruses, such as ENTV-1 and JSRV, occurs intracellularly in association with the microtubule-organizing center (MTOC). The mechanisms and interactions required for betaretrovirus assembly are not well understood, but inclusion of envelope proteins in viral vectors composed of heterologous Gag proteins would most likely require passive incorporation. If there were inhibitory signals in the cytoplasmic tail of Eenv then truncation would eliminate those functions, allowing for more efficient incorporation. Indeed, truncation of the Eenv cytoplasmic tail dramatically increased transduction efficiency of pseudotyped particles. Interestingly, truncation of the Jenv cytoplasmic tail had the opposite effect (246). The increase in transduction efficiency associated with Eenv cytoplasmic tail truncation was not due to a modification of the entry kinetics since the same transduction inhibition profile was observed for all of the cytoplasmic tail truncation mutants as well as for wildtype Eenv after treatment with lysosomotropic agents/V-ATPase inhibitors. Since treatment with lysosomotropic agents/V-ATPase inhibitors leads to an increase in the pH of late endosomes and lysosomes, it is difficult to reconcile the inconsistency observed in NH₄Cl/Bafilomycin A1 and chloroquine inhibition of Eenv. Sensitivity to NH₄Cl and V-ATPase inhibitors and resistance to chloroquine treatment has been reported in other viral systems, including equine infectious anemia virus (26), human foamy virus (25) and Sindbis virus (253). Taken together, this shows that Eenv requires endocytosis and acidification of the endosome for infection but that there is something distinctly different about the mode of action of chloroquine that Eenv can withstand but Jenv cannot.

The surface expression levels, envelope inclusion and subcellular localization of the Eenv cytoplasmic tail truncation mutants were not significantly different from wildtype Eenv. Localization and virion envelope inclusion were not altered by disruption of the dileucine motifs.
in the cytoplasmic tail, indicating that the dileucine motifs at these loci are not active cell sorting motifs. Eenv cytoplasmic tail mutants displayed a similar co-localization with Gag to that of wildtype Eenv. Additionally, all of the Eenv protein mutants displayed prominent cell surface localization when examined by confocal microscopy, mirroring the highly apical staining of Eenv observed in ENA tissues. Clusters of fluorescently labelled Eenv proteins were found to co-localize in distinct puncta with MLV and HIV Gag-GFP fusion proteins both in the cytoplasm and at the plasma membrane. Jenv shared a stricter co-localization with HIV Gag than with MLV Gag, which is supported by the viral envelope inclusion assay that demonstrated more Jenv molecules per capsid molecule in HIV particles than in MLV particles.

The total fusion activity of wildtype Eenv and Jenv was barely above mock implying very inefficient fusion. However, the total number of fusion foci for Eenv I575* and Eenv diLY590* mutants was significantly increased compared to that of Eenv at neutral pH. Therefore, truncation of the cytoplasmic tail of Eenv increases the transduction efficiency of Eenv pseudotyped virus particles by increasing fusion efficiency without significantly affecting localization and virion inclusion. Alternatively, removal or disruption of the membrane proximal structure in the cytoplasmic tail increases the fusogenicity of Eenv.

Jenv and Eenv-mediated transduction of primary sheep lung and nose cells was significantly enhanced compared to murine fibroblasts overexpressing Hyal2 (NIH 3T3/LL2SN). Conversely, a dramatic decrease in transduction efficiency of VSV-G-pseudotyped particles was observed in primary respiratory cells compared to NIH 3T3/LL2SN cells. Therefore, there appears to be something specific about sheep cells that enhances ovine betaretroviral envelope mediated transduction and inhibits transduction mediated by the VSV glycoprotein.
ENTV-1 has been shown to have a widespread tissue distribution, including several tissues anatomically distinct from the respiratory tract, such as kidney and liver (93, 209). Our results show widely distributed transduction of the entire respiratory tract; meaning that nuclear βgalactosidase positive cells were observed in both the lung and nose of animals transduced with Jenv and Eenv I575* pseudotyped virions. Therefore, the entry tropism dictated by the envelope protein is not the determining factor restricting the disease tropism of JSRV to the lung and ENTV-1 to the nose. Eenv and Jenv- pseudotyped viruses transduced similar cells, therefore, it appears that the envelope protein of ovine betaretroviruses is capable of mediating infection of a variety of cell types in the nose as well as the lung. Interestingly, transduction of progenitor cells was observed in both organs, specifically type II alveolar cells of the lung and basal cells of the nose. Basal cells are progenitor cells from which the other cell types of the pseudostratified columnar epithelial cell layer are derived (227). Although basal cells are not known to possess secretory functions they are known to differentiate into cells of the pseudostratified columnar epithelium, which includes the professional secretory cell types: goblet cells and Bowman’s gland cells. Type II alveolar cells are professional secretory cells and are capable of differentiating into type I alveolar cells. Since copious production of exudate is the main clinical sign of OPA and ENA, it is not surprising that the above cell types were transduced in this experiment. Targeting of mitotically active progenitor cells is an advantage for betaretrovirus infection; which requires breakdown of the nuclear envelope during cell division for genome integration and replication. Since virus was administered by nebulization, which would target both the upper and lower airway, it is difficult to reconcile the mechanism by which infection was disseminated to the various cell types observed. From the lumen of the respiratory tract the direct access route to the Bowman’s gland cell clusters is through the Bowman’s gland duct,
which is presumably the route of infection of the cells in the lamina propria. Basal cells are positioned below the pseudostratified columnar epithelial cells, in contact with the basement membrane. The tight junctions maintained between cells of the pseudostratified epithelium should restrict access to basal cells below, unless interrupted by injury or degraded through a virus-mediated mechanism.

