JOURNAL OF VIROLOGY, Aug. 2011, p. 7535–7545 Vol. 85, No. 15
0022-538X/11/$12.00 doi:10.1128/JVI.00400-11

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▽†

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Received 25 February 2011/Accepted 10 May 2011

Jaagsiekte sheep retrovirus (JSRV) induces tumors in the distal airways of sheep and goats, while the closely related enzootic nasal tumor virus type 1 (ENTV-1) and ENTV-2 induce tumors in the nasal epithelium of sheep and goats, respectively. When expressed using a strong Rous sarcoma virus promoter, the envelope proteins of these viruses induce tumors in the respiratory tract of mice, but only in the distal airway. To examine the role of the retroviral long terminal repeat (LTR) promoters in determining tissue tropism, adeno-associated virus (AAV) vectors expressing alkaline phosphatase under the control of the JSRV, ENTV-1, or ENTV-2 LTRs were generated and administered to mice. The JSRV LTR was active in all airway epithelial cells, while the ENTV LTRs were active in the nasal epithelium and alveolar type II cells but poorly active in tracheal and bronchial epithelial cells. When vectors were administered systemically, the ENTV-1 and -2 LTRs were inactive in major organs examined, whereas the JSRV showed high-level activity in the liver. When a putative transcriptional enhancer from the 3′ end of the *env* gene was inserted upstream of the JSRV and ENTV-1 LTRs in the AAV vectors, a dramatic increase in transgene expression was observed. However, intranasal administration of AAV vectors containing any combination of ENTV or JSRV LTRs and Env proteins induced tumors only in the lower airway. Our results indicate that mice do not provide an adequate model for nasal tumor induction by ENTV despite our ability to express genes in the nasal epithelium.

Jaagsiekte sheep retrovirus (JSRV) and enzootic nasal tumor virus (ENTV) are closely related oncogenic betaretroviruses that infect sheep and goats. These viruses share greater than 90% sequence identity and utilize the same cellular receptor for virus attachment and entry (10, 32), yet they cause distinctly different diseases. JSRV transforms secretory epithelial cells of the distal lung leading to ovine pulmonary adenocarcinoma (OPA) (28, 34). ENTV also transforms secretory epithelial cells, although in this case the target cells are located in the nasal mucosa and lead to the formation of a neoplastic disease called enzootic nasal adenocarcinoma (ENA) (9). Two distinct viruses have been implicated in the etiology of ENA, one in sheep (ENTV-1) and one in goats (ENTV-2), and the genomic sequences of both have been determined (7, 26, 38).

It is currently not known how such different diseases result from infection by these closely related retroviruses. Experimental induction of OPA in sheep has been demonstrated using a molecular clone of JSRV (30); however, the lack of a cell culture system capable of propagating ENTV, the fact that an infectious molecular clone of ENTV has not yet been published, and the limitation that experiments using sheep are expensive, have made it difficult to study the mechanism of disease tropism in sheep. Therefore, we developed a model to study JSRV and ENTV tumorigenesis in mice using adeno-associated virus (AAV) vectors (41). AAV vectors made with AAV type 6 (AAV6) capsid proteins were used in this model because they can transfer genes into all airway epithelial cells of mice (14), whereas neither JSRV nor ENTV can infect mouse cells because the mouse ortholog of the retrovirus receptor Hyal2 does not bind to or promote entry of JSRV or ENTV (19). We have previously shown that expression of the JSRV envelope (Env) protein in mouse airway epithelial cells using an AAV vector induces tumors in the distal airway, similar to those observed in JSRV-infected sheep (41, 42). However, when the ENTV Env protein was expressed in a similar manner, it induced lung tumors with a phenotype identical to that of JSRV (40), indicating that tissue specific oncogenesis is not determined by the Env protein.

In the present study, we tested the hypothesis that differences in the long terminal repeats (LTRs) can account for the difference in the locations of tumors induced by JSRV and ENTV. We used AAV vectors to efficiently deliver transgenes that express heat-stable human placental alkaline phosphatase (AP) as a marker for gene expression, or the JSRV or ENTV Env proteins, under the control of the ENTV-1, -2, or JSRV LTRs. We tested intact LTRs because it had previously been shown that elements downstream of the mRNA cap site (the R and U5 regions) were necessary for optimal expression from the JSRV promoter in multiple cell lines, and in some cases,

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† Supplemental material for this article may be found at http://jvi.asm.org/.

▽ Published ahead of print on 18 May 2011.

TABLE 1. Primers used for constructing AAV vectors containing ovine betaretroviral LTRs

Primer	Sequence (5′–3′)[a]	Nucleotide positions in JSRV, ENTV-1, and ENTV-2[b]	AAV construct
JSRV-LTR-Fwd	atatctagaATGCGGGGGACGACCCTGTAAG	7174–7197	AJAP
JSRV-LTR-RV	ataagatctCCTGCCGCGGCCAGCACAAG	109–128	
J_{JE}-LTR-Fwd	atatctagaCTGCATATGAAATATAGAAATATG	7103–7126	A_{JE}JAP
ENTV-1-LTR-Fwd	aatctagaCTGCGGGGGACAACCCGCGGAG	7176–7197	AE_1AP
ENTV-1-LTR-RV	ataagatctCTTGCCGCAGCCAGCACGGAC	101–127	
E_{EE}-LTR-Fwd	aatctagaACATATGAAATATAGATACATG	7107–7128	$A_{EE}E_1AP$
ENTV-2-LTR-Fwd	aatctagaCTGCGGGGGACAACCCGTGAAGG	7171–7193	AE_2AP
ENTV-2-LTR-RV	ataagatctCCTGCCGCGACCAGCACTGACAAGGA	101–126	

[a] Lowercase letters indicate nonviral sequence, e.g., restriction sites.
[b] JSRV, accession number AF105220; ENTV-1, accession number FJ744146; ENTV-2, accession number NC_004994.

deletion of both R and U5 reduced expression to background levels (27). In addition, a region at the 3′ end of the *env* gene that shares poor homology between JSRV and ENTV was incorporated into AAV vectors upstream of the LTR to test whether this sequence possesses promoter-enhancing functions that play a role in determining disease spectrum, as has been shown to occur in other retroviruses (3, 18, 20).

We report here that both the JSRV and the ENTV LTRs are transcriptionally active in the upper and lower airway of mice. Furthermore, we describe a novel role for the 3′ end of the *env* gene as a possible transcriptional enhancer. However, no LTR-Env combination was able to induce nasal tumors in mice, suggesting that a sheep model must be used to determine the mechanism of disease specificity of these highly related viruses.

MATERIALS AND METHODS

Cell culture. Human embryonic kidney 293 (HEK293) cells (12) were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA), 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM L-glutamine in a humidified 5% CO_2 atmosphere at 37°C.

