

Genetic variability and in vitro transcriptional permissibility of primary ovine beta-retrovirus promoter isolates

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Objective—To assess genomic sequence conservation and variation in the proviral promoter of enzootic nasal tumor virus (ENTV) and Jaagsiekte sheep retrovirus (JSRV) in tissue samples from 3 sheep with nasal adenocarcinoma associated with ENTV and 3 sheep with pulmonary adenocarcinoma associated with JSRV and to identify a cell culture system that supports transcriptional activity of the ENTV and JSRV viral promoters.

Animals—6 adult sheep.

Procedures—Standard PCR procedures for detection of the ENTV and JSRV long terminal repeat (LTR) promoter region were performed on samples from the 3 nasal adenocarcinomas and 3 pulmonary adenocarcinomas, respectively. The LTRs were cloned into shuttle vectors, amplified, sequenced, and analyzed. The cloned LTR regions were transferred into reporter plasmids and multiple human and ruminant cell lines, and primary cells were transfected with the promoter-reporter plasmids. The viral promoter activity was evaluated by use of an in vitro β -galactosidase reporter assay.

Results—Each isolate had a unique nucleotide sequence. Single nucleotide polymorphisms were the most common LTR mutation and rarely occurred at transcription factor binding sites. Relative to ENTV, the JSRV promoter isolates had a conserved 66-bp U3 insertion, including the lung-specific transcription factor HNF-3 β binding site. Among the cell lines used, human embryonic kidney (293T) and goat synovial membrane cells supported promoter transcription.

Conclusions and Clinical Relevance—The LTRs of ENTV and JSRV have extensive blocks of sequence conservation. Human 293T and goat synovial membrane cell lines may be suitable in vitro cell culture systems for further research of viral promoter functions. (*Am J Vet Res* 2013;74:1421–1427)

Enzootic nasal tumor virus and JSRV are transmissible oncogenic beta-retroviruses known to induce respiratory tract-specific tumors in sheep and goats.^{1,2}

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ABBREVIATIONS

BHS	Big horn sheep
BT	Bovine turbinate
EE	Enhancer element
ENTV	Enzootic nasal tumor virus
GSM	Goat synovial membrane
JSRV	Jaagsiekte sheep retrovirus
LTR	Long terminal repeat
OFT	Ovine fetal tracheal
OM	Ovine microglial
SNP	Single nucleotide polymorphism

Enzootic nasal tumor virus and the genomically related JSRV are interesting viruses in that they have specific tropisms for the mucosal epithelium of the upper and lower portions of the respiratory tract, respectively, and are associated with the development of adenocarcinomas limited to these locations.³ The 2 viruses have 95% similarity in their amino acid sequences but are dissimilar in 3 genomic loci including the 3' end of *env*, the noncoding U3 region of the LTR, and the *orf-x* gene.⁴ It has been suggested that the tissue specificity conferred

by these viruses is attributable to respiratory cell surface proteins used for viral entry⁵ and viral promoter sequence specificity, which initiates viral transcription.⁶ As with all retroviruses, the ENTV and JSRV LTRs are comprised of the U3, R, and U5 regions; the U3 region is thought to comprise the viral promoter.⁷ The full genomes for both ENTV and JSRV have been previously reported, and many biologically important segments within the LTRs, such as transcription factor binding sites, have been examined extensively.^{4,6,8,9} Despite these reports, studies examining sequence variation in the LTR among animals with naturally occurring tumors are rare.¹⁰

Research on the pathogenesis, specific tissue tropism, and oncogenesis of these viruses has been greatly hindered by the difficulties in propagating the virus *in vitro*.⁸ Cell lines derived from respiratory tract epithelia, such as nasal secretory cells or pulmonary type II pneumocytes, that ENTV and JSRV naturally transform have not been reported, to our knowledge.⁸ Therefore, the purpose of the study reported here was to assess genomic sequence conservation and variation in the proviral promoter of ENTV and JSRV in tissue samples from 3 sheep with nasal adenocarcinoma associated with ENTV and 3 sheep with pulmonary adenocarcinoma associated with JSRV and to identify a cell culture system that supports transcriptional activity of the ENTV and JSRV viral promoters.

Materials and Methods

Animals and tissue samples—To investigate ENTV, tissue and DNA samples were derived from 3 sexually intact rams (designated as sheep ENTVa, ENTVb, and ENTVc) on 3 separate farms in northern and central California between the years of 2005 and 2010. Each of the rams was brought to the University of California Veterinary Medical Teaching Hospital for evaluation of upper airway disease. At the time of the evaluation, 2 of the rams had respiratory distress and the third had chronic nasal discharge. Rhinoscopy and radiography of the rams revealed large destructive space-occupying masses that unilaterally obstructed the nasal passages. Diagnosis of nasal adenocarcinoma was made on the basis of findings of antemortem histologic examination of the mass in 1 case and postmortem histologic examination of the masses in 2 cases. All 3 rams died or were euthanized at the owners' request because of poor prognosis and the suspicion or confirmed diagnosis of sinonasal neoplasia. In all 3 cases, complete necropsies were performed and gross examination confirmed the presence of sinonasal neoplasia (Figure 1).

