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Cloning and Sequence Analysis of Genome from the Inner Mongolia Strain of the Endogenous Betaretroviruses (enJSRV)*

Yu WANG [1], Shu-ying LIU [1]**, Jian-yun LI [1], Min HAN [1] and Zhen-ling WANG [2]

(1.College of Animal Science and Animal Medicine, Inner Mongolia Agricultural University, Huhhot 010018, China; 2.Beijing Vocational College of Agriculture, Beijing 102442, China)

Abstract: In order to amplify the complete genome of enJSRV from the strain of Inner Mongolia (enJSRV-NM), we used enJSRV-specific and JSRV-specific DNA probes in dot blot hybridization. Seven pairs of primers were designed based on Genbank sequences. Seven fragments were obtained by PCR and were cloned into the PMD19-T vectors. The recombinant plasmids were sequenced and analyzed. The results showed that the genome was 7 942 bp in length and contained four overlapping open reading frames corresponding to the gag, pro, pol and env genes as well as an additional open reading frame (orf-x) that overlaps the 3' end of the pol gene. The nucleotide acid sequences of the enJSRV-NM loci were compared with the sequences of South Africa enJS56A1 strain (Accession No. AF153615) and USA JSRV21 strain (Accession No. AF105220). The nucleotide acid identities were 99.2% and 92.3% respectively. Two zinc fingers were found in the NC region in the predicted amino acid sequence. However, the YXXM motif, which is a reliable molecular marker for the infectious exogenous virus, was not found in the TM region. It was found that the enJSRV-NM region was 90%-98% identical at the amino acid level to its exogenous infectious counterparts in most of the retroviral genome. This is the first nucleotide sequence of enJSRV reported in P.R China. The resource work has provided a wide range of information useful not only for expression genomics and annotation of genomic DNA sequence, but also for further research on the clinical diagnosis of OPA.

Key words: Jaagsiekte sheep retrovirus (JSRV); Endogenous betaretroviruses (enJSRV); Ovine pulmonary adenocarcinoma (OPA); Dot blot hybridization

Jaagsiekte sheep retrovirus (JSRV) is the etiological agent of ovine pulmonary adenocarcinoma (OPA) (4, 15, 25) and is a contagious lung cancer of sheep and goats that closely resembles human adenocarcinomas of the deep airways, including bronchioloalveolar carcinoma (BAC) (21). Human lung cancer is the leading cause of human cancer deaths worldwide (12).

Fifteen to 20 copies of endogenous betaretroviruses (enJSRV) are present in the genome of sheep and goats (3, 8, 9, 17, 20). Endogenous retroviruses (ERV) are vertically transmitted as stable mendelian genes in the germline of most eukaryotes and it is believed that

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** Corresponding author. Tel: +86-471-4308913,
E-mai: liushuying_imau@126.com

they derive from integration of exogenous (horizontally transmitted) retroviruses in the germ cells of a specific host (24). In recent years, considerable effort has been directed toward understanding the biological significance of ERV, particularly those present in the human germ line (5, 22). Generally, endogenous proviruses are transcriptionally silent and are often defective, typically differing from the exogenous counterpart by deletions or point mutations that render them incapable of forming infectious virus (12). The biological effects of ERV are largely unknown. Given the long standing presence of ERV in the genome, evolutionary theory would predict they might have more beneficial effects than detrimental effects (12). The incidence of human lung adenocarcinoma has steadily risen and this neoplasm is now responsible for up to one quarter of lung cancer fatalities (2). Ovine pulmonary adenocarcinoma presents a unique opportunity to study the me chanisms of lung carcinogenesis in an animal model (2).

Palmarini *et al* isolated, sequenced and functionally characterized three complete enJSRV proviruses (enJS56A1, enJS5F16 and enJS59A1) derived from a sheep genomic DNA phage library (16). All three proviruses contained open reading frames (ORF) encoding at least one or more structural genes. However, subtle differences in a *gag* region and long terminal repeat (LTR) U3 and envelope transmembrane (TM) nucleotide sequences were found to be useful in differentiating between enJSRV and the pathogenic, exogenous form of JSRV (16). Palmarini *et al*. also observed that enJSRVs can block the exogenous JSRV replication by a novel two-step interference mechanism acting both early and late during the virus replication cycle (19). enJSRVs are highly active and they are abundantly and specifically expressed throughout the reproductive tract of an infected sheep (18). So the sheep enJSRVs system is a model that can be utilized to study many different aspects of ERVs and retrovirus biology (19).