AAV vector plasmid construction. To facilitate cloning into the AAV vector, ARAP4 (1), the vector plasmid was modified as follows. The intron from pSX2.Jenv (31), consisting of the splice donor and acceptor signals from Moloney murine leukemia virus, was inserted upstream of the AP cDNA, and a BglII, NotI, and KpnI multiple cloning site (MCS) was inserted between the Rous sarcoma virus (RSV) promoter and the intron using overlap extension PCR to generate $pAR_{MCS\text{-}intron}AP$. These modifications made it possible to easily replace the promoter in this AAV vector by using an XbaI site upstream of the RSV promoter and one of the restriction sites in the MCS. All ovine betaretroviral promoter sequences were cloned into the XbaI-BglII site of $pAR_{MCS\text{-}intron}AP$ using primers described in Table 1. The JSRV LTR was amplified from the full-length molecular clone of JSRV, $pCMVJS_{21}$ (30), kindly provided by Massimo Palmarini, University of Glasgow, Glasgow, Scotland. The ENTV-1 LTR was amplified from the ENTV-1 NA4 isolate (38). The full-length ENTV-2 LTR was constructed using overlapping clones i and ii (26) generously provided by Marcelo De las Heras, University of Glasgow. Specifically, clone i was digested with NcoI, blunted with Klenow, and then digested with BamHI. The vector fragment was isolated and subsequently dephosphorylated. Clone ii was digested with NdeI, filled in with Klenow, and then digested with BamHI. The BamHI-blunt vector and insert fragments were ligated to generate the full-length ENTV-2 LTR. To create AAV vectors expressing Env, the AP cDNA from AJAP, AE_1AP, and AE_2AP was replaced with the *env* coding region from JSRV or ENTV-1. All vectors contain the AAV2 inverted terminal repeats and all plasmids were propagated in the *Escherichia coli* strain GT116 (InVivoGen). The AAV6 packaging plasmid pDGM6 was kindly provided by David Russell, University of Washington.

AAV vector production and quantification. AAV vectors were made using AAV6 capsid proteins and were produced by cotransfection of HEK293 cells with vector and packaging plasmids as described previously (13). AAV vector titers were determined by Southern blot (15) and by competitive PCR. For competitive PCR, DNA was extracted from AAV particles using a novel heat-based method based on the finding that AAV genomes can be uncoated at temperatures of 71°C or greater (24). A portion (5 μl) of an AAV preparation was treated with 2 U of DNase I (Boehringer Mannheim) for 15 min at 37°C in a final volume of 20 μl. To terminate the reaction, EDTA (Gibco-BRL) was added to a final concentration of 5 mM and heated at 75°C for 15 min to completely inactivate the DNase I enzyme. With the vector genomes liberated from the capsid, a competitive PCR method was conducted as previously described (43). The concentration of competitor DNA, containing a 26-bp insertion in the AP gene, was determined by using a Nanovue spectrophotometer (GE Healthcare, Waukesha, WI). Competitive PCR was conducted with various concentrations of competitor DNA and static amounts of target DNA in order to quantify AAV vector genomes. The primer pair Fwd-ext-AP (5′-TACGCAGC TCATCTCCAACA-3′) and Rev-ext-AP (5′-TCCAGGCTCAAAGAGACCCA-3′) was used under the following PCR conditions: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 63.5°C for 30 s, and 72°C for 30 s. A final extension of 5 min at 72°C concluded the program.

AAV vector delivery. Mouse experiments were performed in accordance with the guidelines set forth by the Canadian Council on Animal Care (CCAC). Eight-week-old C57BL/6/J mice were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada), and eight-week-old C57BL/6/J-Rag2 mice were obtained from Taconic (Hudson, NY). Lightly anesthetized mice received 10^{10} vector genomes (vg) intranasally by the administration of drops to the nose; these drops were spontaneously inhaled. For systemic administration, mice were given 2×10^{10} vg intravenously via tail vein injection and 8×10^{10} vg intraperitoneally. Mice were euthanized 1 month after vector administration. The lungs were perfused by way of the heart with 20 ml of phosphate-buffered saline (PBS), and individual lung lobes were separated. For consistency, the same lobe from each mouse was either flash frozen in liquid nitrogen, preserved in RNAlater (Qiagen), or fixed in 2% paraformaldehyde-PBS for 2.5 h at 22°C. All major organs were preserved in the same manner with the exception that fixation was conducted for 24 h at 22°C. Tissues were washed extensively with PBS (5×10 min) prior to the inactivation of endogenous AP by incubation at 65°C for 1 h. Tissues were stained for vector-encoded heat-stable AP as described previously (15, 16). Immunohistochemical staining of mouse tissues for Env expression was performed as described previously (42).

Determination of AP enzymatic activity. Mouse tissues were harvested 1 month after vector administration, snap-frozen in liquid nitrogen, and stored at −80°C until assayed. Tissues were homogenized in TMNC lysis buffer (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 100 mM NaCl, and 4% [wt/vol] CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}) using a hand-held homogenizer (PRO200; Diamed). For extraction of total protein from the nasal cavity, one half of the nasal cavity (cut sagittally) was placed in a pulverizer on a bed of dry ice, pulverized into a fine dust, and reconstituted in TMNC buffer. Tissue homogenates were placed in a water bath at 65°C for 1 h to inactivate endogenous heat-labile AP activity and subsequently clarified by centrifugation at $17{,}900 \times g$ for 15 min at 4°C to remove cell debris. The protein content of each sample was determined by the method of Bradford, and the AP activity in tissue lysates was determined by a fluorometric assay (39) using the 4-methylumbelliferyl phosphate (MUP) (Sigma, St. Louis, MO) substrate. Briefly, AP activity was measured by mixing 100 μl cell lysate or purified AP protein, 100 μl of 2× SEAP buffer (2 M diethanolamine, 1 mM $MgCl_2$, 10 mM L-homoarginine), and 5 μl of MUP solution (11.4 mg of MUP [Sigma] per ml in dimethyl

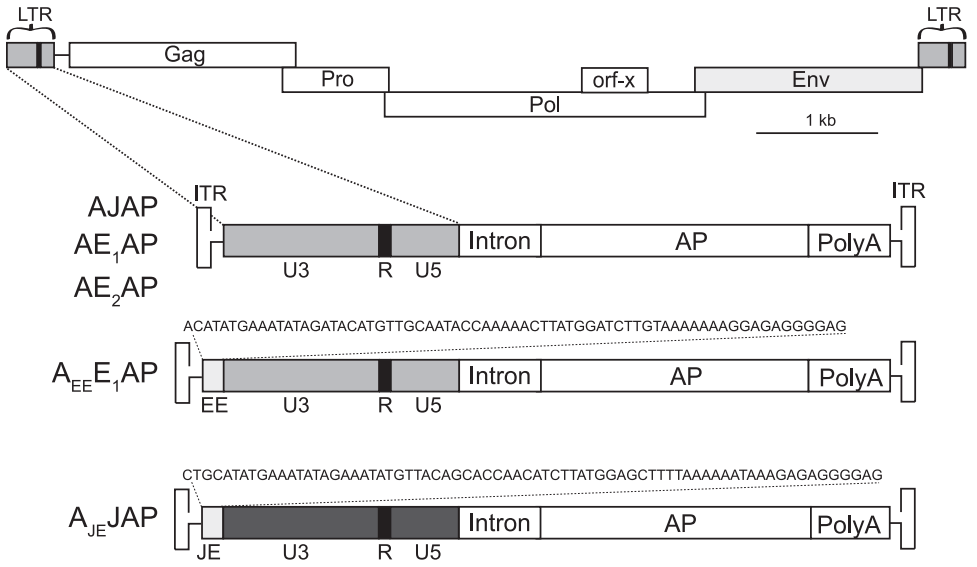

FIG. 1. Schematic of AAV vector genomes containing LTRs and upstream enhancing elements from the JSRV, ENTV-1, or ENTV-2 genomes. The nucleotide sequence of the enhancing element (EE or JE) is shown above. Abbreviations: ITR, AAV inverted terminal repeat; PolyA, polyadenylation signal; kb, kilobase.

sulfoxide) in wells of an opaque black 96-well plate. The plate was incubated at 23°C, and fluorescence due to production of 4-methylumbelliferone was measured every 10 min for 1 h with a fluorometer (Fluostar Optima). Fluorescence over time was plotted, and the maximum linear reaction rates were determined. AP protein present in the tissue samples was calculated by comparison of enzymatic rates to that of the purified human placental AP standard (Sigma catalog no. P3895) and is expressed as the amount of AP per μg of total protein.