Since ENTV and JSRV are the only retroviruses known to infect the respiratory epithelium, there is interest in utilizing these envelopes for lung gene therapy. However, for this therapy to be cost-effective, improvements in vector yield would have to be devised.
Chapter Seven:

General Discussion

The ENTV-1 provirus sequence is extremely stable among North American isolates

The ENTV-1 provirus sequences determined in our studies show an extremely high level of conservation, sharing 99% to 100% nucleotide identity among isolates across the entire viral genome. Calculation of the Ka/Ks ratio for each of the open reading frames in the genome of ENTV-1NA isolates demonstrated a bias to conserve the amino acid sequence, which implies the involvement of negative selection pressure or perhaps that the ENTV-1 reverse transcriptase (RT) possesses higher fidelity than other RT enzymes. Unfortunately, we were unable to evaluate the fidelity of the ENTV-1 RT due to many failed attempts at expressing recombinant RT in bacteria (data not shown) leading us to conclude that there is some fundamental incompatibility of RT with this expression system. However, since we were able to detect six nucleotide differences in the genomic sequence derived from the experimentally induced ENA (ENTV-1OVC) compared to its parental sequence (ENTV-1NA9), it would seem that the RT has an average or even lower than average fidelity compared to other retrovirus RTs (254, 255).

The clonal nature of ENA tumor growth could provide an alternative explanation for the high degree of genetic stability of ENTV-1. Our in vivo infections, and observations by other groups regarding JSRV infections, have shown that tumor development is actually an uncommon outcome of ENTV-1 and JSRV infection (89). Consequently, only a minority of infection events induces disease, resulting in tumors derived from a single infection event and clonal growth of the tumor. The lack of intra-tumor variability we observed in sequences recovered from ENA samples supports this hypothesis. According to this hypothesis, inter-tumor variants between successive transmission events would be expected, as mutations arising during replication would
be present in the progeny tumors. This, however, was not reflected in our observations as we observed 100% nucleotide identity in ENTV-1 sequences extracted from ENA from the same flock (ENTV-1NA6 and 7).

Finally, the immune system could be the aforementioned selection force that would be modulated by the tolerance of sheep for antigens similar to the endogenous ovine betaretroviral sequences. Therefore, maintenance of an exogenous sequence similar to that of endogenous sequences is critical for evasion of the ovine immune response such that viruses bearing any variant sequences would be eliminated. The increase in variance observed between ENTV-1 and the endogenous ovine betaretroviruses at four distinct loci (the hypervariable regions outlined in Chapter 2) implies that the three of these hypervariable regions that are within coding regions are non-immunogenic and thus, mutations are more tolerated by the immune system in these regions.

**ENTV-1 infection is the causative agent of ENA**

In order to determine the etiology of a disease and conclusively prove that a specific pathogen is the causative agent of the associated disease, Koch’s postulates (120) must be fulfilled. This would have been achieved if ENA were observed after infection with virus generated in cell culture from the molecular clone in Chapter 4. Although some virus expression was observed in a limited nasal cell population of JSRV complemented ENTV-1 virus infected sheep, no ENA was observed. This does not reject ENTV-1 as the causative agent of ENA because there were other factors that were not fully accounted for in our experiments.