In this study, dot blot hybridization of genomic DNA with novel DNA probes derived from JSRV *U3* domains, JSRV *env* TM domains, enJSRV *gag* domains and enJSRV *env* TM domains were used for the first time to identify the enJSRVs provirus in China. Cloning and sequence analysis of a intact enJSRV proviruses are also described in this report. The results provide insight into the genomic structure of enJSRVs, its phylogeny and its relationship to known enJSRV and JSRVs.

MATERIALS AND METHODS

Sources and preparation of samples

In this study, healthy domestic sheep were collected from flocks in Inner Mongolia and defined as "normal" animals if they appeared normal by macroscopic and histological examination of the lungs. OPA-infected sheep were defined as animals which showed typical clinical signs, particularly the production of abundant lung fluid from the nostrils when the rear limbs were elevated above the head. Diagnosis was confirmed by macroscopic and histological examination of the lungs. Sections of lungs from sheep with OPA were used as positive controls.

DNA extraction

Genomic DNA was extracted as described previously (17). Briefly, uterus and lung tissues from healthy domestic sheep were ground to a powder in liquid nitrogen, lysed by addition to TES (0.01 mol/L Tris, 0.1 mol/L EDTA, 0.5% SDS), digested with 100

μg of RNase per ml for an hour at 37℃, then 4 μL (20 mg/mL) of proteinase K was added and incubated at 55℃ overnight and extracted with phenol, pH 8.0. DNA was precipitated with sodium acetate and isopropanol, washed twice with 70% ethanol, dried, resuspended in TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0) and stored at 4℃. DNA was also isolated from SPA-affected sheep lung tumor tissues for use as a positive control. The integrity of DNA was examined by electrophoresis on 1% agarose.

Dot blot hybridization

Four probes were designed within a highly variable area between enJSRV and JSRV. The positions of the probes relative to the genome are shown in Table1. The four digoxigenin-labeled probes were synthesized by TaKaRa Biotechnology Co., Ltd. (TaKaRa, Dalian, China).

The dot blot hybridization procedure used was performed essentially as described previously (23). Briefly, DNA was denatured by boiling at 100℃ for 10 min and immediately cooling on ice for 10 min. Then DNA specimens were dotted onto nylon membrane (TaKaRa, Dalian, China). Dotted nylon membranes were sealed in plastic bags with 10 mL of a prehybridization mixture containing 5×SSC, 50% formamide, 25 mmol/L Na_2HPO_4 (pH 6.5), 5× Denhardt solution, and 250 μg of sheared denatured salmon sperm DNA per mL. The membranes were incubated at 42 ℃ for 2 to 4 h in a shaking water bath. The prehybridization mixture was removed and replaced with 10 mL of hybridization mixture containing 5×SSC, 45% formamide, 25 mmol/L Na_2HPO_4 (pH6.5), l×Denhardt solution, 250 μg of sheared denatured salmon sperm DNA per mL, 10% dextran sulfate, and 1.5 μL of 20 μmol/L DNA probe. The plastic bags were resealed and incubated for 18 to 22 h at 42℃ in a shaking water bath. The hybridization mixture was removed and stored at -20 ℃ for reuse. The nylon membranes were washed in (a) 250 mL of 2×SSC-1.0% (wt/vol) SDS for 3 to 5 min at room temperature. (b) 250 mL of 0.2×SSC-1.0% (wt/vol) SDS for 3 to 5 min at room temperature, and (c) 250 mL of 0.16×SSC-1.0% SDS for 15 min at 50°C twice and then were rinsed briefly in 2× SSC-0.1% SDS at room temperature. Finally these membranes were dried and radiographed.