RNA extraction, RT-PCR, and 5′ RACE. Total RNA was extracted from mouse tissues by homogenization in TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcriptase PCR (RT-PCR) was conducted on RNA extracted from transduced mouse liver and lung. First-strand cDNA synthesis was performed using the reverse primer, AAV INT R (5′-CTTCCAGAC CTCTCGTTGTA-3′), and SuperScript III (Invitrogen) according to the manufacturer's directions. PCR amplification of cDNA originating from the first-strand synthesis was performed using a primer in the U5 region of the LTR, U5 JSRV/ENTV F (5′-CTGATCCTCTCAACCCCATC-3′), and a primer in the AAV vector immediately upstream of the AP gene, AAV INT R (5′-CTTCCA GACCTCTCGTTGTA-3′), using 5 PRIME MasterMix (5 PRIME, Gaithersburg, MD) under conditions previously described (38). 5′ RACE (rapid amplification of cDNA ends) was performed on 5 μg of total RNA isolated from vector transduced mouse livers using the 5′ race system for rapid amplification of cDNA ends (Invitrogen). First-strand synthesis was carried out using SuperScript III (Invitrogen) and a gene-specific primer, 5RACE AP OUT R (5′-TGCAGC CCAAGCTGATCCAC-3′), as directed by the manufacturer. The nested gene-specific primer, 5RACE AP IN R (5′-CAAGTTCTTAGTTCTGGTGCCG-3′), and the abridged anchor primer (5′-GGCCACGCGTCGACTAGTACGGGIIG GGIIGGGIIG-3′) provided with the kit were used to carry out amplification of dC-tailed cDNA. A final nested PCR was performed using the abridged universal amplification primer (5′-GGCCACGCGTCGACTAGTAC-3′) and the aforementioned reverse primer, AAV INT R, under conditions previously described (37). Products from the final nested PCR were cloned into pGEM-T Easy (Promega) and sequenced.

RESULTS

The LTR promoters of ovine betaretroviruses show similar activities in the noses and lungs of mice. AAV vectors expressing the AP cDNA under the control of the JSRV (AJAP), ENTV-1 (AE_1AP), or ENTV-2 (AE_2AP) LTRs were constructed (Fig. 1), and 10^{10} vg of each vector was administered intranasally to lightly anesthetized mice. A total of six mice were infected in two independent experiments (three mice per experiment). To minimize variability and to ensure a more equitable distribution of vector throughout the lung, each mouse received the same total volume of vector divided into four doses of 50 μl each. Mice were euthanized 1 month later, and tissues from the upper and lower respiratory tract were harvested for analysis of AP expression. The lung, nose, and tracheal sections from mice given saline did not express AP (Fig. 2A, B, and C). Those given the AJAP vector expressed AP in the nasal epithelium, the trachea, and the lung as indicated by the dark staining in all three tissues (Fig. 2D, E, and F). As predicted, the AE_1AP and AE_2AP vectors expressed AP in the nasal epithelium (Fig. 2G and J, respectively). When looking at the sagittal view of the nasal cavity with the nasal septum removed, it appeared that all three vectors transduced the maxilloturbinate and the nasal turbinate, but only the AE_1AP vector transduced the ethmoid turbinate to any significant extent (data not shown). Examination of the trachea revealed that the three LTRs displayed differing levels of transcriptional activity, with JSRV being the strongest, followed by ENTV-1 and, finally, ENTV-2 (Fig. 2E, H, and K, respectively). In contrast to the staining in the lung parenchyma, where all three LTRs were active (Fig. 2F, I, and L), the AE_1AP vector gave minimal AP staining in the large airways (Fig. 2I), whereas the AE_2AP vector gave no staining (Fig. 2L).

Histologic analysis showed that instillation of the AJAP vector resulted in abundant AP staining in all respiratory epithelium including the trachea (Fig. 3C), the alveolar type II and Clara cells of the distal lung, as well as the large airway (Fig. 3D). Infection with the AE_1AP vector resulted in moderate AP expression in the tracheal epithelium (Fig. 3E), sparse staining in the Clara cells lining the bronchioles, and fairly robust expression in the alveolar type II cells of the distal airway (Fig. 3F). Infection with the AE_2AP vector resulted in very low