To investigate JSRV, tissue and DNA samples were derived from 3 sexually intact female Rasa Aragonesa ewes (designated as sheep JSRVa, JSRVb, and JSRVc) from Spain. Diagnosis of pulmonary adenocarcinoma was made on the basis of findings of postmortem his-

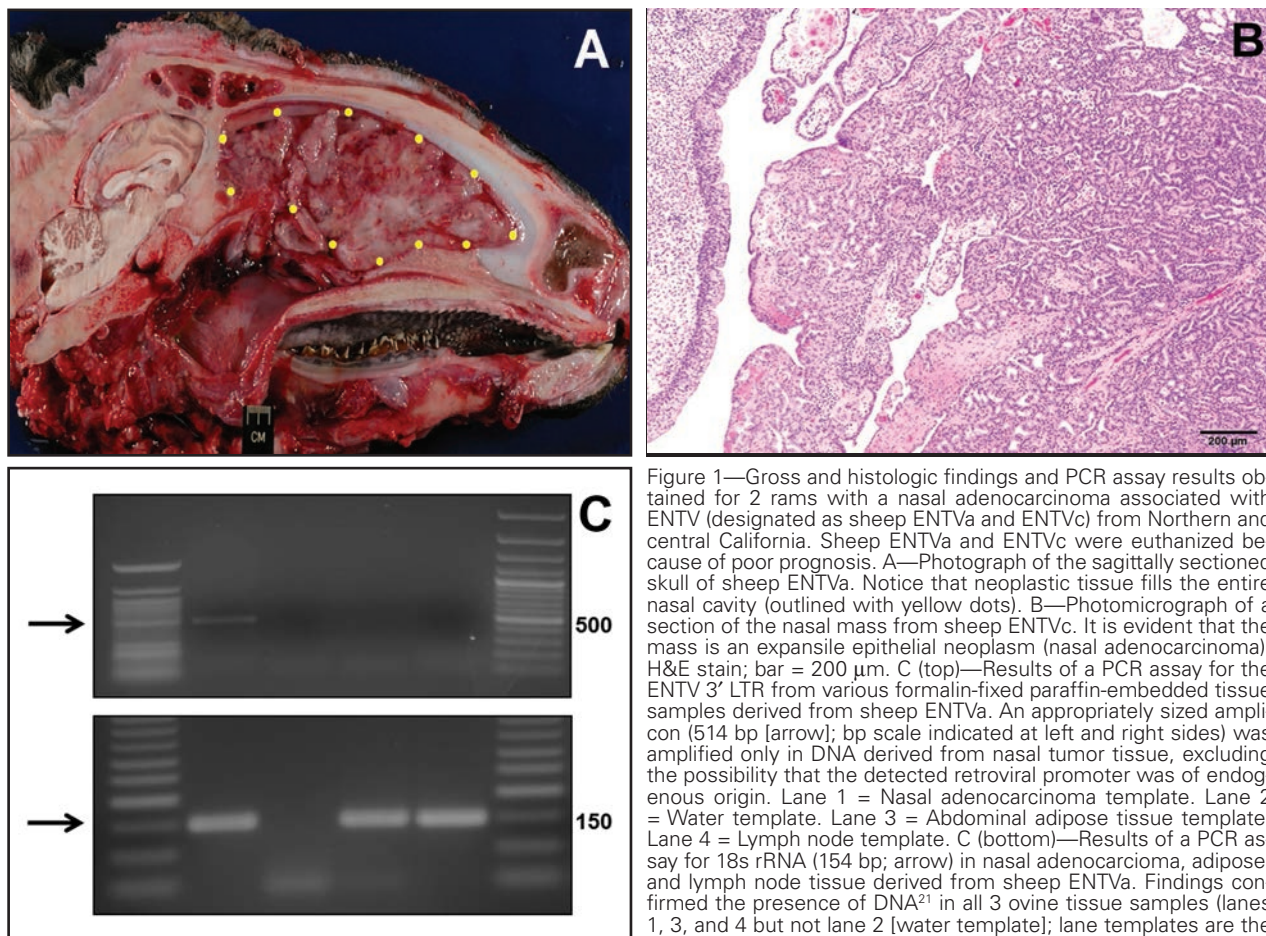


Figure 1—Gross and histologic findings and PCR assay results obtained for 2 rams with a nasal adenocarcinoma associated with ENTV (designated as sheep ENTVa and ENTVc) from Northern and central California. Sheep ENTVa and ENTVc were euthanized because of poor prognosis. A—Photograph of the sagittally sectioned skull of sheep ENTVa. Notice that neoplastic tissue fills the entire nasal cavity (outlined with yellow dots). B—Photomicrograph of a section of the nasal mass from sheep ENTVc. It is evident that the mass is an expansile epithelial neoplasm (nasal adenocarcinoma). H&E stain; bar = 200 µm. C (top)—Results of a PCR assay for the ENTV 3' LTR from various formalin-fixed paraffin-embedded tissue samples derived from sheep ENTVa. An appropriately sized amplicon (514 bp [arrow]; bp scale indicated at left and right sides) was amplified only in DNA derived from nasal tumor tissue, excluding the possibility that the detected retroviral promoter was of endogenous origin. Lane 1 = Nasal adenocarcinoma template. Lane 2 = Water template. Lane 3 = Abdominal adipose tissue template. Lane 4 = Lymph node template. C (bottom)—Results of a PCR assay for 18s rRNA (154 bp; arrow) in nasal adenocarcinoma, adipose, and lymph node tissue derived from sheep ENTVa. Findings confirmed the presence of DNA²¹ in all 3 ovine tissue samples (lanes 1, 3, and 4 but not lane 2 [water template]; lane templates are the same as those in the top panel).

tologic examination of formalin-fixed, paraffin-embedded tissues stained with H&E stain. Detailed clinical history for these 3 ewes was not available.

