JOURNAL OF VIROLOGY, Sept. 2010, p. 9078–9085 Vol. 84, No. 18
0022-538X/10/$12.00 doi:10.1128/JVI.00950-10
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Viral Particles of Endogenous Betaretroviruses Are Released in the Sheep Uterus and Infect the Conceptus Trophectoderm in a Transspecies Embryo Transfer Model▽†

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Received 1 May 2010/Accepted 26 June 2010

The sheep genome contains multiple copies of endogenous betaretroviruses highly related to the exogenous and oncogenic jaagsiekte sheep retrovirus (JSRV). The endogenous JSRVs (enJSRVs) are abundantly expressed in the uterine luminal and glandular epithelia as well as in the conceptus trophectoderm and are essential for conceptus elongation and trophectoderm growth and development. Of note, enJSRVs are present in sheep and goats but not cattle. At least 5 of the 27 enJSRV loci cloned to date possess an intact genomic organization and are able to produce viral particles *in vitro*. In this study, we found that enJSRVs form viral particles that are released into the uterine lumen of sheep. In order to test the infectious potential of enJSRV particles in the uterus, we transferred bovine blastocysts into synchronized ovine recipients and allowed them to develop for 13 days. Analysis of microdissected trophectoderm of the bovine conceptuses revealed the presence of enJSRV RNA and, in some cases, DNA. Interestingly, we found that RNAs belonging to only the most recently integrated enJSRV loci were packaged into viral particles and transmitted to the trophectoderm. Collectively, these results support the hypothesis that intact enJSRV loci expressed in the uterine endometrial epithelia are shed into the uterine lumen and could potentially transduce the conceptus trophectoderm. The essential role played by enJSRVs in sheep reproductive biology could also be played by endometrium-derived viral particles that influence development and differentiation of the trophectoderm.

Retroviruses have the unique ability to integrate their proviral DNA into the genome of most eukaryotes (6). As a result of their integration step, retroviruses can be found in nature as either "exogenous" or "endogenous" viruses. Exogenous retroviruses are transmitted, like any other virus, horizontally from infected to uninfected host. Occasionally, exogenous retroviruses gain access to the germ line by infecting the germ cells of the host, resulting in the stable integration of the viral genome (termed provirus) into the host genome. In these cases endogenous retroviruses (ERVs) are transmitted from generation to generation in a typical Mendelian fashion. ERVs are present in the genome of all animal species, and a complete ERV provirus has the same general structure as an exogenous retrovirus: *gag*, *pro*, *pol*, and *env* genes flanked by two noncoding long terminal repeats (LTRs). The *gag* gene encodes matrix, the major capsid protein, and nucleocapsid proteins necessary for viral particle formation. The *pro* gene encodes the viral protease, while the *pol* gene encodes the enzymes reverse transcriptase, integrase, and RNase H. The *env* gene encodes the envelope glycoprotein (Env), consisting of both surface and transmembrane domains, that is necessary to interact with the cellular receptor for virus entry (51). Most ERVs are defective for viral replication due to either mutations, substitutions, insertions, and/or deletions that alter the provirus genome, thereby preventing these elements from producing infectious viral particles and being horizontally transmitted (6). However, ERVs with an intact genomic structure are present in various animal species and, in general, represent proviruses that have integrated in their host relatively recently from the evolutionary point of view (6, 16, 24).

Sheep offer an interesting model to study the biological impact of ERVs and their interactions with exogenous retroviruses and their host (3). The ovine genome contains multiple copies of endogenous betaretroviruses highly related to two oncogenic exogenous retroviruses, jaagsiekte sheep retrovirus (JSRV) and the enzootic nasal tumor virus (ENTV) (3, 9, 11, 12, 34, 35). The infectious and pathogenic JSRV and ENTV are tropic for the respiratory tract of sheep, while endogenous JSRVs (enJSRVs) are predominantly expressed in the female reproductive tract (32–35, 46). There are at least 27 enJSRV proviruses that have integrated in the host genome throughout the last 5 to 6 million years during the evolution of Caprinae (including sheep and goats and their wild relatives) (3). Inter-

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

▽ Published ahead of print on 7 July 2010.

estingly, endogenization of enJSRVs is still occurring, and there are several proviruses (known as insertionally polymorphic) that have integrated in the last few thousand years and are present in only some domestic sheep (3, 10). The enJSRVs possess several biological features that helped and shaped host evolution (4, 43–45, 50). For example, some enJSRV loci are able to block viral exit of related exogenous and endogenous retroviruses at late stages of the replication cycle (3, 30, 31). The enJSRVs can also block entry of related exogenous retroviruses by receptor competition, as JSRV and enJSRVs can utilize hyaluronidase 2 (HYAL2) as a cellular receptor (1, 36, 44).