Sequence of the enJSRV-NM genome

Sequencing of the complete genome of enJSRV-NM was achieved using overlapping PCR amplification. The positions of the fragments relative to the genome are shown in Fig 1. The primers and annealing temperature used to generate these fragments are shown in Table 2. PCR was performed using 0.5 μg of DNA of Uterus tissues identified, 0.5 μmol/L concentrations of each primer, 200 μmol/L concentrations of each deoxynucleoside triphosphate, 10 mmol/L Tris-HCl (pH 8.8), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 2.5 U of Taq polymerase (TaKaRa, Dalian, China) in a total volume of 100 μL. The PCR cycles were 95℃ for 5 min, then 30 cycles

Table 1 Probes used in this study

Probes	Name	Derives from	Sequence(5′→3′)
1	enJSRV	enJSRV	GTTCCCGAGGGGGTTAAAAGCGACCCTCCGT
2	JSRV	JSRV	GAAATGCTGCATATGAAATATAGAAAT
3	enJSRV	enJSRV	GTATAATTGCTATGGGCGCGGTTCTGACAG
4	JSRV	JSRV	ATACCAGGAAATCTGATTATATAAGAATCCGG

were performed of 60 s at 94℃ (denaturation), 60 s annealing (for temperature see Table 1) and 120 s at 72℃, with a final extension of 5 min at 72℃. The amplified mixture was electrophoresed on agarose gels, and the specific bands were cut and purified. Then fragments were ligated into PMD19-T vector (Promega) as specified by the manufacturer. At least three clones from two independent PCR reactions were sequenced for each PCR fragment. The full-length sequence was assembled by using the DNA-STAR software package. Finally, the full-length sequence was annotated according to enJS56A1 (AF153615) and submitted to GenBank.

Computer-assisted analysis

Sequence data was analyzed using the programs of the DNASTAR software package (Lasergene). Sequences were aligned by using the MEGALIGN program DNASTAR1.59. Three phylogenetic trees were constructed by using the Kimura method of the DNAMAN software package (version 6.0.3.48), and 1000 bootstraps were run to validate the three phylogenetic threes.

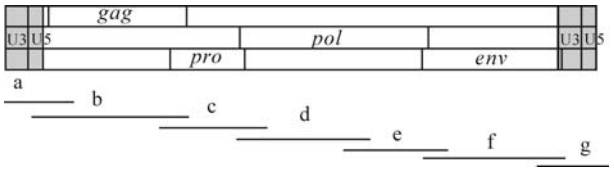

Fig.1. enJSRV-NM sequencing scheme. The clones to obtain the complete sequence of enJSRV-NM are show.

Nucleotide sequence accession number

The nucleotide sequence of enJSRV-NM has been deposited in GenBank under accession number DQ838493.

RESULTS

Four probes sensitivity test

Quantities of DNA were denatured and spotted onto a nylon membrane in the indicated pg amounts. Denatured salmon sperm was spotted as a negative control (NC). The four probes show a clear positive reaction with 10 pg and is weakly reactive with 5 pg of target DNA. The endpoint was approximately 5 pg in every test and at this concentration the color was

Table 2. Primers and PCR condition for amplification used for sequencing of enJSRV-NM

Fragment	Primer pair	Sequence (5′→3′)	Annealing Tem. (℃)	Product length（bp）
a	enJSRV LTR PCR		59	618
	P1-1	CTGCGGGGGACGACCCGTGAA		
	P1-2	ATGCACAAACAACTGACGACTATG		
b	enJSRV *gag* PCR		55	2006
	P2-1	TCTCCTCGCCACTACTCTT		
	P2-2	GGTAAAGGCCCAAACACT		
c	enJSRV *pro* PCR		55	1106
	P3-1	ACGGATGTTCAAGGTAAT		
	P3-2	CAGAGGTAGAGGGTTCAA		
d	enJSRV *pol* PCR		57	1613
	P4-1	TATGGGTCGATCAGTGGC		
	P4-2	AGGAAGGGTGGAATGTGC		
e	P4-3	GTCGGTGCCTTACAGATGA	60	1145
	P4-4	AGGTGTCACTGGAGGTTGT		
f	enJSRV *env* PCR		56	1861
	P5-1	ACAGGATGCCGAAGCGCCGCG		
	P5-2	CGTGAAGGGTTAAGTCTTGGGAGCT		
g	enJSRV LTR PCR		53	613
	P6-1	TAATTGCTATGGGCGCGGTTCTGA		
	P6-2	TGCCGCGGCCAGCACAAGCAA		

faintly visible. Therefore, the actual sensitivity limit of our probes with DNA extracted from uterus and lung tissues of healthy and infected domestic sheep was probably 7 to 12 pg. The negative control spot was included to ensure probe specificity (Fig not shown).