Experimental procedures—For each of the 6 sheep, DNA was individually isolated from formalin-fixed, paraffin-embedded tissue samples.^a Standard PCR assays confirmed the presence of proviral LTR DNA of ENTV in all 3 nasal tumors and JSRV in all 3 of the pulmonary tumors by use of primers designed (on the basis of published sequences⁴) to flank the 3'LTR promoter regions. Similar PCR assays were performed on additional tissues from sheep ENTVa, including samples of lymph node and adipose tissue, to determine whether the amplified product represented an endogenous retrovirus. Extratumoral tissue samples were not available from the other animals. An additional set of primers was developed to include the EE upstream from the ENTV promoter at the 3' end of *env*.⁶ Primer sequences were designed as follows: ENTV_{forward} (5'-TCT TTT GGT TCC TTG CCT CA) and ENTV_{reverse} (5'-CCA GCA CGG AAC AAA AGT TAC A); ENTV-EE_{forward} (5'-CCC GGA TCC ACA TAT GAA ATA TAG ATA CAT GTT GCA ATA CC) and ENTV-EE_{reverse} (5'-CCC GAA TTC TGC CGC AGC CAG CAC GGA CAA AAG TTA CA); JSRV_{forward} (5'-CTG CCG CGG CCA CG) and JSRV_{reverse} (5'-CTG CGG GGG ACG ACC). The PCR-amplified proviral DNA segments from all 6 tumors were individually cloned into plasmid vectors^b by complementary bp hybridization (thymine-adenine/TA cloning) and transformed into *Escherichia coli*.^b Five

to 10 transformed clones from each sheep were selected at random, and the retroviral LTRs were subsequently amplified by a PCR procedure. Clones were selected for genomic sequencing on the basis of the presence of an appropriately sized product detected by means of gel electrophoresis. Single clone sequences derived from each of the 3 ENTV infected sheep (designated ENTVa-c) were aligned with a published ENTV⁴ sequence. As well, clone sequences from each of the 3 JSRV infected sheep (designated JSRVa-c) were aligned with a published JSRV⁸ LTR sequence and analyzed for LTR sequence variability^c (Figure 2). Three clone sequences from 2 of the JSRV infected animals (JSRVa and JSRVc) were included in the alignment (designated JSRVa1-3 and JSRVc1-3) to assess variation between clones derived from a single animal.

From the plasmid shuttle vector,^b the ENTV LTRs derived from sheep ENTVa, ENTVb, and JSRVa as well as the ENTV LTR with the upstream EE from sheep ENTVa were moved into reporter plasmids.^d The reporter plasmids were used to assess the basal transcriptional activity of the inserted promoters in different cell types. This was achieved by inserting the promoter upstream of the plasmid's *lacZ* gene, which encodes for the enzyme β -galactosidase. Transcriptionally active promoters drove the amount of β -galactosidase produced, which was measurable with a commercially available β -galactosidase colorimetric assay.¹¹ The constructed reporter plasmids were transiently transfected into a single human and multiple ruminant cell lines: 293T human embryonic kidney cells,^e GSM cells,^e OM