Importantly, enJSRVs have essential roles in sheep reproduction (14). In the female reproductive tract, the epithelia of oviduct, uterus, cervix, and vagina express enJSRVs (33, 34, 46). Of particular note, the enJSRV *env* RNA is very abundant in the endometrial luminal and glandular epithelia during the estrous cycle and pregnancy (33). Maximal levels of enJSRV RNA in the endometrial epithelia coincides with the onset of conceptus (embryo and associated extraembryonic membranes) elongation, when the mononuclear trophectoderm cells are rapidly proliferating and producing interferon tau (IFNT), the pregnancy recognition signal that maintains ovarian progesterone production (40). In sheep, the conceptus elongates from an ovoid or tubular shape on day 11 to a filamentous form by day 14 in a process that involves proliferation and migration of mononuclear trophectoderm cells. Beginning on day 14, some mononuclear trophectoderm cells begin to differentiate into trophoblast giant binucleate cells that comprise 15 to 20% of the conceptus trophectoderm by day 18 (23). The binucleate cells migrate and fuse initially with the luminal epithelium as well as with each other to form multinucleated syncytial plaques, which comprise the cotyledonary portions of the placenta that interdigitate into the endometrial caruncles of the uterus and form placentomes essential for supplying maternal nutrients to the developing fetus (23).

In the conceptus, enJSRV *env* RNA is first detected beginning on day 12 as it begins to elongate and is most abundant in the binucleate cell and multinucleated syncytia throughout gestation (13). Indeed, *in vivo* loss-of-function experiments in sheep found that conceptus elongation and binucleate cell formation were compromised when the production of enJSRV Env was inhibited, which supported the idea that enJSRV Envs play a role in conceptus development (15). Sixteen of the 27 enJSRV loci contained an *env* gene with an intact open reading frame, and 5 of the 27 enJSRV loci isolated so far have an intact genomic structure and can produce viral particles *in vitro*, as well as utilize ovine HYAL2 as a receptor for cell entry (3). The intact enJSRV proviruses integrated in the host genome after sheep domestication and are insertionally polymorphic in the host (3).

Available evidence supports the hypothesis that the endometrial epithelia of the ovine uterus expresses intact enJSRV proviruses that produce viral particles, which are subsequently shed into the uterine lumen and can transduce the conceptus. This study tested that hypothesis and found that enJSRVs produce viral particles that are released into the uterine lumen of the ewe. Further, enJSRVs were found to potentially infect the conceptus trophectoderm when *in vitro* produced bovine embryos were transferred into the ovine uterus. Interestingly, only the evolutionarily young enJSRV proviruses, which integrated around or after sheep domestication, were found to be consistently expressed in the uterine endometrium and form the genomes packaged into the released viral particles present in the uterine lumen, which could influence development and differentiation of the conceptus trophectoderm.

MATERIALS AND METHODS

Animals. Ewes of Suffolk and Texel breed (*Ovis aries*) were observed daily for estrus in the presence of vasectomized rams and used in experiments after they had exhibited at least two estrous cycles of normal duration (16 to 18 days). All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Blastocyst production and embryo transfer. Bovine ova were obtained from a local abattoir and matured *in vitro* using methods described previously (18). The ova were fertilized *in vitro* by using standard procedures and then cultured *in vitro* by using G_1/G_2 embryo culture medium (vitrolife, Engelwood, CO) at 38.5°C in an atmosphere of 5% CO_2–5% O_2–90% N_2. Recipient ewes were synchronized to estrus via a 12-day treatment with progesterone using an intravaginal insert (controlled internal drug release [CIDR]) (Pfizer, New York, NY). On day 12, the CIDR was removed, and each ewe was administered 20 mg of dinoprost tromethamine (Lutalyse; Pfizer) intramuscularly. Estrus was detected using vasectomized rams fitted with a marking harness. Two *in vitro* produced bovine blastocysts were transferred into each synchronized ovine recipient ($n = 30$) on day 7 postestrus. All ewes received a CIDR at transfer. On day 20 of pregnancy (13 days posttransfer), bovine conceptuses were recovered by gently flushing the ovine uterus with 20 ml of 10 mM Tris (pH 7.0). Recovered bovine conceptuses were frozen in Tissue-Tek Optimal Cutting Temperature Compound (OCT; Pelco International, Redding, CA) and stored at −80°C. The ovine uterus was then obtained, and the endometrium was removed by physical dissection and stored at −80°C. Uterine flushes were clarified by low-speed centrifugation ($3{,}000 \times g$) immediately following collection, aliquoted, and stored at −80°C.

LCM. All instruments and reagents used for laser capture microdissection (LCM) and nucleic acid extraction were obtained from MDS Analytical Technologies (Sunnyvale, CA) unless otherwise noted. The conceptuses frozen in OCT were sectioned at 8 μm using a cryostat. Sections (at least six per conceptus) were placed on polyethylene naphthalate (PEN) membrane slides and stored at −80°C until use. Slides were stained using a HistoGene LCM Frozen Section Staining Kit. Immediately following the staining, LCM was performed using a Veritas Microdissection System (Molecular Devices, Sunnyvale, CA) to isolate conceptus trophectoderm cells. Trophectoderm cells were transferred to CapSure Macro LCM Caps for RNA and DNA extraction using a PicoPure Extraction Kit. The PicoPure RNA or DNA Extraction kit was used according to the manufacturer's recommendations, including DNase treatment using an RNase-free DNase Set from Qiagen (Valencia, CA).