To determine whether the four DNA probes were sufficient for differentiation between enJSRVs and JSRVs, digoxigenin-labeled DNA probes of enJSRV-specific (probe 1 and 3) and JSRV-specific (probe 2 and 4) were tested by hybridization with a known amount of purified chromosomal DNA of enJSRVs and JSRVs (Fig.2). JSRV-specific probes (probe 2 and 4) hybridized with homologous DNA of lung tumor tissues of OPA-affected sheep identified by the wheelbarrow test and histological examination of the lungs. No hybridization was obtained with Uterus and lungs tissues of healthy domestic sheep identified by macroscopic and histological examination of the lungs DNA (Fig.2). Although the sequence of enJSRVs and JSRVs have higher homolog, the results indicated that the four DNA probes were adequate for determination of enJSRV and JSRV using this method with 70～120 pg of target DNA fixed onto the nylon membrane.

The complete genomic sequence of enJSRV-NM loci was obtained using a shotgun sequencing approach. The complete assembled nucleotide sequence is 7 942 bp in length and contains the four overlapping open reading frames of the *gag, pro, pol,* and *env* genes as well as an additional open reading frame (*orf-x*) that overlaps *pol* (Fig.3). This is the first nucleotide sequence of enJSRVs reported in China. The G+C content of the enJSRV genome averages 41.68%, which is very similar to the 41.78% G+C content of enJS56A1.

LTRs

The LTRs comprises 435bp of sequence at each end of the enJSRV-NM loci. The enJSRV-NM LTRs are subdivided into three adjacent sequences blocks consisting of a 319 bp U3 region, a 15bp R region and a 101 bp U5 region. The upstream and downstream

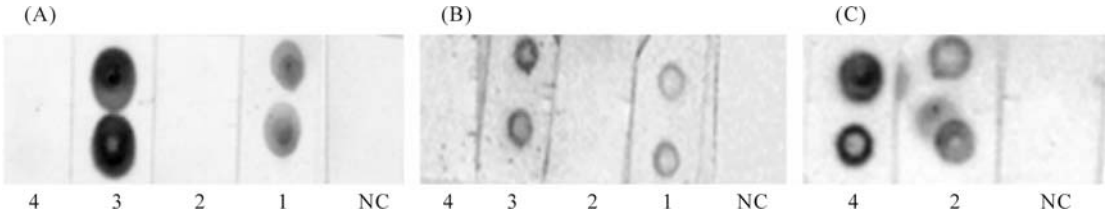

Fig.2. Autoradiographs after hybridization of DNA probes with purified viral DNA. A: DNA of uterus tissue from healthy domestic sheep. B: DNA of lung tissue from healthy domestic sheep. C: DNA of lung tissue from OPA-affected sheep. NC, negative control. Sections of lungs from sheep with OPA were used as positive controls. The probe name is indicated at the bottom of each column.

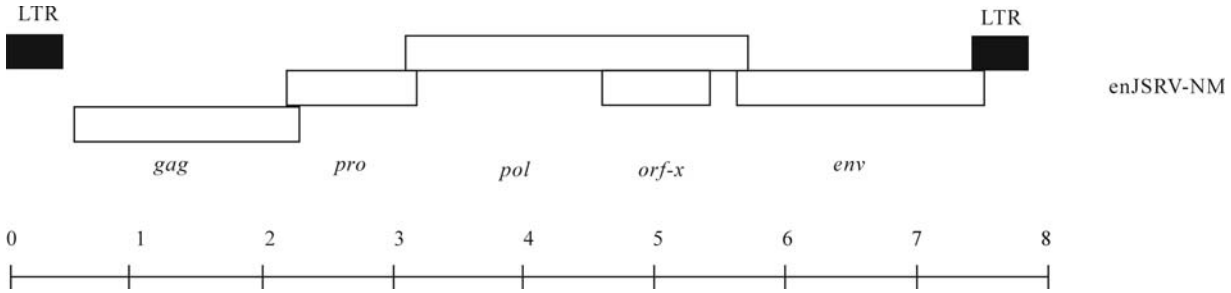

Fig.3. Schematic representation of the genome of enJSRV-NM. LTR, long terminal repeat. The numbered bar at the bottom indicates distances in kilobases.