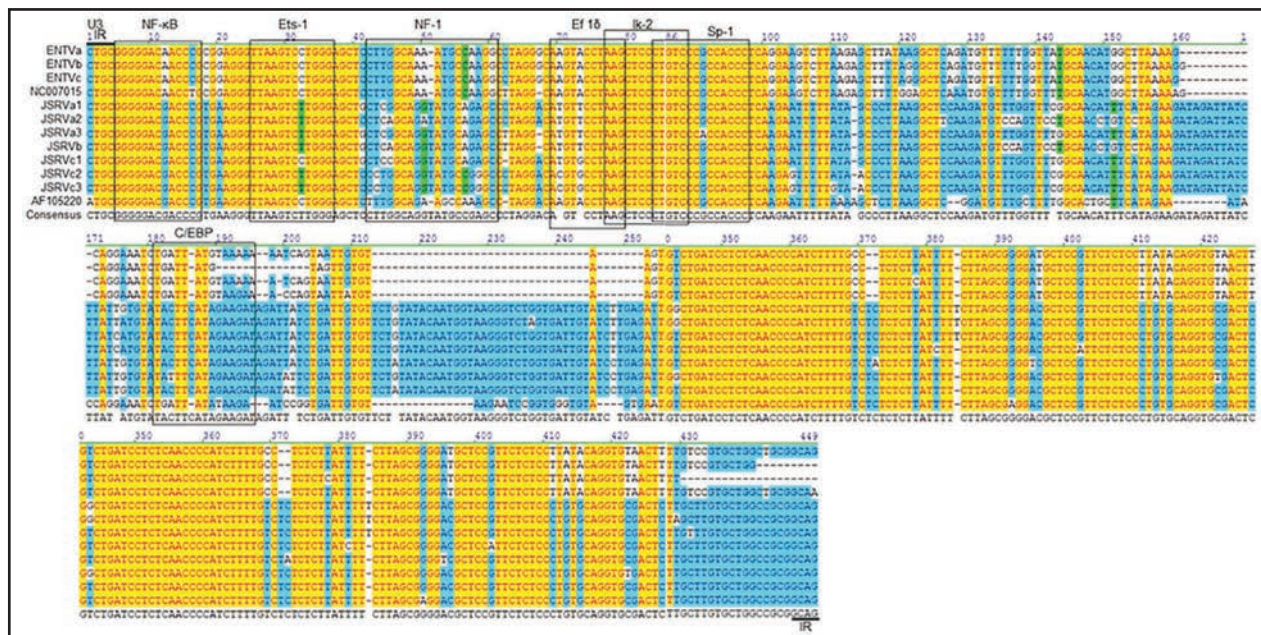


Figure 2—Sequence alignment of the ENTV and JSRV 3' LTRs detected in tumor tissue derived from 3 sheep with nasal adenocarcinoma (sheep ENTVa, ENTVb, and ENTVc), 3 sheep with pulmonary adenocarcinoma (designated as sheep JSRVa, JSRVb, and JSRVc), 1 published ENTV LTR sequence (GenBank accession No. NC007015), proviral DNA derived from nasal fluid from a sheep with naturally occurring nasal tumor, and 1 published JSRV LTR sequence (GenBank accession No. AF105220), proviral DNA derived from a sheep with pulmonary adenocarcinoma. All sheep died except sheep ENTVa and ENTVc, which were euthanized. Each sequence is unique. Three clones from sheep JSRVa (JSRVa1, JSRVa2, and JSRVa3) and JSRVc (JSRVc1, JSRVc2, and JSRVc3) were sequenced, which also revealed sequence variability. The inverted repeats (IR) flanking the LTR are underlined by solid lines. The putative locations of transcription factor-binding sites are identified with boxes. The U3, R, and U5 regions are delineated with vertical lines. The consensus sequence for all of the clones is indicated at the bottom of the sequences. The numbers along the top of the sequences represent nucleotide position. Yellow indicates conservation of nucleotides over all sequences. Blue and green boxes indicate positions of nucleotide variability between sequences with color highlighting the consensus base.

cells,⁸ OFT cells,¹² BHS fetus-derived lung cells,¹³ and BT cells.¹² The 293T cells were transfected by 2 commercially based methods: a lipid-mediated method^h and a calcium phosphate-mediated method.ⁱ This was done to compare the efficiency between methods. Transfection was clearly more efficient with the lipid-mediated method (data not shown); therefore, all additional cell line transfections were performed by the lipid-mediated transfection method following the manufacturer's protocol.^h All experiments were performed in triplicate in 6-well tissue culture plates as described by Murphy et al.¹⁴ For each experiment, positive and negative control plasmids were used in parallel with each cell line. The positive control (referred to as pCMV blue) contained a constitutively active cytomegalovirus promoter upstream from the β -galactosidase reporter gene^j and was used with at least 1 negative control (with no plasmid [control 1], the β -galactosidase reporter plasmid lacking a promoter insert [control 2],¹⁴ or the reporter plasmid with the sheep ENTVa LTR inserted in reversed direction [control 3]). Results for transfected wells were evaluated by ANOVAs followed by Tukey multiple comparison tests to determine significant differences; a value of $P < 0.05$ was considered significant.^k

Because weak or absent β -galactosidase expression might indicate that the ovine cell lines were suboptimally transfected, a cotransfection assay was performed with a green fluorescent protein-expressing plasmid^l and the cytomegalovirus promoter-driven β -galactosidase plasmid to assess cell line transfectability.¹⁵ This experiment was performed in all of the investigated cell lines.

Results

The ENTV LTR was amplified from sinonasal tumor tissues only and not from the control tissues (adipose and lymph nodes) from sheep ENTVa (Figure 1). This experiment was important because it distinguished ENTV provirus from an endogenous retrovirus sequence. Viral promoter sequences from sheep ENTVa, ENTVb, and ENTVc were aligned. Each sequence was determined to be unique (GenBank accession No. KF119136 through KF119145; Figure 2).

The sequences from the ENTV LTR isolates in the present study had a total of 6 SNPs among the ENTV LTR isolates within the U3, R, and U5 regions of the promoter. Most SNPs were located outside the described transcription factor binding sites and in the U3 region. There were fewer SNPs present within the isolates, compared with the published ENTV LTR sequence (for which there are 9 SNPs). The loci of the SNPs were not identical among ENTV isolates. For the clone ENTVb1, there was a 12-nucleotide deletion in the region of the

C/EBP transcription factor-binding site. Two single nucleotide additions (nucleotides 162 and 200) were present in the ENTV isolates.