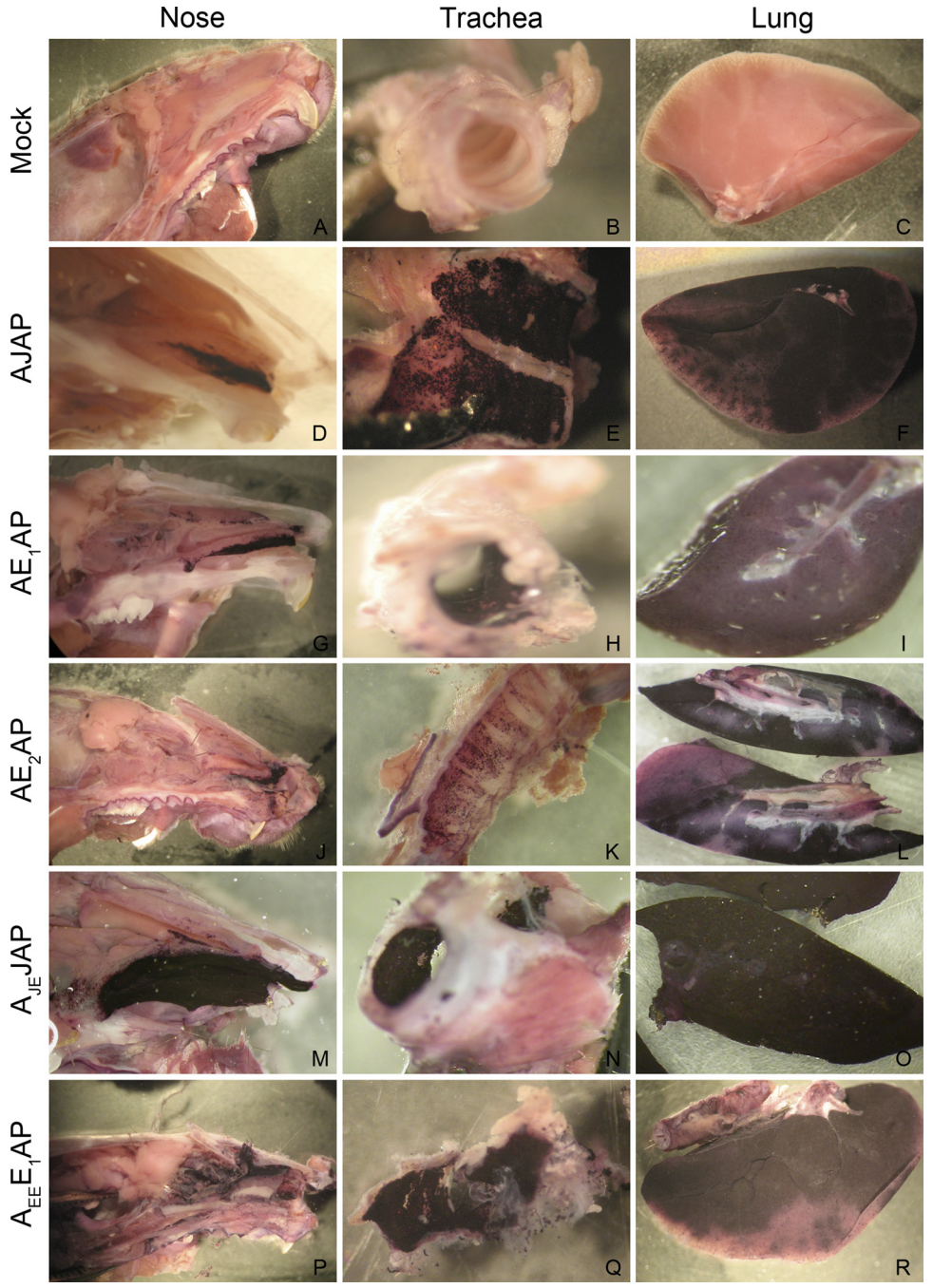

FIG. 2. AP expression in mouse airway tissues following intranasal administration of AAV vectors. Portions (10^{10} vg) of the indicated AAV vectors or saline only (mock) were administered to the noses of mice. One month later the mice were euthanized, and the indicated tissues were removed and stained for AP.

levels of AP expression in the trachea (Fig. 3G) and no expression in Clara cells of the distal airway (Fig. 3H), but rather robust AP expression in the alveolar type II cells of the distal airway (Fig. 3H), nearing that of AJAP. For higher-resolution images, see Fig. S1 in the supplemental material.

The amount of heat stable AP protein produced as a result of vector transduction was further evaluated using a quantitative enzymatic assay. The results from these experiments revealed that the JSRV LTR produced nearly six times more AP protein in the nose than the ENTV LTRs (Fig. 4A). Interestingly, the JSRV and ENTV-2 LTRs produced nearly equivalent amounts of AP protein in the lung (Fig. 4B). Due to the small size of the trachea, it was not possible to quantify the amount of AP expression in this tissue.

In summary, these results show that the JSRV LTR is highly active in epithelial cells of both the upper and lower airway, suggesting that at least in mice, JSRV LTR activity is not limited to the lung. Similarly, the ENTV LTRs are active in

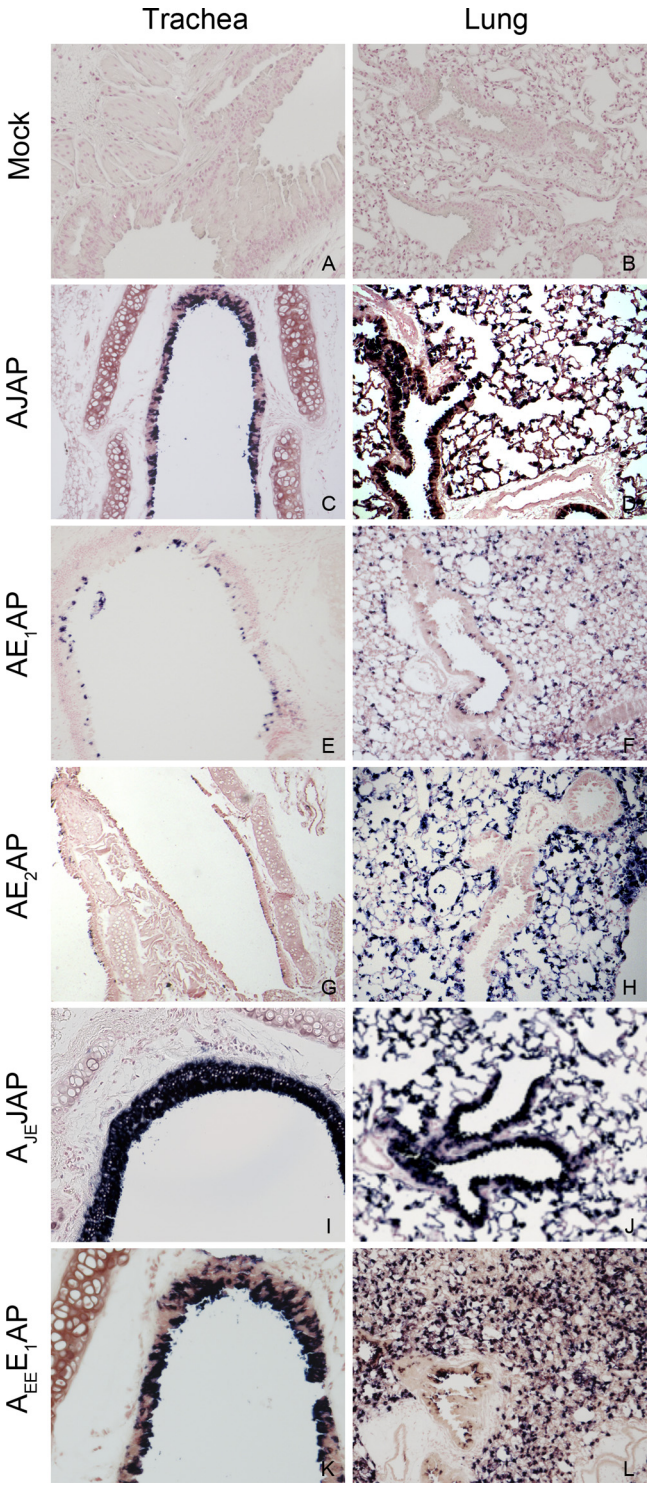

FIG. 3. Histological analysis of AP expression in mouse airway tissues following intranasal administration of AAV vectors. Mice were treated and analyzed as described in Fig. 2.

both the noses and the lungs of mice, albeit to a lesser extent than the JSRV LTR in the lung. One notable difference between JSRV and ENTV LTR activity in mice was in the tracheal and airway epithelium, indicating that the LTRs do display differential activity *in vivo* but only in a specific subset of cells.

The variable region at the 3′ end of the *env* gene increases transgene expression from the JSRV and ENTV LTRs after intranasal administration of AAV vectors. Results from the previous experiment suggested that the LTRs are not entirely responsible for the cell-type-specific oncogenicity observed in naturally infected sheep and that perhaps other regions of the ovine betaretroviral genome contribute to the disease spectrum. The genomic sequences of JSRV and ENTV differ at the 3′ end of the *env* gene, just upstream of the 3′ LTR (29), in a region that has been shown to be dispensable for transformation (17), but possesses enhancer functions in other retroviruses (3, 18). To test the potential role of this region in determining disease tropism, we incorporated the ~75-bp sequence upstream of the JSRV and ENTV-1 3′ LTRs into AJAP and AE_1AP to create $A_{JE}JAP$ and $A_{EE}E_1AP$, where JE stands for JSRV element and EE stands for ENTV element (Fig. 1). The putative enhancer was cloned upstream of the LTR because an RNA export function has been ascribed to a region containing this domain (2, 25). Because our interest was in testing the enhancer function of this domain exclusively, we wanted to ensure that this sequence was not present in the mRNA. These vectors were administered intranasally to 8-week-old C57BL/6 mice, and 1 month later the mice were euthanized.