LTRs of enJSRV-NM and enJS5F16 were identical, while those of enJS56A1 and enJS59A1 displayed two- and four-base changes respectively. Thus, intra-genomic variability of the LTRs can be used as a molecular clock to estimate time of integration (16). By this analysis, the integration events of enJS56A1 and enJS59A1 happened ca. 0.9 to 1.8 million years ago, and the enJSRV-NM and enJS5F16 might have integrated less than 500,000 years ago, based on sequence identity of the upstream and downstream LTRs. These numbers are subject to a wide margin of error (16). and do not take into account the possibility of gene conversion (9). The entire LTR sequence were 85-98% identical among enJSRV-NM and other enJSRVs and JSRVs, particularly in the R and U5 regions. However, there was only 49.3% sequence identity between enJSRV-NM loci and JSRVs in the U3 region because of deletions and point mutations in JSRVs. This region of divergence in U3 has been widely exploited in distinguishing JSRV-infected cells and tissues from sheep enJSRV sequence by PCR and by southern hybridization. Another region of divergence in the TM region of env is discussed below.

gag

The enJSRV-NM *gag* (nt 583 to 2433) encodes a 617-amino-acid (aa) polypeptide. There are two sequences of Cys-X2-Cys-X4-His-X4-Cys, separated by 13 residues, in the nucleic acid gag protein. This is typical of a zinc finger domain and is thought to mediate nucleocapsid binding to the genomic RNA. The presence of the Sca Ⅰ site at position 1724～1729 in *gag* of $JSRV_{21}$ which is a molecular marker for JSRVs, was not found in *gag* of enJSRV loci (10). Two short regions (VR1 and VR2) were analyzed in enJSRV-NM in the *gag* and found to contain major differences between endogenous JSRVs and exogenous JSRVs but are absent in the corresponding regions of the enJSRV proviruses.

pro

The *pro* open reading frame (nt 2325 to 3194) encodes a 326-residue polypeptide. The protein encoded by *pro* is thought to comprise two domains, a pseudo protease (or protease-like) domain which exhibits a dUTPase activity, as has been suggested for other retroviruses by both computer analysis and functional studies (19), and an active protease (Pr). Alignment of deduced amino acid sequences showed high homology between enJSRV-NM and known endogenous and exogenous JSRVs.

pol

The *pol* open reading frame (nt 3 440 to 5 785) encodes a 781-residue polypeptide. The *pol* gene of enJSRV-NM and known endogenous and exogenous JSRVs had greater than 97% sequence identity. However, there are only 95% deduced amino acid sequences identity between the enJSRV-NM and enJS56A1 in the *pol* region and because In enJS56A1 there was a 2 bp deletion at the 3′ end of the *pol* gene, the last 33 aa have no similarity due to the frame shift (16).

orf-x

The second ORF in *pol* region was identical to the *orf-x* of unknown function previously described for the JSRV from South Africa (25). The orf-x extended from nt 4 938 to 5 354 and encoded 166 amino acids. The enJSRV-NM loci was different from the JSRV-SA strain in only two amino acids, but was different from $JSRV_{21}$ in 13 amino acids indicating that the JSRV strain from South Africa was more closely related to ESRV within the *orf-x* region, which is in agreement with results of an earlier study (1).

env

The *env* open reading frame (nt 5 682 to 7 517) encodes a peptide of 611a.a. giving a predicted molecular mass of 69 kDa. As shown in Table 3, the *env* of enJSRV-NM loci and other enJSRVs are very similar, with nucleotide and amino acid identities greater than 98%. Alignment of the *env* gene of enJSRV-NM loci with JSRVs (type I and type Ⅱ) shows that they share 88%-93% amino acid identity. The TM region of *env* is the most divergent of the coding regions of enJSRV-NM loci and JSRVs. They are 96% identical in SU and 85% identical in TM at the amino acid level. The TM region is also the most divergent of the coding regions of other enJSRVs and JSRVs and for this reason was chosen for selection of dot hybridization probes. The enJSRV-NM *env* lacked the YXXM motif present in the VR3 region of the TM domain of the exogenous JSRV *env* that has been found to be critical for transformation (18).