When the JSRV LTR sequences were aligned with one another, multiple sites of sequence variability were

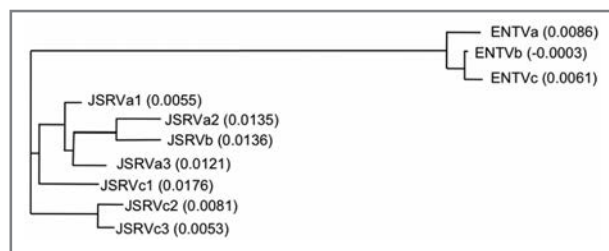


Figure 3—Dendrogram of the ENTV and JSRV LTR sequences obtained from tumor samples derived from sheep ENTVa, ENTVb, ENTVc, JSRVa (3 clones: JSRVa1, JSRVa2, and JSRVa3), JSRVb, and JSRVc (3 clones: JSRVc1, JSRVc2, and JSRVc3). Notice the clustering of isolates among viral families. Numbers in parentheses represent the degree of divergence between LTR clone sequences (distance calculated by use of the neighbor-joining method²²).

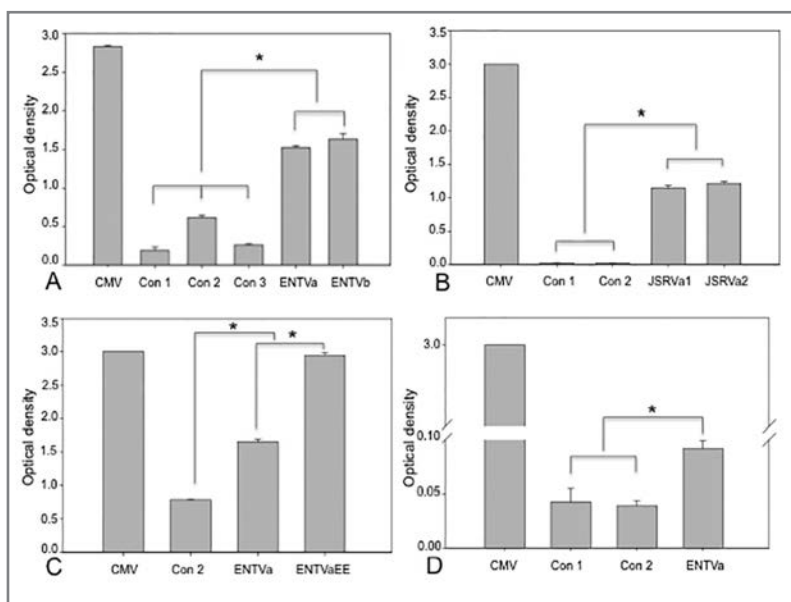


Figure 4—Results of β -galactosidase assays to characterize the transcriptional activity of the ENTV LTRs (identified in nasal tumor tissues from 3 sheep) and JSRV LTRs (identified in pulmonary adenocarcinomas from 3 sheep). Data are shown for sheep ENTVa, ENTVb, and JSRVa (clones JSRVa1 and JSRVa2); in addition, data for the ENTVa EE is included. For each assay, a positive control (CMV) containing a constitutively active cytomegalovirus promoter upstream from the β -galactosidase reporter gene was used and at least 1 negative control (with no plasmid [Con1], the β -galactosidase reporter plasmid lacking a promoter insert [Con2], or the reporter plasmid with the sheep ENTV1a LTR inserted in reversed direction [Con3]). Transfections were performed in triplicate cell cultures. In each panel, the x-axis denotes the sample (the column equal to the mean and the error bars representing the SD) and the y-axis is the β -galactosidase optical density in absorbance units. A—Human 293T cell transfection with 2 ENTV LTR isolates (ENTVa and ENTVb) by means of lipid-mediated transfection yielded significant ENTV-promoted β -galactosidase expression relative to the findings for each of the 3 negative controls. B—Human 293T cell transfection with 2 JSRV LTR clones (JSRVa1 and JSRVa2) by means of lipid-mediated transfection yielded significant JSRV-promoted β -galactosidase expression relative to each of the 2 negative controls. C—Human 293T cell transfection with the ENTV LTR (with and without the EE) by lipid-mediated transfection yielded a significant increase in promoter driven β -galactosidase production. D—Goat synovial membrane cell transfection with ENTV LTR by lipid-mediated transfection yielded significantly increased β -galactosidase expression relative to each of the 2 negative controls. The asterisks and brackets indicate significant ($P < 0.05$) differences in optical density between treatment groups; for instance, in panel A, both ENTVa and ENTVb had significantly higher promoter driven expression of β -galactosidase as measured by spectrophotometry, compared with each of the 3 negative controls (Con A, B, and C).