PCR. Total RNA was analyzed by reverse transcription-PCR (RT-PCR) using primers and cycling conditions as provided in Table S1 in the supplemental material. The cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Input RNA could not be quantified accurately due to the nature of the extraction process from the microdissected cells. The cDNA was resuspended in 20 μl of double-distilled H_2O (ddH_2O), and 2 μl was used in the subsequent PCRs. The resulting cDNA was amplified using the primers in standard PCRs using Ex *Taq* DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's directions on an MJ Research PTC-200 machine. Each reaction mixture contained 2 μl of cDNA template, 2 μl of Ex *Taq* buffer, 1.6 μl of Ex *Taq* deoxynucleoside triphosphates (dNTPs), 0.4 μl of each 10 μM primer, 0.25 μl of Ex *Taq* DNA polymerase, and water to 20 μl.

Genomic DNA was analyzed by PCR using Ex *Taq* DNA Polymerase and an MJ Research PTC-200 machine (see Table S1 in the supplemental material for primer sequences and cycling conditions). Each genomic DNA PCR mixture contained 5 μl of DNA template, 5 μl of Ex *Taq* buffer, 4 μl of Ex *Taq* dNTPs, 1 μl of each 10 μM primer, 0.5 μl of Ex *Taq* DNA polymerase, and water to 50 μl. RT-PCR and PCR products (10 μl aliquots) were visualized using a 1% agarose gel in Tris-acetate-EDTA (TAE) buffer containing ethidium bromide.

Western blot analyses. Uterine flush samples (2 ml per animal) were diluted approximately 4-fold in Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich) and centrifuged over a 1.5-ml 29% (wt/vol) sucrose-PBS cushion at

100,000 × *g* at 4°C for 1 h. The resulting pellets were resuspended in 20 μl of 2× SDS-PAGE loading buffer (5) for subsequent Western blot analyses or in 30 μl of Dulbecco's PBS for RNA extraction and transcriptional profiling. For Western blot analyses, concentrated viral particles were denatured, separated using SDS-PAGE, and transferred to nitrocellulose. Western blot analyses were performed using methods described previously (41). Enhanced chemiluminescent detection (Immun-Star Western C Kit; Bio-Rad, Hercules, CA) on X-OMAT AR X-ray film (Kodak, Rochester, NY) was performed according to the manufacturer's recommendations. The presence of Gag proteins was analyzed with rabbit polyclonal serum against the JSRV major capsid (CA) protein (8) diluted at 1:10,000 in PBS containing 1% bovine serum albumin (BSA).

enJSRV transcriptional and phylogenetic analysis. Total RNA was isolated from the ultracentrifuge pellets of uterine flushes and resuspended in PBS using a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was isolated from the LCM-isolated bovine conceptus trophectoderm samples using a PicoPure RNA Extraction kit, according to the manufacturer's recommendations, including DNase treatment using an RNase-free DNase Set from Qiagen (Valencia, CA). Total RNA was also extracted from samples of Texel endometrium using Trizol (Invitrogen). Texel ewes were used for this phylogenetic analysis in order to be consistent with previous analyses (3). RT-PCR was conducted using total RNA from individual samples of viral particles purified from each of the uterine flushes, the LCM-isolated bovine conceptus trophectoderm cells, and the ovine endometrium. Primers designed to amplify enJSRV *env* and the U3 region of the LTRs (see Table S1 in the supplemental material for sequences and cycling conditions) were utilized for RT-PCR analysis. PCR products were cloned into pCRII Dual Promoter vector using a T/A Cloning Kit (Invitrogen). For each individual ewe, a total of 100 individual plasmid clones containing *env* cDNAs were sequenced for each endometrial sample, and 50 individual plasmid *env* cDNAs were sequenced for samples of purified uterine flush viral particles and LCM-isolated bovine conceptus trophectoderm samples. Sequencing was carried out using an ABI Prism Dye Terminator Cycle Sequencing Kit and ABI Prism automated DNA sequencer (Perkin-Elmer Applied Biosystems, Forest City, CA).

For each animal, the 200 partial *env* sequences from endometria, uterine flush, and conceptus, as well as homologous sequences from the 27 known enJSRV loci, were aligned using the Clustal W algorithm (49) as implemented in the program Geneious. From these alignments (566 bp), Bayesian phylogenies were estimated in MrBayes, version 3.1.2 (38), under a general time-reversible model with among-site variation (GTR+G). Initial analyses indicated that sampled trees had unrealistically high branch lengths, a known problem with some MrBayes analyses (7). Final runs were therefore not started from random trees but, rather, from neighbor-joining (NJ) trees calculated under a GTR+G model in PAUP* (47). Branch length priors were rescaled using the formula supplied by Brown et al. (7) based on average branch length from the NJ tree. Two independent chains were run for 5 million generations, from each of which 1 million was later removed as burn-in. Parameters and trees were sampled every 2,000 steps, and convergence among runs was assessed visually using Tracer, version 1.5 (A. Rambaut and A. J. Drummond [http://beast.bio.ed.ac.uk/Tracer]). Majority rule consensus trees and posterior support for individual branches were then found based on the final sample of 4,000 trees.