Phylogenetic analysis and evolution

We generated rooted neighbor-joining phylogenetic trees to assess the phylogenetic relationships between the enJSRV-NM cloned in this study and with other known sequences of endogenous and exogenous type D retroviruses of sheep. We generated a tree for the *U3* region, one for *env* and one for *gag* (Fig.4). In each tree it was possible to distinguish two major branches: one for the endogenous loci, and one for the exogenous JSRV sequence, confirming previous analyses with limited *gag* sequences. The exogenous JSRVs could be further divided into two branches corresponding to sequences derived from Africa (type Ⅰ) or from the United States and the United Kingdom (type Ⅱ) as previously described. The endogenous loci also could be further divided into at least two phylogenetic groups, enJSRV-A and -B. These phylogenetic trees show that the enJSRV-NM loci is most closely related to enJSRVs, especially enJS56A1 (enJSRV-B) and enJS5F16 (enJSRV-B). So the enJSRV-NM loci belong to type-B.

DISCUSSION

We have sequenced and cloned the enJSRV genome from Inner Mongolia. Sequence analysis identified

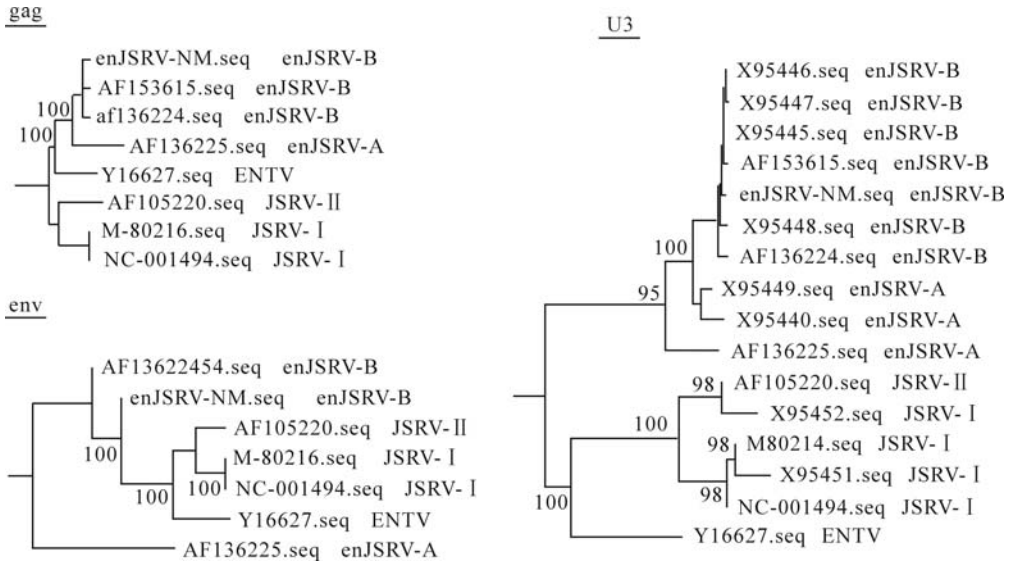

Fig.4. Phylogenetic analysis of sheep type D retroviruses of sheep. Rooted phylogenetic trees for *gag*, *env*, and *U3* were derived by the neighbor joining method. All the reference sequences in this study are from the GenBank database.