Gross analysis of AP staining in the lungs, nose, and trachea revealed a significant increase in transgene expression from the JSRV JE-LTR containing vectors (Fig. 2M, N, and O) relative to the vectors encoding the JSRV LTR alone in all three tissues (Fig. 2D, E, and F). Histological analysis of AP staining showed that the level of AP expression was dramatically increased in the epithelium lining the trachea (compare Fig. 3I to 3C) in the tissues exposed to the JE-LTR containing vector. The increase in AP expression in the lungs of JE-LTR containing vectors was not as obvious histologically (Fig. 3D and J); however, based on the intensity of AP staining, it is possible that a dose of 10^{10} vg saturated the lung tissue. For higher-resolution images, see Fig. S1 in the supplemental material.

Results from the intranasal administration of $A_{EE}E_1AP$ were not as dramatic. Upon gross analysis of AP stained tissues, it appeared that the staining intensity was elevated in the lungs of mice that received $A_{EE}E_1AP$ compared to the wild-type LTR containing vector, AE_1AP (Fig. 2R and I). This difference was more obvious upon histological examination of AP stained lung sections where in alveolar type II cells, AP expression was much stronger (compare Fig. 3L to F). These results indicate that sequence elements immediately upstream of the 3′ LTR of JSRV and ENTV increase transgene expression from the JSRV and ENTV LTRs following intranasal delivery.

The variable region at the 3′ end of the *env* gene expands the tissue tropism of the ENTV LTR after systemic administration of AAV vectors. To examine the transcriptional activity of ovine betaretroviral LTRs in nonrespiratory tissue, the AJAP, AE_1AP, AE_2AP, $A_{JE}JAP$, and $A_{EE}E_1AP$ vectors were administered to mice via tail vein (2×10^{10} vg) and intraperitoneal (8×10^{10} vg) injections. At 1 month postinfection, the mice were euthanized, and a full-body necropsy was conducted. Staining for heat-stable AP expression revealed that the JSRV LTR was highly active in the liver (Fig. 5A), mod-

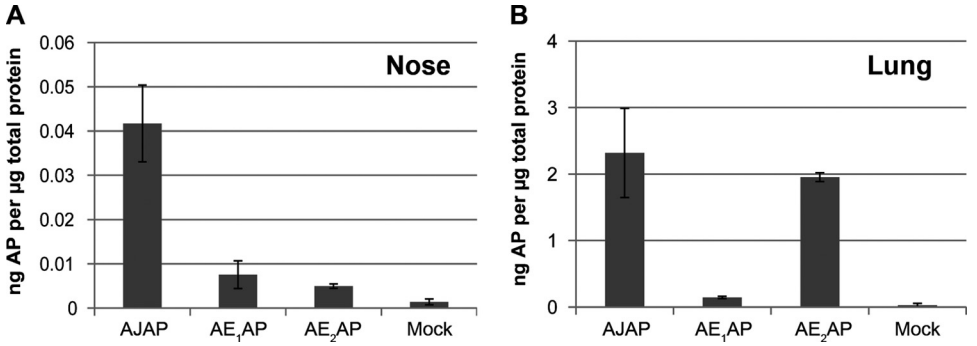

FIG. 4. Quantitative analysis of AP enzymatic activity in mouse nose (A) and lung (B) tissues transduced with AAV6 vectors expressing AP from the JSRV (AJAP), ENTV-1 (AE_1AP), or ENTV-2 (AE_2AP) LTRs. Mice were treated as described in Fig. 2. The results are means ± the standard deviations from six mice; three mice in each of two separate experiments. AP activity measurements for each mouse were performed in triplicate.

erately active in the spleen (Fig. 5B), and poorly active in the kidney (Fig. 5C). Other than a few AP^+ foci in the livers of AE_2AP transduced mice (data not shown), the ENTV LTRs were inactive in all tissues examined (liver, spleen, and kidney [Fig. 5D, E, and F] and heart, pancreas, and brain [data not shown]). Systemic administration of the $A_{JE}JAP$ vector showed stronger AP expression in the liver, spleen, and kidney (Fig. 5G, H and I) compared to the WT JSRV LTR in the same tissues (Fig. 5A, B, and C). Similarly, systemic administration of the $A_{EE}E_1AP$ vector led to high levels of AP expression in the liver (Fig. 5J), spleen (Fig. 5K), and pancreas (data not shown), while virtually no AP expression could be detected in these same tissues when the WT ENTV-1 LTR was used (Fig. 5D and E). Unlike the JE containing vectors, there was very little visible AP expression in the kidneys of mice transduced with the $A_{EE}E_1AP$ (Fig. 5L).

Histological examination of AP expression in the liver of $A_{JE}JAP$- and $A_{EE}E_1AP$-transduced mice demonstrated intense apical staining of the hepatocytes in both cases (Fig. 5M and P, respectively), with $A_{JE}JAP$ producing a significantly greater level of AP expression. AP expression from both $A_{JE}JAP$ and $A_{EE}E_1AP$ was restricted to the red pulp and the occasional artery in the spleen (Fig. 5N and Q, respectively). In the kidney, AP expression could readily be detected in the glomerulus and glomerular arterioles of $A_{JE}JAP$-transduced mice (Fig. 5O) and to a lesser extent in the $A_{EE}E_1AP$-transduced mice (Fig. 5R). These data suggest that the upstream sequence elements that enhanced transgene expression from the JSRV and ENTV LTR in the mouse respiratory tract have similar activity in other organs, with the most dramatic effect observed in the liver and spleen.

To more accurately evaluate the difference in transgene expression from JE- and EE-containing vectors, the level of AP expression was quantified by using an enzymatic assay. The liver and lung were chosen since these tissues demonstrated the greatest increase in AP expression when JE and EE were present. Using this assay, we were able to demonstrate that the amount of AP protein expressed from the $A_{JE}JAP$ vector was more than four times greater in the lung (Fig. 6A) and nearly two times greater in the liver than the AJAP vector (Fig. 6B). Similarly, AP expression from the $A_{EE}E_1AP$ vector was ~3-fold higher than its WT AE_1AP counterpart in the lung (Fig. 6C) and 8-fold higher in the liver (Fig. 6D). Taken together, these data confirm what was observed grossly, that the 3′ end of the *env* gene contains sequences that enhance the activity of the JSRV and ENTV LTRs *in vivo*, particularly in the lung and liver.

The upstream enhancing elements are not present in AAV vector mRNA. The JE and EE elements contain part of a putative RNA export element termed the signal peptide-responsive element (SPRE) (2) or Rej-responsive element (RejRE) (25). To determine whether the mRNA transcripts produced from the $A_{JE}JAP$ and $A_{EE}E_1AP$ vectors *in vivo* might contain the upstream JE- and EE-enhancing elements, 5′ RACE was conducted. Total RNA extracted from the liver of vector-transduced and mock-infected mice were subjected to 5′ RACE. The resultant RT-PCR products were cloned and sequenced. One dominant band was amplified from both the $A_{JE}JAP$- and the $A_{EE}E_1AP$-transduced livers, and sequencing of the cloned cDNA revealed that both transcripts initiated within the R region of the LTR (data not shown), in the same region as the WT LTR, indicating that the upstream enhancing elements are not incorporated into the mRNA and thus do not function in *cis* to enhance mRNA export, stability, or translation.