Koch’s postulates have since been redefined to take into account pathogens that cannot be isolated and cultured and for whom PCR based detection methods are required (121–123). Despite the lack of disease induction observed in our studies, we believe that the evidence presented in this thesis demonstrates ENTV-1 is the causative agent of ENA. ENTV-1
sequences and antigens have been shown to be present in all samples of ENA tested. We have also shown the presence of reverse transcriptase activity and virions with betaretroviral morphology in sucrose gradient analysis of ENA homogenate in the fraction with the expected buoyant density of a retrovirus. In addition, we have demonstrated transmission of ENA to a healthy lamb from a flock with no history of ENA after inoculation with cell-free filtrate from ENA tumor homogenate. Finally, ENA tumor tissue from the experimentally induced tumor showed robust ENTV-1 envelope staining and contained an ENTV-1 provirus sequence that was derived from a sequence represented in the ENA homogenate. Since an area of the experimentally induced ENA tumor had fibropapilloma-like appearance, a morphology commonly associated with papillomavirus induced neoplasms (256), genomic DNA was screened for papilloma virus co-infection using OvPV-3-specific primers (257) as well as degenerate broadly cross-reactive papilloma virus primers (258). Papilloma virus PCRs were negative so ENTV-1 was the only oncogenic virus implicated in the tumor. Cumulatively, the above evidence establishes an etiologic relationship between ENTV-1 infection and ENA induction.

**Tropism dictated by the ENTV-1 envelope does not explain ENA disease tropism**

Immunohistochemical staining of the experimentally induced ENA showed positive pan-cytokeratin and CK7 staining of the adenosquamous area of the tumor. The pseudo-stratified epithelium in the nasal luminal space and the cells of the Bowman’s gland in adjacent normal tissue from the same animal also stained positive for the same markers. Therefore, it would seem that the experimentally induced ENA tumor was derived from one or both of these cell populations. This is interesting as both populations contain a subset of cells that generate exudate (227). The Bowman's gland cells produce serous exudates but the goblet cells in the
pseudostratified epithelium produce mucoid exudates. Glycohistochemical analysis performed by Scocco et al. (259, 260) demonstrated that ENA cells produce both serous and mucous exudate, indicating that tumors are comprised of more than one cell type. The cell transduction pattern observed in our pseudotype infection experiments revealed a propensity for Eenv and Jenv to direct infection of stem or progenitor cell populations in the nose and lung, namely basal and type II alveolar cells, respectively. These cells maintain a secretory function and/or have the capability to differentiate into the cells that have secretory functions, such as Bowman's gland cells and goblet cells (227). Infection of these cells is advantageous for a betaretrovirus like ENTV-1 as these cells have an increased replication potential and cell division is required for betaretrovirus infection. As well, extracellular matrix modifying proteins, such as Hyal2, are upregulated in stem/progenitor cells (261, 262) in order to allow motility of newly differentiated cells to their requisite destination. In addition, progenitor/stem cells have an increased or unlimited reproductive potential, which is a hallmark of cancer (263) and an advantage for envelope mediated neoplastic transformation (264). Therefore, disease tropism of ENA is not strictly dictated by the entry tropism of Eenv, but certain progenitor cell types are preferentially targeted for infection in both the lung and nose by Eenv.

Since pseudotyped vectors were utilized in the in vivo infection experiments described in Chapter 6, the results do not take into account the impact of other viral proteins on determining the cell specificity of infection; possibly through interactions with restriction factors leading to inhibition of viral replication. Transduction, indicated by envelope expression, of alveolar type II and basal cells was observed in the lambs infected with JSRV complemented ENTV-1, which closely matches the transduction pattern of the pseudotyped lentivirus vectors. Therefore, it
appears that the pseudotyped lentivirus transduction results are reflective of ENTV-1 and JSRV transduction.

It has been noted in several studies that Hyal2 is the receptor for Jenv and Eenv mediated infection (95, 187). The evidence for this interaction is convincing, but incomplete. Although evidence of direct binding has been demonstrated and overexpression of Hyal2 makes relatively resistant cell lines more susceptible to infection, it is not known whether Hyal2 is required for infection or if it is only a contributing factor, such as an attachment or processing factor. While the expression pattern for Hyal2 is widespread, the majority of Hyal2 has been noted to localize to the lysosome rather than the cell surface and the total expression level is relatively low. Therefore, it is possible that Hyal2 plays more of a supportive role in ovine betaretrovirus infection rather than a required role. It is also interesting and noteworthy that inhibition of acidification of endosomes and lysosomes, which is a required step for Hyal2 enzymatic activation, by NH\textsubscript{4}Cl and bafilomycin A2 but not chloroquine inhibited Eenv-mediated entry. It is possible that the inhibition of virus infection was a result of inhibition of Hyal2 activation and that chloroquine is unable to achieve the same inhibition. More studies are required to probe this model of the interaction between Eenv and Hyal2 before a conclusion can be made.