Table 3. Comparison of enJSRV-NM and other known enJSRV /JSRV genes and proteins

Isolate	Identical residues between enJSRV-NM and isolate (%)									
	U3		*gag*		*pro*		*pol*		*env*	
	NA	AA	NA	AA	NA	AA	NA	AA	NA	AA
enJS56A1	99.5	—	98.9	98.4	99.7	98.6	99.3	95	99.2	98.9
enJS5F16	98.1	—	99	98.9	99.2	99.0	99.1	98	98.7	98.5
$JSRV_{21}$	49.3	—	89.6	94.8	97.4	98.6	97	98.6	88.5	92.6
JSRV-SA	50.4	—	91.4	94.0	95.1	97.6	98.8	98.8	88.9	92.2

NA: nucleic acids; AA: amino acids.

three highly variable regions between JSRV and enJSRV in the gag region, the transmembrane domain of env (TM) and the U3 region of LTR, from which JSRV-specific DNA probes and enJSRV-specific DNA probes were derived. By using these DNA probes in dot hybridization, we have now identified these probes for distinguishing between healthy and infected specimens. The four probes were highly specific for differentiation between enJSRVs and JSRVs in the specimens tested (Fig.2). For the first time we successfully identified enJSRV proviral sequences from uterus and lung genomic DNA in P.R.China, validating the use of these specific DNA probes in the identification of endogenous and integrated exogenous proviral sequences. The dot hybridization assay described here could be employed as a primary means for the detection of enJSRVs and JSRVs, and it should also be useful in confirming the presence of JSRVs in specimens identified by other techniques.

In this study we molecularly cloned one enJSRV-NM and investigated its proviral structure, phylogeny. It was found that the non-pathogenic enJSRV-NM are 90%-98% identical at the amino acid level to their exogenous infectious counterparts in most of the retroviral genome and that, in addition to the previously reported differences in the *U3* region, enJSRVs consistently differ from JSRV isolates in three variable regions (VRs). VR1 and VR2 are found in *gag*, whereas VR3 is at the the transmembrane domain of *env,* which is in agreement with results of an earlier study by Palmarini *et al* (16).

In previous studies of exogenous and endogenous JSRV proviruses Palmarini *et al*. observed a difference between wild-type JSRV and one of the endogenous clones (enJS56A1) in particle production. In transiently transfected cells, enJS56A1 is unable to release viral particles, even when expressed under control of the CMV immediate early promoter (pCMV 2enJS56A1) (16). The use of JSRV/enJS56A1 chimeras determined that the main defect for particle formation resided in the first two-thirds of *gag* (19). Two short regions (VR1 and VR2) were identified in the enJS56A1 *gag* gene that contained major differences between ovine endogenous and exogenous betaretroviruses. Since these two variable regions harbor the major sequence differences between endogenous and exogenous JSRV in this part of *gag*, it was postulated that either or both of them are responsible for the observed particle formation/release defect. However, Hallwirth *et al*. found that the endogenous JSRV particle formation/release defect is not exclusively determined by either VR1 or VR2. They found that either Cys98 or Val102 (or both in combination) are involved in the particle release defect of enJS56A1, however all other enJSRV *gag*

sequences known to us resemble the exogenous isolates in these positions, i.e. they have Arg98 and Leul02 (6). If the Cys98 and/or Val102 are involved in the enJS56A1 particle release defect, this would imply that the defect is not a general one for enJSRVs. After this work was submitted, Mura *et al*. reported that combined mutation of R98C and L102V led to loss of particle production for wild-type JSRV, and that the other endogenous *gag* proteins do not show the particle production defect of enJS56A1 (23). Whether the enJSRV-NM loci at position 98 (Arg) and 102 (Leu) can release virus particle, requires further study.

A third region that is divergent between exogenous and endogenous sequences was located in the transmembrane protein (VR3) and was previously reported (16). All the enJSRVs *env* genes lacked the YXXM motif that is present in the VR3 region of the JSRV *env* gene and that is critical for transformation in vitro of rodent fibroblasts (13). Transformation of cells by the *env* gene of JSRV can be Hyal2 receptor independent and involve the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway (18).

The enJSRV model is perfectly suited to address many biological questions on ERVs and retroviral biology with practical applications of great relevance. The study of enJSRV also has implications for the design of strategies to control JSRV infection. OPA is one of the major infectious diseases of sheep. Given the extensive homology between JSRV and enJSRV it is difficult to hypothesize a vaccine that can elicit a strong immune response in the sheep, as most viral epitopes would be recognized as self-antigens. Moreover, enJSRV proteins are highly expressed in the sheep genital tract, and consequently even if a hypothetical effective JSRV vaccine was to be found this could have adverse effects on normal host cells (19).

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