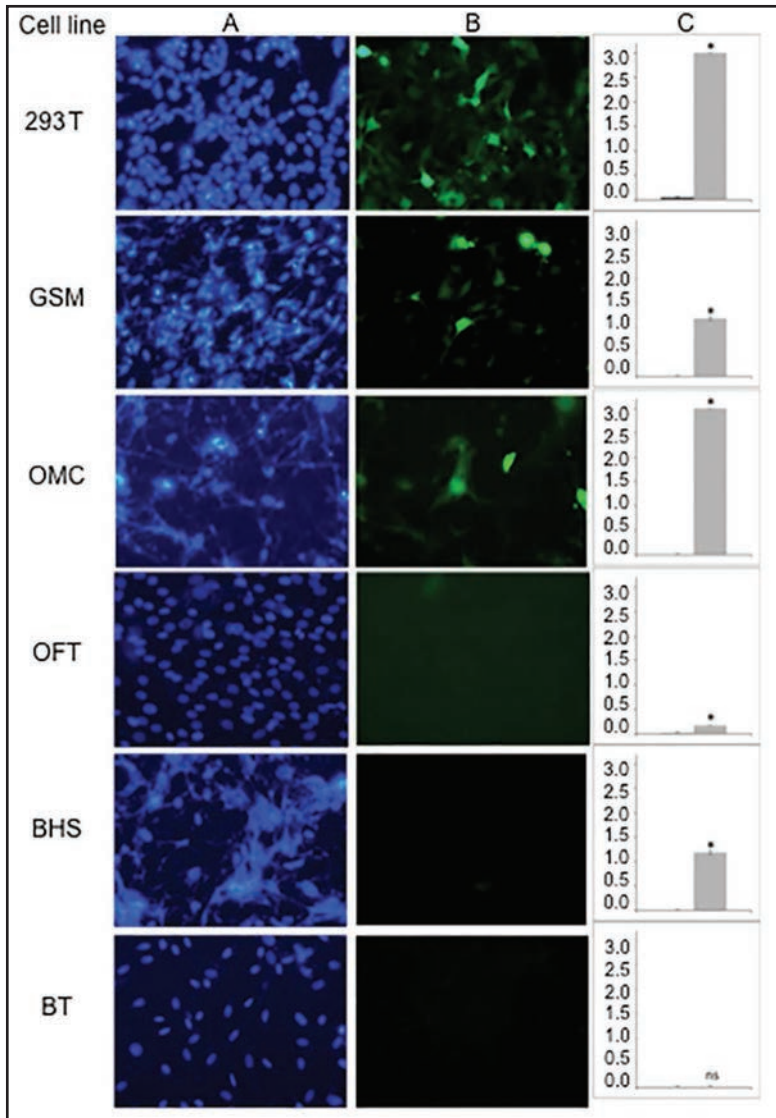


Figure 5—Findings following co-transfection of multiple cell lines with green fluorescent protein-expressing plasmid and the cytomegalovirus promoter-driven β -galactosidase plasmid to assess cell-specific transfection efficiency. A lipid-mediated transfection method was used. Column A—Representative fluorescent microscopic image obtained after 4',6-diamidino-2-phenylindole staining for DNA for each of the cell lines. The blue fluorescence reveals cellular nuclei. Column B—Representative image illustrating cytoplasmic green fluorescence (column B) indicative of cytomegalovirus promoter-driven green fluorescent protein expression for each of the cell lines. Notice evidence of successful transfection in 293T, GSM, and OM cells. Expression of green fluorescent protein is undetectable in OFT, BHS, and BT cells, suggesting that these cells are refractory to lipid-mediated transfection. Column C—Results of β -galactosidase assays in each of the cell lines (light gray bars) transfected with a cytomegalovirus promoter-driven plasmid expressing β -galactosidase, compared with a negative control (ie, with no plasmid [dark grey bars]). The 293T, GSM, OM, OFT, and BHS cells have variable degrees of β -galactosidase expression. The x-axis denotes the sample (the column equal to the mean and the error bars representing the standard deviation) and the y-axis is the β -galactosidase optical density in absorbance units.

identified including 49 SNPs. Similar to the ENTV LTR, most JSRV SNPs were located outside known transcription factor binding-sites and within the U3 region. Unlike ENTV, SNPs in the JSRV LTRs were commonly at the same nucleotide positions among isolates and similar to the published JSRV LTR sequence. Overall, 5 single nucleotide additions and 2 deletions were present within the JSRV isolates. There were 34 addi-

tional SNPs present in the published JSRV LTR sequence, compared with the isolates obtained in the present study. Fifty-two single nucleotide additions, which were often contiguous, and a single deletion were present in the JSRV LTR isolates, compared with the published sequence.

Comparison of the ENTV and JSRV LTRs revealed a relatively contiguous and conserved 66-nucleotide insertion (nucleotides 214 to 280) only in the JSRV isolates. There was very little nucleotide variation in the R and U5 regions of either ENTV or JSRV and strong sequence conservation between the 2 viruses. An LTR sequence dendrogram revealed clustering of ENTV samples and JSRV samples and stark genomic variability that distinguished the 2 viruses (Figure 3). Although all ENTV clones from the 3 sheep were clustered together under the ENTV virus family, there was consistent individual LTR sequence variation among the clones. The ENTVb and ENTVc LTRs had the greatest sequence similarity, whereas the ENTVa LTR sequence was divergent from the other 2 sequences. The sequence similarity between the LTR clone JSRVc1 and clones JSRVa and JSRVb was greater than that between the LTR clone JSRVc1 and any other JSRVc clone.

Of the cells tested, only 293T, GSM, and OM cells were efficiently transfected and only the 293T and GSM cells supported ENTV promoter-driven expression of β -galactosidase to levels significantly greater than expressions in the negative controls (Figure 4). Similar results (ie, significantly greater β -galactosidase expression relative to that in the negative controls) were observed when 293T cells were transfected with the JSRV promoter-driven plasmid or the plasmid with ENTV promoter containing the upstream EE by means of the lipid-mediated transfection method. Comparison of 293T cells transfected with the ENTV LTR alone (ENTVa) and with the ENTV LTR containing the upstream EE (ENTVa EE) revealed significantly greater promoter-driven β -galactosidase expression in the ENTVa EE-transfected cells. However, no such augmentation was identified in any of the tested ruminant cell lines (data not shown). In the OM, OFT, BT, and BHS cells, β -galactosidase expression driven by the ENTV promoter was not significantly different from the expression in the negative controls (data not shown); however, it should be noted that the latter 3 cell lines had poor transfection efficiency, which hindered interpretation of the β -galactosidase assay. When 293T cells were transfected with the ENTV promoter-driven plasmid by use of the calcium phosphate transfection method, there was a significant yet minimal increase in β -galactosidase