Administration of AAV vectors expressing the JSRV or ENTV Env proteins from the JSRV or ENTV LTRs results in lung tumor induction with no evidence of nasal tumors. In previous experiments, administration of 5×10^{10} vg of AAV vectors expressing the JSRV or ENTV Env protein from a strong RSV promoter resulted in robust tumor formation in the distal lung of immunodeficient mice by 5 months postinfection (40, 41). We hypothesized that expressing ENTV Env from its native LTR instead of the previously used RSV promoter might lead to nasal tumor formation. We therefore constructed a series of AAV vectors expressing the ENTV-1 Env from the ENTV-1, ENTV-2, and JSRV LTRs and, similarly, the JSRV Env from the ENTV-1, ENTV-2, and JSRV LTRs. Note that these vectors carry the JE or EE enhancer elements as part of the Env coding regions. Intranasal administration of 5×10^{10} vg of Env expressing AAV vectors, along with 5×10^9 vg of the AP-expressing vector, ARAP4 (1), into immunodeficient C57BL/6-Rag2 mice (three mice per vector) resulted in robust and rapid tumor formation for all of the

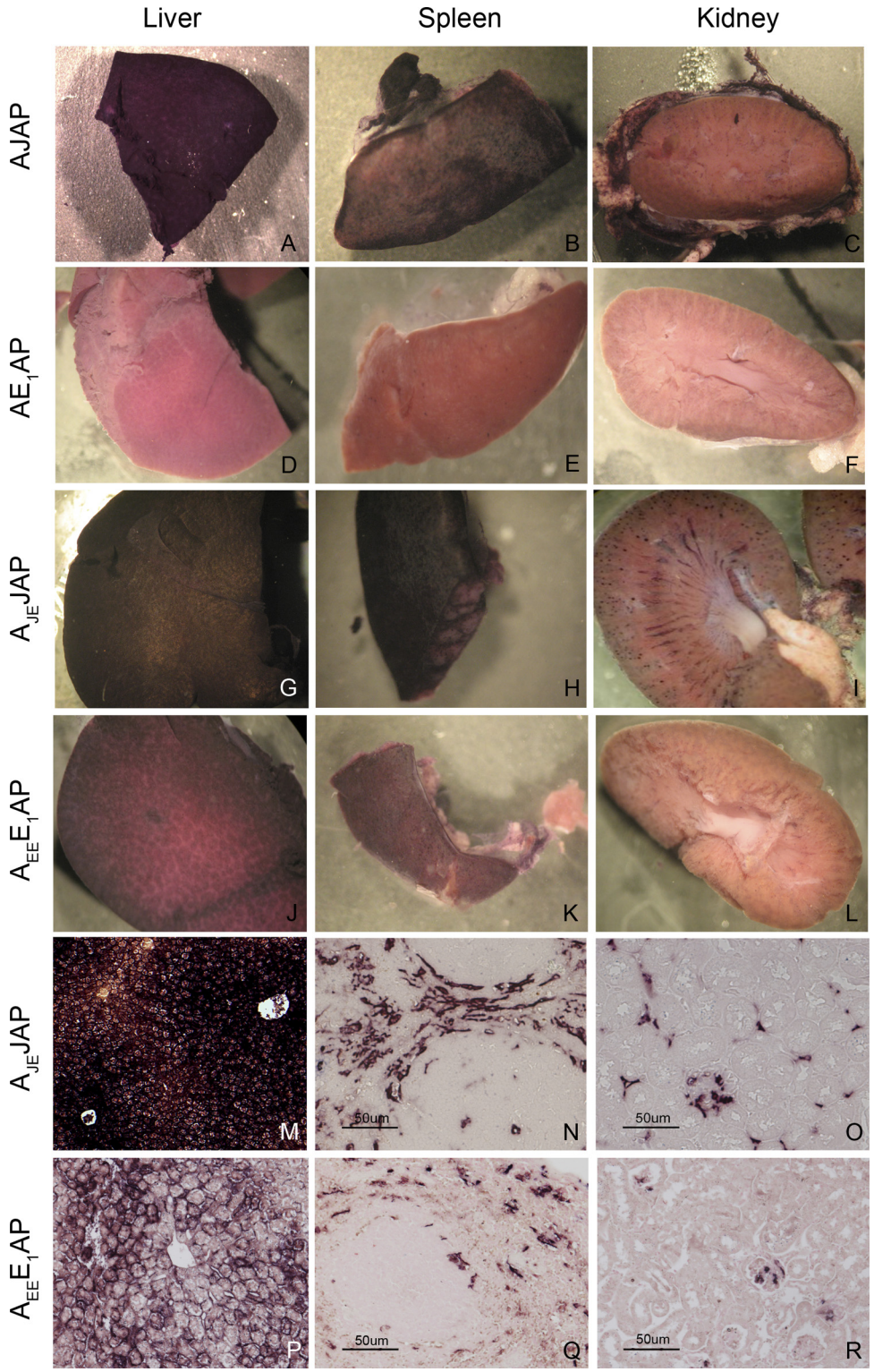

FIG. 5. Representative gross images of AP expression in mouse tissues following combined intravenous and intraperitoneal administration of AAV vectors. The indicated AAV vectors were administered to mice via combined tail vein (2×10^{10} vg) and intraperitoneal (8×10^{10} vg) injections. At 1 month postinfection, the mice were euthanized, and the indicated tissues were removed and stained for AP. The very low AP staining seen in the livers, spleens, and kidneys of mice exposed to the AE_1AP vector (panels D, E, and F) is essentially the same as that of mock-infected mice (not shown).