RESULTS

Mature enJSRV particles are released in the uterine lumen. Western blot analysis of uterine flush samples, clarified and ultracentrifuged over a sucrose cushion, detected the enJSRV major capsid protein (CA) from all ewes tested in this study ($n = 16$) (Fig. 1). As expected, the immunoreactive protein identified was ~25 kDa in size, which is the predicted size of the mature enJSRV CA derived from the cleavage of Gag by the viral protease. These data strongly support the hypothesis that intact enJSRV particles are assembled in the uterine endometrial epithelia and released into the uterine lumen.

enJSRVs *env* mRNA is present in bovine conceptus trophectoderm. In order to test whether the enJSRV particles released in the uterus could potentially infect the developing conceptus, we conducted a transspecies embryo transfer experiment in which *in vitro* produced bovine embryos (37, 48) were transferred into the ovine uterus, allowed to gestate for 13 days, and

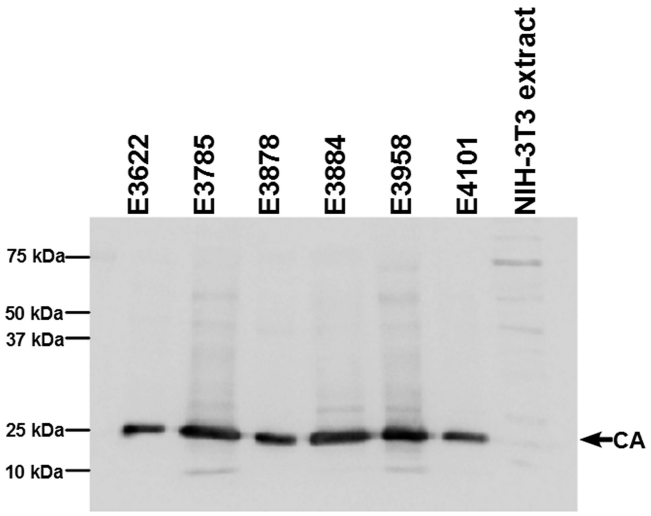

FIG. 1. Western blot analysis of viral particles isolated from the lumen of the ovine uterus. Viral particles were purified by ultracentrifugation of uterine flush samples over a sucrose cushion. Proteins were separated by 12% SDS-PAGE, and Western blot analysis was conducted using a rabbit antiserum toward the major capsid protein of JSRV. An immunoreactive protein of ~25 kDa in size, which is the correct size of the mature enJSRVs capsid (CA), was detected in uterine flushings from all ewes ($n = 16$). Representative results from six ewes are presented. Cell lysates from NIH 3T3 cells were used as a negative control.

then recovered and analyzed for the presence of enJSRVs (Fig. 2). The use of bovine embryos was necessary because these species do not harbor enJSRV loci in their genome (3, 22). The background of enJSRVs in sheep embryos would make exceedingly difficult the detection of uterine-derived enJSRVs.

The spherical blastocyst changes morphology during early pregnancy that involves proliferation and outgrowth of the trophoblast, a process termed conceptus elongation (42). Sixteen fully elongated and filamentous conceptuses (8 to 10 cm in length) were recovered at 13 days postimplantation in 16 of the 30 recipient ewes that received two *in vitro* produced bovine blastocysts. Trophectoderm cells from the bovine conceptuses were isolated by laser capture microdissection, and RNA was analyzed by RT-PCR for *IFNT*, *LGALS15*, *HYAL2*, and *CSH1* mRNAs. *IFNT* is expressed exclusively in the mononuclear trophectoderm cells of the conceptus (17). As expected, all of the bovine trophectoderm samples contained *IFNT* mRNA (Fig. 3A and Table 1). *LGALS15* is a gene expressed specifically in the endometrial luminal epithelium in the sheep and goat uterus but not in the conceptus (19, 28). Although the *LGALS15* gene is present in cattle, *LGALS15* mRNA is not detectable in the bovine uterus or conceptus or in other bovine tissues (28). *CSH1* is expressed specifically by the trophoblast giant binucleate cells in both cattle and sheep but is not expressed in the uterus (23). Thus, the presence of *LGALS15* mRNA from the microdissected bovine trophectoderm cell samples would indicate contamination by cells of ovine endometrial luminal epithelium origin, whereas the presence of *CSH1* mRNA would indicate the presence of differentiated trophoblast giant binucleate cells in the conceptus trophectoderm. In total, we detected *LGALS15* and/or *CSH1* mRNA in