expression, relative to the negative control expressions (data not shown). To assess the transfectability of the various cell lines, cells were cotransfected with pCMV blue and a green fluorescent protein-expressing plasmid. β -Galactosidase expression was variably detectable in 293T, GSM, OM, and BHS cells and was detected to a minimal extent in OFT cells (Figure 5). There was no β -galactosidase expression detected in the BT cells. Green fluorescent protein expression was detected by fluorescence microscopy in 293T, GSM, and OM cells but was not detectable in the OFT, BHS, and BT cells.

Discussion

Variation in retroviral LTR sequences has been reported for many retroviruses including equine infectious anemia virus,¹⁶ HIV,¹⁷ avian retroviruses,¹⁸ caprine arthritis encephalitis virus,¹⁹ and FIV.²⁰ This variation has been hypothesized to be a result of complex selective pressures and may function to generate cell specificity or influence transcription.¹⁶

In the present study, there was moderate overall sequence variability evident in the ENTV and JSRV LTR DNAs when data for the ENTV-affected sheep were compared and when data for the JSRV-affected sheep were compared. It was interesting to note that individual sheep had LTR sequence variations among clones, as illustrated by findings for sheep JSRVa and JSRVc. The JSRV promoter variation within a single animal (sheep JSRVc) was sometimes greater than the viral promoter variation among study animals. Single nucleotide polymorphisms were the most common mutation identified in both ENTV and JSRV LTR sequences. The SNPs were most commonly located outside described transcription factor binding sites in the U3 region, suggesting the possibility that these regions of the U3 promoter are more tolerant of mutations. There was a 66-nucleotide insertion in the JSRV LTR (relative to the ENTV LTR), which included the lung-specific transcription factor HNF-3 β binding site, consistent with the previously suggested hypothesis⁸ that the tissue-specific expression of this transcription factor may be associated with the JSRV's pulmonary tumor specificity. Interestingly, sequence variation within the R and U5 regions was minimal both within and among the various ENTV and JSRV isolates. This suggests both an evolutionary link between these viruses and strong conservation of function within these regions.

In the present study, we attempted to identify a ruminant respiratory tract- or ovine-derived cell line that supported ENTV LTR transcription by transfecting multiple primary cell lines derived from domestic sheep, BHS, goats, and oxen, but had limited success. However, 2 cell culture systems in which the ENTV promoter is transcriptionally active, human-derived 293T cells and caprine-derived GSM cells, were identified. Given that the ENTV virus cannot currently be cultured *in vitro*, such culture systems could be used for further investigations into the mechanisms of disease development and promoter function. These cell lines could also be used as potential coculture or explant systems for viral propagation from biopsy- or necropsy-derived ovine nasal carcinoma tissue.

In the present study, 293T, GSM, OM, OFT, BHS,

and BT cells had variable transfection efficiencies by means of the lipid-mediated transfection method and the cytomegalovirus promoter-driven plasmids. Low to absent transfectability partially explains the lack of detectable LTR transcription in the OFT, BHS, and BT cells. It remains undetermined why the OM cells did not support ENTV promoter-driven transcription. These results may be attributable to limitations in the transfection efficiency of lipid-mediated transfection. Other transformation methods such as electroporation or the use of a retroviral vector may allow for introduction of the viral promoter into these ruminant cell lines.

Weak ENTV or JSRV-based transcription could alternatively be explained by the differences in the cellular milieu that support transcription or available transcription factors within these cell types. Multiple previously described binding domains for transcription factors including nuclear factor- κ B, C/EBP, Ets-1, Ef-1 δ , IK2, and SP-1 were identified in the viral sequences from the ENTV and JSRV isolates.⁸ Given that these binding domains are ubiquitous among many cell types, it is possible that an as-yet unidentified nasal-specific transcription factor is required for ENTV promoter function, which was not present in the tested cell lines. It is also possible that if a nasal-specific transcription factor does exist, its binding domain was not present in the plasmid construct (potentially, the transcription factor binding site might lie outside of the LTR region [eg, within the gag leader region]). For this reason, the 3' ENTV EE was cloned and placed proximal to the ENTV promoter. Although this enhanced the expression of the ENTV promoter in human 293T cells, no such augmentation was identified in any of the tested ruminant cell lines (data not shown). Further research to identify nasal secretory cell-specific transcription factors would be informative to address this concern.

Results of the present study have provided evidence of proviral LTR sequence conservation and variability in ENTV and JSRV derived from different naturally infected tumor-bearing sheep. Single nucleotide polymorphisms were the most common mutation and were most commonly found in the U3 regions of the promoter, as previously reported.⁴ The 2 *in vitro* cell culture systems used in the present study supported transcription of the ENTV and JSRV promoters and could facilitate further research efforts into viral cell specificity, promoter function, and oncogenesis.