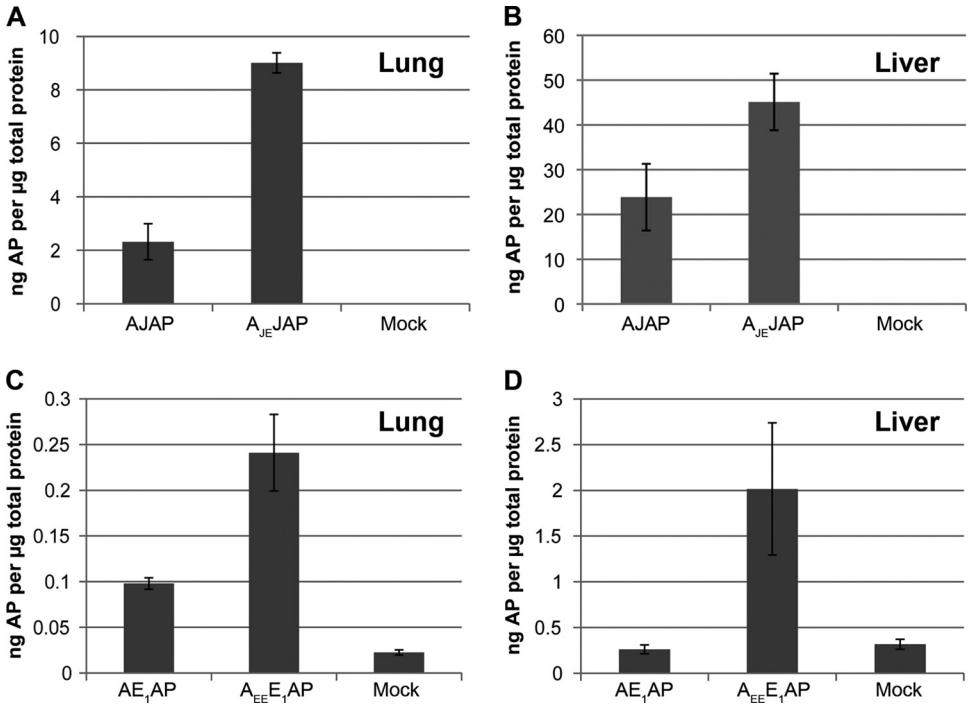

FIG. 6. Quantitative analysis of AP enzymatic activity in mouse tissues transduced with AAV6 vectors containing JSRV and ENTV-1 LTRs with or without enhancing elements. (A) AP expression in the lungs of mice transduced intranasally with 10^{10} vg of JSRV LTR-bearing vectors. (B) AP expression in the liver of mice transduced intravenously (2×10^{10} vg) and intraperitoneally (8×10^{10} vg) with JSRV LTR-bearing vectors. (C) AP expression in the lungs of mice transduced intranasally with 10^{10} vg of ENTV-1 LTR-bearing vectors. (D) AP expression in the liver of mice transduced intravenously (2×10^{10} vg) and intraperitoneally (8×10^{10} vg) with ENTV-1 LTR-bearing vectors. The results are means ± the standard deviations from six mice; three mice in each of two separate experiments. AP activity measurements for each mouse were performed in triplicate.

AAV vectors tested. By 2.5 months postinfection, the mice had developed signs of respiratory distress necessitating euthanasia. Upon gross inspection, the lungs of mice receiving AAV vectors with any combination of LTR and Env looked very similar. The lungs had nearly doubled in size and were filled with an extensive array of tumor nodules that permeated the entire lung parenchyma (Fig. 7A, B, and C). There was no evidence of tumor formation or foci of hyperplasia in either the nasal epithelium or the trachea in any of the mice examined (data not shown). Histologic analysis of lung tumor sections revealed that nearly all of the normal lung tissue had been replaced by adenomatous tissue (Fig. 7D, E, and F) that stained positive for Env (Fig. 7G, H, and I). Additional staining revealed that all of the tumors were positive for the alveolar type II specific maker, surfactant protein C (SPC), and negative for the Clara cell maker, CC10 (data not shown). The cognate promoter-Env combination exacerbated tumorigenesis in the lung but did not result in tumorigenesis in the nose. Both JSRV and ENTV *env* genes gave similar results irrespective of which ovine betaretroviral LTR was used.

DISCUSSION

In this report we studied the tissue and cell type specificity of the JSRV and ENTV LTRs in a mouse model. Intranasal delivery of AAV vectors expressing AP from JSRV, ENTV-1, and ENTV-2 LTRs revealed that all three LTRs were active in the nose and lung. Although the ENTV-1 LTR displayed somewhat broader tissue tropism in the nose, quantitative analysis of AP activity showed that the JSRV LTR elicited the highest level of AP expression in this tissue. Differences in transcriptional activity were most striking in the epithelium lining the trachea and in Clara cells lining the bronchioles. In these cells, the JSRV LTR was the most transcriptionally active. Interestingly, in the AE_2AP-transduced mice there was no sign of AP expression in the Clara cells, and expression in the trachea was weak. The fact that the ENTV-2 LTR was poorly active in the trachea and Clara cells suggests that ENTV-2 is not capable of replicating in the upper airway and thus cannot disseminate to the distal lung. However, if the nasal tropism of ENTV-2 was purely a matter of limited ability to disseminate to the lung, it would follow that JSRV would induce tumors in the nasal mucosa, trachea, and lungs based on the transcriptional activity of its LTR. Despite the weaker activity of the ENTV-1 LTR in the lung, expression of Env from the ENTV-1 LTR induced lung tumors with the same frequency and intensity as the JSRV and ENTV-2 LTRs, suggesting that expression from this LTR exceeds the threshold necessary for tumorigenesis.

The activity of the JSRV LTR was not restricted to the respiratory tract, since AP expression following systemic injection was detected in the liver, spleen, kidney, and pancreas. It is not surprising that the JSRV LTR displayed such a high level of transcriptional activity in the liver given that two hepatocyte nuclear factor-3β (HNF-3β) transcription factor binding sites

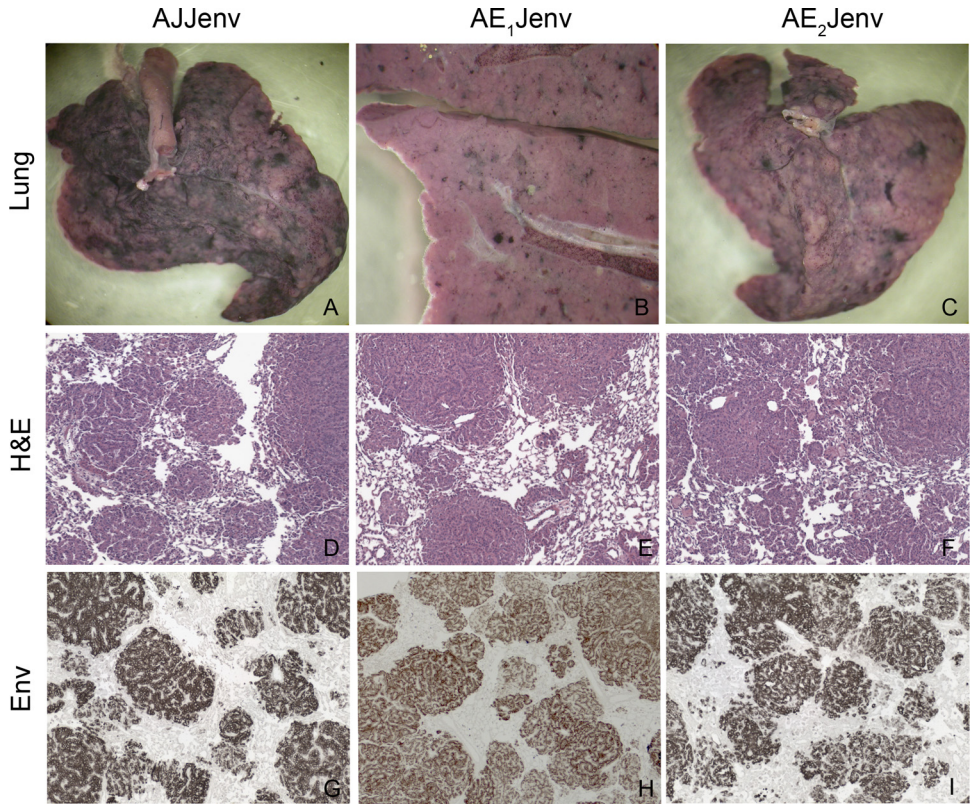

FIG. 7. Tumor induction in mouse lungs following intranasal administration of AAV6 vectors expressing the JSRV Env protein from the JSRV (A, D, and G), ENTV-1 (B, E, and H), or ENTV-2 (C, F, and I) LTR. A total of 5×10^{10} vg of the indicated AAV vectors plus 5×10^{9} vg of ARAP4 was administered to the noses of C57BL/6/J-Rag2 mice. Ten weeks later, mice were euthanized, and the tissues were removed and stained for AP and Env. (A, B, and C) Representative gross images of mouse lungs stained for AP (purple staining). (D, E, and F) Hematoxylin-and-eosin-stained histologic sections. (G, H, and I) Immunohistochemical staining of transduced mouse lungs with an Env-specific monoclonal antibody.

reside within the JSRV LTR, and both were previously shown to be important for gene expression in mouse lung cell lines (23). Since the HNF-3β binding sites are not conserved in the ENTV LTRs (22), this could explain why there was no AP expression in the livers of mice transduced with AE_1AP and AE_2AP vectors.