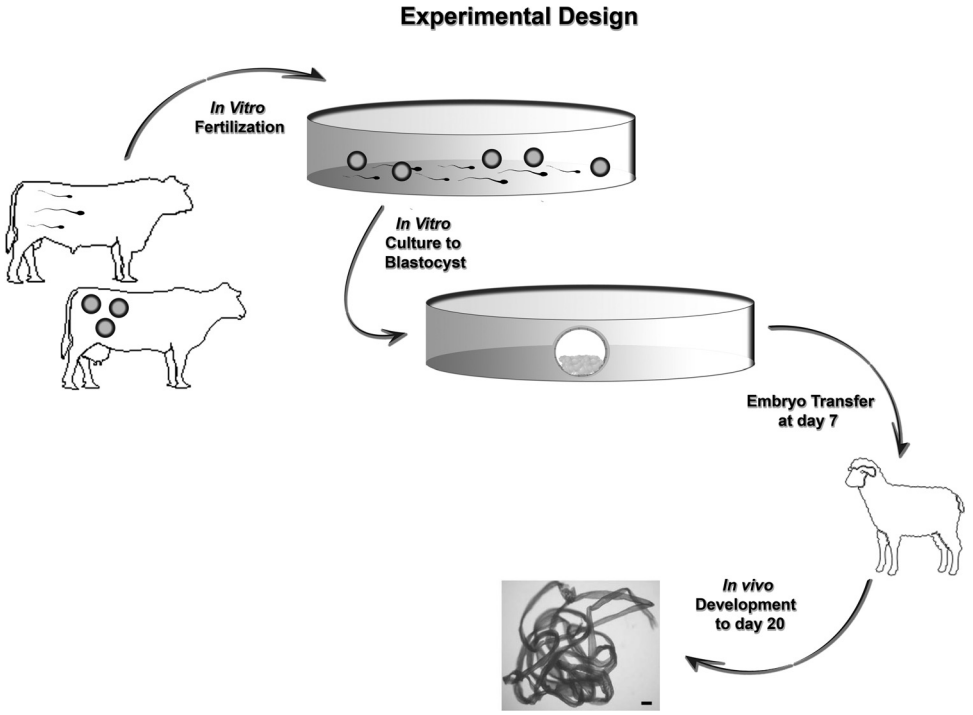

FIG. 2. Transspecies embryo transfer model. Schematic illustrating experimental design of transspecies embryo transfer conducted to test the working hypothesis that enJSRV particles are released from the uterine endometrial epithelia and infect the conceptus trophectoderm.

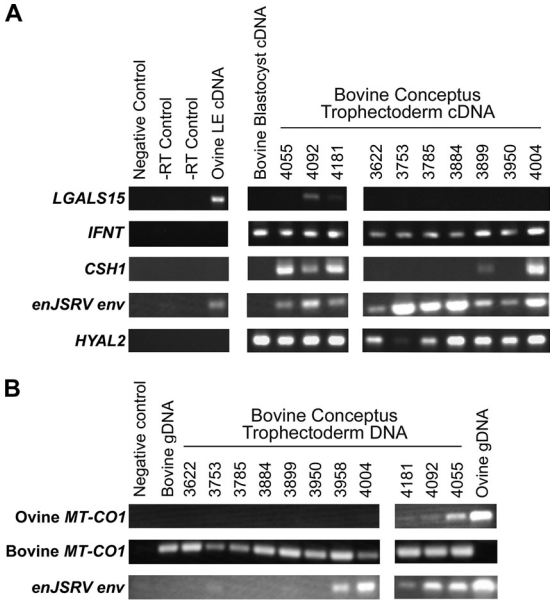

FIG. 3. Analysis of microdissected bovine conceptus trophectoderm for presence of enJSRV RNA and DNA. (A) RT-PCR analysis of bovine conceptus trophectoderm. RNA was extracted from microdissected bovine conceptus trophectoderm cells and analyzed by RT-PCR for *LGALS15*, *IFNT*, *CSH1*, *HYAL2*, and enJSRV mRNAs. Representative results are shown from 10 different bovine conceptuses. Negative controls included water as template and no reverse transcriptase (−RT) to assess genomic DNA contamination. (B) PCR analysis of bovine conceptus trophectoderm. DNA was extracted from microdissected bovine conceptus trophectoderm cells and analyzed by PCR for ovine and bovine *MT-CO1* genes as well as enJSRV *env*. Representative results are shown from 11 bovine conceptuses. The negative control used water as a template, and the positive controls were bovine or ovine genomic DNA (gDNA).

six of the microdissected bovine trophectoderm samples (Fig. 3A and Table 1); these samples were excluded from subsequent analysis. *HYAL2* mRNA was detected in all of the bovine trophectoderm samples. Importantly, enJSRV *env* RNA was present in all bovine trophectoderm RNA samples (16/16), including the 10 samples with no evidence for contamination by ovine uterine endometrial luminal epithelium.

Analysis of DNA from bovine conceptus trophectoderm. Collectively, the results presented so far support the ideas that (i) enJSRVs release viral particles from the endometrial epithelium into the lumen of the uterus, and (ii) these particles can enter the trophectoderm of the transplanted bovine conceptuses. The trophectoderm nourishes the developing conceptus in the early stages of pregnancy by phagocytosis of secreted products from the uterine epithelia that are present in the uterine lumen (21). Thus, the presence of enJSRV *env* RNA in the conceptus trophectoderm could arise from either nonspecific trophoblast phagocytosis or true receptor-mediated entry. In order to differentiate these processes, we tested whether we could detect enJSRV *env* DNA in bovine trophectoderm, indicative of reverse transcription. We purified DNA from the microdissected bovine trophectoderm and ruled out the presence of ovine DNA contamination by PCR using primers that amplify the ovine mitochondrial gene *MT-CO1* (27). As illustrated in Fig. 3B and summarized in Table 1, all of the bovine conceptus trophectoderm samples were positive for bovine *MT-CO1* (27). On the other hand, ovine *MT-CO1* was amplified from only four samples (animals 4055, 4092, 4181, and 7919) (data not shown). Thus, 10 conceptuses had no indication of ovine cell contamination as tested by RT-PCR of the *CSH1* and *LGALS15* mRNAs and PCR of ovine *MT-CO1*.