**ENTV-1 infection and the sheep immune response**

We observed an extremely low disease incidence in our animal infection experiments, especially in comparison to the frequency of disease observed in the flocks analyzed in Chapter 5. All animals used in our infection experiments were from the same closed flock, suggesting that there could be a genetic element involved in ovine betaretrovirus infection and disease induction. Detection of ENTV-1 antigen-reactive antibodies in Chapter 5 and observation of clusters of immune cells in the lungs of animals infected with JSRV complemented ENTV-1 in Chapter 4,
indicates that the immune response is a factor in the development of ENA after ENTV-1 infection. Therefore, the genetics contributing to the immune response could be the aforementioned element responsible for determining the disease outcome of ENTV-1 infection, meaning that development of an immune response against ENTV-1 proteins would lead to elimination of productively infected cells and prevention of disease. Recent evidence has shown neutralizing antibodies (226) and T-cell immune responses (172) specific to JSRV in infected sheep, albeit this finding is uncommon, even in animals experimentally vaccinated against JSRV (172). Indeed spontaneous regression of a JSRV positive lung tumor was reported in a sheep co-infected with a small ruminant lentivirus (170).

Studies in sheep have shown an inverse relationship between antibody levels and reproductive success, presumably because ewes prone to strong immune responses are more likely to develop an antibody response against the fetus and cause abortion (265). The exact complement of antigens involved in this process is unknown, but endogenous ovine betaretrovirus proteins are intimately involved in establishing the placenta and maintaining the conceptus to term (78, 266). Therefore, preservation of tolerance to endogenous betaretrovirus antigens is paramount to reproductive success and the genetics to support a robust immune response would be developed only at the expense of higher reproductive fitness. In contrast, a robust immune response and the associated genetics would be an important factor for resistance to pathogens, such as ENTV-1, thus these traits are likely maintained at equilibrium.

The immune tolerance of sheep to endogenous ovine betaretrovirus antigens is a benefit for ENTV-1 infection as this tolerance extends to ENTV-1 antigens via a mechanism known as molecular mimicry. In this manner, the immune system of the natural host is already modulated for ENTV-1 infection. Co-assembly of ENTV-1 and endogenous ovine betaretrovirus
polyproteins and packaging of ENTV-1 genomic RNA, as postulated in Chapter 4, would be another mechanism by which ENTV-1 could utilize the immune tolerance of sheep to endogenous ovine betaretroviruses. Thus, incoming virions would be coated with endogenous ovine betaretrovirus proteins and would be less immunogenic. Antibodies reactive with ENTV-1 envelope and capsid can develop in infected sheep as was shown in Chapter 5. Implicit with these results is that endogenous ovine betaretrovirus tolerance has been broken. Presumably, immune tolerance breakage occurs when lymphoid cells displaying receptors with low affinity for endogenous ovine betaretroviruses escape deletion during thymic selection. When antigen-presenting cells displaying ENTV-1 antigens as well as the correct complement of co-stimulatory molecules are encountered, these lymphocytes may proliferate and an anti-ENTV-1 immune response is developed. Therefore, the antibodies detected in Chapter 5 are likely low affinity antibodies and it is difficult to determine how effective this response is in elimination of tumors. In the end, we were unable to replicate ENA in our experimental infections and so were unable to evaluate the development of an immune response to ENTV-1 during infection and disease development.

In conclusion, the evidence presented in this thesis demonstrates that ENTV-1 is the causative agent of ENA; however, induction of ENA is not the most common outcome of infection with ENTV-1. The Gag-Pro-Pol polyprotein of the ENTV-1 molecular clone, which is derived from a circulating ENTV-1 isolate, is unable to proteolytically process itself but can be processed by a co-packaged complementing polyprotein. Furthermore, the tropism of infection dictated by the ENTV-1 envelope protein does not determine the tissue tropism of disease. Finally, contrary to previous reports, antibodies specific to ENTV-1 envelope and capsid antigens can be detected in sheep with ENA and those sheep housed in proximity to ENA-affected sheep. Our results
suggest that the immune system is a large factor in the pathogenesis of ENTV-1 infection affecting the stability of the genome sequence and affecting the disease outcome of infection. This warrants further study concerning the interplay between ENTV-1 and the sheep immune system.
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