In previous studies, the JSRV LTR was found to be preferentially active in MLE-15 and mtCC1-2 mouse cell lines derived from alveolar type II pneumocytes (ATII) and Clara cells, respectively (22, 23, 27), while in transgenic mice carrying a bacterial β-galactosidase gene driven by the JSRV LTR, expression was limited to ATII cells and not detected in Clara cells or in any other tissues (8). However, none of these studies examined the function of the JSRV LTR in the nasal epithelium. Our study reveals that the LTRs of JSRV and ENTV are broadly active in the upper and lower respiratory tract of mice in the context of an AAV vector where expression is assessed by detection of an enzymatic protein. So, while it may be difficult to extrapolate these data to what happens in cells where only one or a few proviruses are stably integrated, our data do suggest that the disease specificity of JSRV for secretory lung epithelial cells and ENTV for nasal epithelial cells may not entirely be a function of the transcriptional specificity of the viral LTRs.

One of the major objectives of the present study was to determine whether expressing ENTV Env from its cognate LTR would promote tumor formation in the nasal cavity. Surprisingly, mice exposed to all LTR-Env combinations developed lethal lung tumors in a relatively short period of time (2.5 months) without evidence of nasal tumors. ENTV Env was as oncogenic as the JSRV Env, and the nature of the LTR did not affect the extent of tumor formation. It was remarkable how quickly the mice succumbed to the disease. In previous experiments where the RSV promoter was used to drive expression of the JSRV or ENTV Env (ARJenv and AREenv), lethal lung tumor induction took 5 to 6 months (40, 41). One reason why the JSRV/ENTV LTR-Env containing AAV vectors were so potently oncogenic could be the fact that these promoters are more transcriptionally active in the distal lung, specifically in ATII cells, than the RSV promoter. Despite the acutely oncogenic nature of the JSRV/ENTV LTR-containing AAV vectors, there was no evidence of tumor formation or signs of hyperplasia in the nasal epithelium. It is possible that because lung tumors formed so rapidly that mice succumbed to lung cancer before nasal tumors had a chance to develop. However, the nasal epithelium was clearly targeted by these vectors, and one would expect an epithelial cell in the nose to be as susceptible to transformation as one in the lung. Because it was not feasible to quantify the amount of Env protein expressed, it remains plausible that the threshold level of Env expression

needed for tumorigenesis was not achieved in the nasal epithelium. Lastly, it is also possible that differences in sheep and mouse biology account for the difference in disease location. We plan to address this issue by using the same AAV vectors described here to express the Env proteins in sheep.

We demonstrate here that when sequences derived from the 3′ end of the *env* gene are included in the AAV vector upstream of the LTR promoter, a marked increase in transgene expression is observed, notably in the respiratory tract, liver, and spleen. The region of the genome that comprises JE has been shown to function as part of an RNA export element in JSRV, termed SPRE or RejRE (2, 25). Repeated attempts at RT-PCR and 5′ RACE failed to identify mRNA transcripts initiating anywhere other than the R domain, leading us to conclude that the upstream JE/EE sequence was not incorporated into the mRNA and thus unable to act as a *cis*-acting RNA export signal. These data indicate that the upstream sequence may be functioning as an enhancer. Previously, Sinn et al. reported in 2005 that incorporation of JSRV proviral sequences flanking the *env* gene dramatically increased Env protein expression (35). This increase in Env expression resulted from a combination of RNA splicing and increased steady-state RNA levels. While a subset of these *cis*-acting enhancing elements are present in our AAV Env expressing constructs, they are in a different context than those described by Sinn et al., and thus it is unlikely that the same mechanism is at play in our system.

There is precedent for sequences located immediately upstream of the 3′ LTR (end of the *env*) functioning as enhancers and potentially as determinants of disease spectrum in other retroviruses. Early studies with RSV found that viral sequences immediately preceding the 3′ LTR of the Prague (PrRSV) and Schmidt-Ruppin strains of RSV (SR RSV) enhanced reporter gene expression (18, 20). Interestingly, the enhancer domain in PrRSV, termed the exogenous virus-specific region (XSR) (18), was shown to be responsible for differences in oncogenicity (36), and the same region of the avian leukosis virus, subgroup J (ALV-J), termed E, has been shown to contribute to oncogenicity in certain genetic lines of chickens, potentially through an enhancing mechanism (3).

Another possibility is that the JE/EE sequence forms part of an internal promoter that allows for synthesis of a small RNA transcript that transactivates the JSRV or ENTV LTR and/or other cellular genes. Both Moloney murine leukemia virus and feline leukemia virus express transactivating noncoding RNAs from their 3′ LTRs, and these have been shown to transactivate cancer-related signaling pathways (4, 5, 11). The Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html) strongly predicts a polymerase II promoter in the JE/EE upstream sequence that could potentially synthesize a transcript of ~200 bp [provided transcription stopped at the poly(A) in the 3′ LTR]. The molecular mechanism by which this putative noncoding RNA transcript could regulate transcription remains to be determined, but noncoding RNA sequences have been shown to regulate gene expression by influencing chromatin architecture, transcription, alternative splicing, translational efficiency, and mRNA stability (21).

Lastly, it is possible that the enhanced gene expression that was observed from the JE- and EE-containing vectors is the result of some ability to overcome one of the rate-limiting steps involved in AAV vector transduction such as binding, internalization, endosomal processing, nuclear targeting, nuclear import, or more likely, conversion from single-stranded DNA to the duplex form (33). In addition, it is conceivable that increasing the distance from the inverted terminal repeat (ITR) to the promoter may somehow improve promoter activity, although there are currently no reports to support this claim.

In summary, we have shown that the LTRs of JSRV and ENTV are broadly active in the upper and lower respiratory tracts of mice. Examination of several candidate elements (Env, LTR, and a putative enhancer) suspected to influence the disease tropism of JSRV and ENTV in sheep failed to reveal the mechanism of tissue-specific tumorigenesis, indicating that further studies on tropism need to be conducted in sheep.

ACKNOWLEDGMENTS

This study was supported by the Natural Sciences and Engineering Research Council of Canada. D.L.Y. was supported by an OVC Doctoral Fellowship, S.R.W. was supported by an OVC Graduate Fellowship, and C.L.H. and A.D.M. were supported by funding from the Fred Hutchinson Cancer Research Center.

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