TABLE 1. Summary of RNA and DNA analysis of microdissected bovine conceptus trophectoderm samples[a]

Conceptus (animal no.)	mRNA detection					DNA detection		
	IFNT	*LGALS15*	*CSH1*	enJSRV *env*	*HYAL2*	Bovine *MT-CO1*	Ovine *MT-C01*	enJSRV *env*
3622	+	−	−	+	+	+	−	−
3753	+	−	−	+	+	+	−	+
3785	+	−	−	+	+	+	−	−
3884	+	−	−	+	+	+	−	−
3899	+	−	+	+	+	+	−	−
3950	+	−	−	+	+	+	−	−
3958	+	−	−	+	+	+	−	+
4004	+	−	+	+	+	+	−	+
4055	+	−	+	+	+	+	+	+
4092	+	+	+	+	+	+	+	+
4181	+	+	+	+	+	+	+	+
7900	+	−	−	+	+	+	−	+
7912	+	−	−	+	+	+	−	−
7919	+	−	+	+	+	+	+	+
7935	+	−	−	+	+	+	−	−
7937	+	−	−	+	+	+	−	−
Total positive	16	2	6	16	16	16	4	8

[a] Presence (+) or absence (−) of mRNA or DNA was determined by PCR analyses of microdissected bovine conceptus trophectoderm samples (see Table S1 in the supplemental material for primer sequences and cycle conditions and Materials and Methods for protocols).

Interestingly, enJSRV *env* DNA was amplified in total DNA isolated from 3 of the 10 bovine conceptus trophectoderm samples (animals 3753, 3958, and 7900) that were not contaminated with ovine DNA or RNA. These results support the idea that enJSRV viral particles from the ovine uterus entered into the bovine conceptus trophectoderm and that, in some cases, their genome was reverse transcribed, which is indicative of receptor-mediated cell entry.

Transcriptional profile of enJSRV loci in the ovine endometrium. We next analyzed the transcription profile of the enJSRV loci that were (i) expressed in the ovine endometrium, (ii) packaged in viral particles released in the lumen of the uterus, and (iii) present in the bovine conceptuses. We performed RT-PCR analysis of RNA extracted from the endometrium, viral particles purified from flushes of the uterine lumen, and microdissected trophectoderm of the bovine conceptuses from four ewes (animals 7900, 7912, 7935, and 7937). PCR products were then cloned and, from each animal, 100 colonies derived from ovine endometrium, 50 from viral particles purified from uterine flushes, and 50 from microdissected bovine conceptus trophectoderm were sequenced, and Bayesan phylogenies were estimated as described in Materials and Methods. The enJSRV loci have a very high degree of sequence similarity between each other, which makes it difficult to identify each provirus with certainty (3). In addition, some of these loci are insertionally polymorphic and entered the host genome only in the last few thousand years. Consequently, different individuals may possess different enJSRV proviruses with similar/identical sequences while the current nomenclature is based on enJSRV loci cloned from a single bacterial artificial chromosome (BAC) library isolated from a single Texel ram. Nonetheless, sequence differences in the amplified region (consisting of the transmembrane domain of Env and the U3 region of the LTR) are sufficient at least to distinguish proviruses with similarity to either evolutionarily younger (after domestication) or older (before domestication) enJSRV loci.

As illustrated in Fig. 4, all four of the ewes analyzed showed a consistent transcriptional profiling. The great majority of the enJSRV sequences amplified from the endometrium of the four ewes clustered with sequences amplified from the viral particles present in the uterine flushes and from the transferred bovine embryos. In all cases, the majority of sequences clustered with the youngest enJSRV loci that are present only in the domestic sheep (and not with the wild relatives within the *Ovis* genus). From three of the four ewes, the majority of the enJSRV sequences expressed clustered with the insertionally polymorphic proviruses (such as enJSRV-16 and enJSRV-18). In one of the ewes (7912), the majority of the expressed sequences appeared to cluster more specifically with enJSRV-8. The enJSRV-8 was detected in our previous study (3) in domestic sheep but not in their wild relatives. The enJSRV-8 provirus contains a stop codon in *env* that was not present in the enJSRV sequences recovered from ewe 7912. Sequences similar to some of the oldest proviruses, such as enJSRV-3, were detected at a variable proportion in all the endometrial samples. Interestingly, these sequences were not recovered in the viral particles isolated from uterine flushes.

The enJS56A1 locus, which has a transdominant mutation in *gag*, is able to block viral exit of all the intact enJSRV proviruses with the exception of enJSRV-26 that escapes this viral restriction (3). The enJS56A1-like sequences were found rarely expressed in the endometrium of the Texel sheep analyzed in this study, suggesting weak expression in these animals of this transdominant proviral locus compared to the other enJSRVs. Sequences similar to enJSRV-26 were detected in the conceptuses of two animals (7912 and 7935). The enJSRV-26 is the only provirus that is able to escape restriction induced by enJS56A1. Overall, these results suggest that recently integrated enJSRV proviruses are abundantly expressed in the sheep endometrium and are able to release viral particles. The low abundance of enJS56A1 and the presence of enJSRV-26-like escape mutants might allow those enJSRVs to release efficiently infectious viruses that subsequently enter/infect the bovine conceptus trophectoderm.