- a. QIAamp DNA FFPE tissue kit, Qiagen, Gaithersburg, Md.
- b. TA Cloning Kit with pCR 2.1 Vector and One Shot Top 10F Chemically Competent *E coli*, Life Technologies Corp, Carlsbad, Calif.
- c. *AlignX* function, Vector NTI, Life Technologies Corp, Carlsbad, Calif.
- d. pBlue TOPO TA Expression Kit, Life Technologies Corp, Carlsbad, Calif.
- e. American Type Culture Collection (ATCC), Manassas, Va.
- f. Provided by W. Cheevers, Washington State University, Pullman, Wash.
- g. Provided by T. Baszler, Washington State University, Pullman, Wash.
- h. Lipofectamine 2000 Transfection Reagent, Life Technologies Corp, Carlsbad, Calif.
- i. ProFection Mammalian Transfection Systems, Promega Corp, Madison, Wis.

- j. pcDNA 3.1D/V5-His/lacZ, Life Technologies Corp, Carlsbad, Calif.
- k. GraphPad, version 3.1, InStat3, La Jolla, Calif.
- l. pcDNA3.1/NT-GFP, Life Technologies Corp, Carlsbad, Calif.

References

1. De las Heras M, Garcia de Jalon JA, Minguijon E, et al. Experimental transmission of enzootic intranasal tumors of goats. *Vet Pathol* 1995;32:19–23.
2. De las Heras M, Garcia de Jalon JA, Sharp JM. Pathology of enzootic intranasal tumor in thirty-eight goats. *Vet Pathol* 1991;28:474–481.
3. Palmarini M, Mura M, Spencer TE. Endogenous betaretroviruses of sheep: teaching new lessons in retroviral interference and adaptation. *J Gen Virol* 2004;85:1–13.
4. Cousens C, Minguijon E, Dalziel RG, et al. Complete sequence of enzootic nasal tumor virus, a retrovirus associated with transmissible intranasal tumors of sheep. *J Virol* 1999;73:3986–3993.
5. Dirks C, Duh FM, Rai SK, et al. Mechanism of cell entry and transformation by enzootic nasal tumor virus. *J Virol* 2002;76:2141–2149.
6. Yu DL, Linnerth-Petrik NM, Halbert CL, et al. Jaagsiekte sheep retrovirus and enzootic nasal tumor virus promoters drive gene expression in all airway epithelial cells of mice but only induce tumors in the alveolar region of the lungs. *J Virol* 2011;85:7535–7545.
7. Rabson AB, Graves BJ. *Retroviruses*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1997.
8. McGee-Estrada K, Fan H. Comparison of LTR enhancer elements in sheep beta retroviruses: insights into the basis for tissue-specific expression. *Virus Genes* 2007;35:303–312.
9. York DF, Vigne R, Verwoerd DW, et al. Nucleotide sequence of the Jaagsiekte retrovirus, an exogenous and endogenous type D and B retrovirus of sheep and goats. *J Virol* 1992;66:4930–4939.
10. Walsh SR, Linnerth-Petrik NM, Laporte AN, et al. Full-length genome sequence analysis of enzootic nasal tumor virus reveals an unusually high degree of genetic stability. *Virus Res* 2010;151:74–87.
11. Klevjer-Anderson P, Cheevers WP. Characterization of the infection of caprine synovial membrane cells by the retrovirus caprine arthritis-encephalitis virus. *Virology* 1981;110:113–119.
12. Foreyt WJ. Fatal *Pasteurella haemolytica* pneumonia in bighorn sheep after direct contact with clinically normal domestic sheep. *Am J Vet Res* 1989;50:341–344.
13. Shanthalingam S, Topliff C, Kelling CL, et al. Bighorn sheep fetal lung cell line for detection of respiratory viruses. *Can J Vet Res* 2010;74:75–77.
14. Murphy B, Hillman C, Castillo D, et al. The presence or absence of the gamma-activated site determines IFN gamma-mediated transcriptional activation in CAEV promoters cloned from the mammary gland and joint synovium of a single CAEV-infected goat. *Virus Res* 2012;163:537–545.
15. Sambrook J, Russell DW. *Molecular cloning: a laboratory manual*. 3rd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 2001.
16. Lichtenstein DL, Craigo JK, Leroux C, et al. Effects of long terminal repeat sequence variation on equine infectious anemia virus replication in vitro and in vivo. *Virology* 1999;263:408–417.
17. Corboy JR, Garl PJ. HIV-1 LTR DNA sequence variation in brain-derived isolates. *J Neurovirol* 1997;3:331–341.
18. Ju G, Skalka AM. Nucleotide sequence analysis of the long terminal repeat (LTR) of avian retroviruses: structural similarities with transposable elements. *Cell* 1980;22:379–386.
19. Gomez-Lucia E, Rowe J, Collar C, et al. Diversity of caprine arthritis-encephalitis virus promoters isolated from goat milk and passaged in vitro. *Vet J* 2012;196:431–438.
20. Duarte A, Tavares L. Phylogenetic analysis of Portuguese Feline Immunodeficiency Virus sequences reveals high genetic diversity. *Vet Microbiol* 2006;114:25–33.
21. Murphy B, McElliott V, Vapniarsky N, et al. Tissue tropism and promoter sequence variation in caprine arthritis encephalitis virus infected goats. *Virus Res* 2010;151:177–184.
22. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406–425.