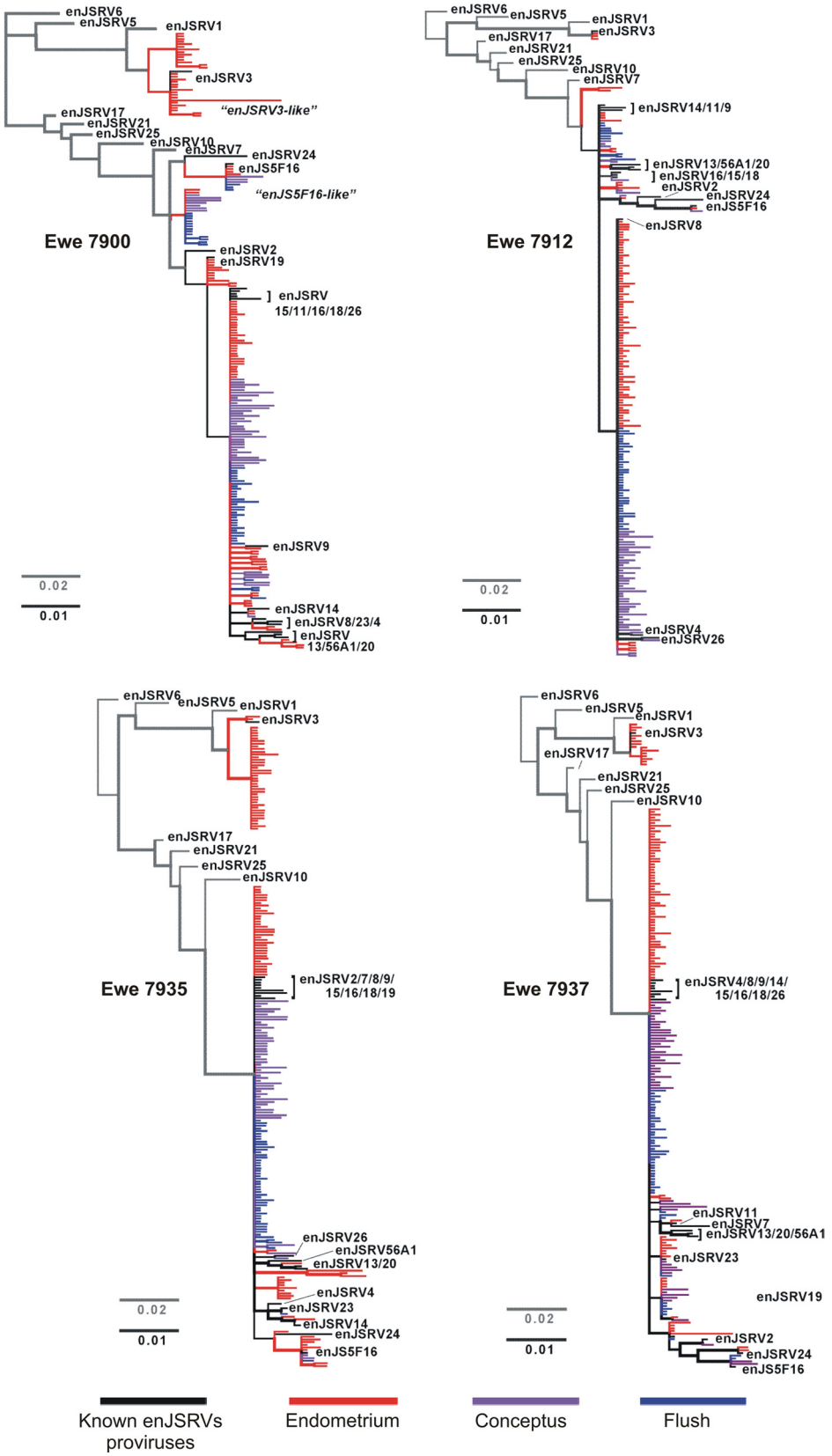

FIG. 4. Phylogenetic analysis of enJSRVs expressed in the uterus. Bayesian majority rule consensus trees of 200 partial *env* sequences from endometrium (red), uterine flush (blue), and conceptus (purple) of four ewes receiving bovine blastocysts. Sequences from 26 known endogenous betaretroviruses (enJSRVs) were also included (in black); enJSRV-6 was used to root the tree as it is the oldest enJSRV provirus integrated in the sheep genome containing the sequences analyzed in this figure. Branch width represents the level of posterior support, with most internal branches receiving 100% support. Scale bars represent the expected substitutions per site. For easier visual representation, deeper parts of the phylogeny (gray branches) are shown at a different scale.

DISCUSSION

Collectively, results presented here strongly support the idea that enJSRVs are expressed in the sheep endometrium and release viral particles into the uterine lumen that have the potential to infect the trophoblast of the developing conceptus. In fact, the presence of viral particles in mammalian placentae has been documented in many species, including humans, baboons, and mice (25, 26, 29, 39). Although it has been always assumed that these particles derive from ERVs expressed in the placenta, it is also possible, as shown in this study, that these particles are released from the uterine endometrium and then enter into the mononuclear trophectoderm cells of the gestating conceptus.

The present study utilized a transspecies embryo transfer model in which *in vitro* derived bovine blastocysts were transferred to recipient ovine uteri. Utilization of bovine blastocysts provided a unique opportunity to assess the presence of the enJSRV RNA/DNA in the bovine conceptus, as enJSRVs are not present in the bovine genome (3, 22). The bovine conceptuses gestated in the ovine uterus developed appropriately because they were elongated, filamentous in nature, and contained *IFNT* mRNA. While the bovine genome does not contain enJSRV sequences, the presence of enJSRV RNA and, in some cases, DNA in the bovine conceptuses is evidence that enJSRV viral particles, released by the ovine uterine endometrial epithelia, are able to enter the mononuclear trophectoderm cells of the bovine conceptus.

The mechanisms by which the enJSRV particles enter the conceptus trophectoderm are not clear. One possibility is that they use HYAL2, the cellular receptor for JSRV and enJSRVs. Although *HYAL2* mRNA was detected in all of the bovine conceptus trophectoderm samples, bovine HYAL2 is much less efficient at mediating entry of the enJSRV particles than ovine HYAL2 (3). Alternatively, enJSRV particles could enter the mononuclear trophectoderm cells via a nonreceptor-mediated process such as phagocytosis. Indeed, the trophectoderm is highly phagocytic in nature due to reliance on substances secreted by the endometrium and present in the uterine lumen for conceptus development (20, 52). Although enJSRV RNA was detected in all 10 conceptuses, enJSRV DNA was detected in only in 3 of the 10 microdissected trophectoderm samples of bovine conceptuses. These data suggest that receptor-mediated entry and subsequent reverse transcription did not occur in all of the bovine conceptuses and that enJSRVs in the trophectoderm could be derived from both receptor-mediated entry and from phagocytosis/pinocytosis. Further, the small amount and frequency of enJSRV DNA detected in bovine conceptuses may also be indicative of the presence of restriction factors blocking the early steps of replication of these viruses in bovine cells.

We recently found that BST2/tetherin is upregulated in the ovine endometrium during early pregnancy by conceptus IFNT (2). The BST2/tetherin is a restriction factor that can inhibit enJSRV exit *in vitro*; however, it is upregulated in only the stromal cells of the endometria of pregnant ewes and is not present in the endometrial luminal epithelium (2). Thus, the host appears to have evolved mechanisms that favor (or at least do not hamper) enJSRV expression and release of viral particles from the uterine luminal epithelium. It is tempting to speculate that the enJSRV particles are beneficial to the developing conceptus because the enJSRVs Env regulates trophectoderm cell proliferation and perhaps differentiation. Previous research from our laboratory supports this hypothesis as *in utero* enJSRV Env knockdown resulted in growth-retarded conceptuses that exhibited reduced mononuclear trophectoderm cell proliferation and an absence of binucleate cell formation (15). In that study, morpholinos were used to inhibit the splicing and translation of enJSRV *env* RNA in the conceptus trophectoderm, but the *in utero* injections also reduced the amount of enJSRV Env in the uterine luminal epithelium. Thus, it is possible that the morpholino knockdown of enJSRV Env in the uterine luminal epithelium reduced the amount of enJSRV viral particles shed by the uterine luminal epithelium, thereby impacting mononuclear trophectoderm cell proliferation and binucleate cell differentiation. Therefore, enJSRVs expressed in the developing conceptus after day 12 may be derived from proviruses expressed in the trophoblast itself and/or from those expressed in the maternal uterus that are subsequently transmitted to the trophoblast.

It is feasible to hypothesize that at least some enJSRV loci present in the ovine genome may be the result of integration of new proviruses derived from enJSRVs produced by the uterine epithelia that infect the inner cell mass of the developing embryo that gives rise to the embryo/fetus proper. However, one would expect that transmission of enJSRVs from the trophectoderm to the inner cell mass is not readily achieved by enJSRVs; otherwise, there would be an acquisition of too many proviruses in each generation. From our results, the enJSRVs abundantly expressed in the uterus and producing functional Env proteins are those recently integrated in the *Ovis aries* genome. Therefore, the ongoing endogenization and *de novo* integrations in the genome of intact enJSRVs might render redundant the function provided by older proviruses; however, enJSRVs are still entering the sheep genome, and, therefore, the host may not need yet to select expression of a specific provirus(es) in the ovine reproductive tract generation after generation.

Collectively, findings from the current study provide evidence that fully intact proviral loci are present within the sheep genome and are capable of producing viral particles that are shed from the uterine epithelia expressing enJSRV loci. Further, the resultant viral particles enter the cells of the conceptus, undergo reverse transcription, and potentially integrate in the bovine trophectoderm cells in a transspecies embryo transfer model.

ACKNOWLEDGMENTS

We thank the members of the Laboratory for Uterine Biology and Pregnancy of Texas A&M University and the Laboratory of Viral Pathogenesis of the University of Glasgow Faculty of Veterinary Medicine for their help and stimulating discussions.

This work was supported by NIH Grant HD052745 to T.E.S. and M.P. and a Strategic Research Developmental Grant by the Scottish Funding Council to M.P.

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