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Unique Long Terminal Repeat U3 Sequences Distinguish Exogenous Jaagsiekte Sheep Retroviruses Associated with Ovine Pulmonary Carcinoma from Endogenous Loci in the Sheep Genome

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Ovine pulmonary carcinoma (OPC) is a contagious lung cancer of sheep that is presumed to be caused by an exogenous retrovirus of sheep, jaagsiekte sheep retrovirus (JSRV). The sheep genome carries 15 to 20 copies of endogenous sheep retrovirus (ESRV) loci that hybridize to JSRV DNA probes. In order to clarify the etiologic roles of ESRV and an exogenous JSRV-like retrovirus (*ex*JSRV) in OPC, we assessed sequence differences between ESRV and JSRV. Molecular characterization of six ESRV loci revealed restriction sites specific for JSRV. Nucleotide sequences of ESRVs from sheep of different breeds were similar to those of JSRV in structural genes but divergent in U3. Therefore, primers specific for the U3 sequences of *ex*JSRV were designed for use in the PCR. Of 13 tumor DNAs tested by PCR with these exogenous-virus U3 primers, 8 produced DNA fragments that hybridized with the JSRV *gag* probe, but neither lung DNAs from healthy sheep nor DNAs from nontumor tissues of diseased sheep produced similar DNA fragments. *ex*JSRV PCR products from tumor DNAs of sheep with OPC from three continents had restriction profiles similar to each other but different from those of ESRVs upon digestion with *Eco*RI, *Hin*dIII, *Nde*I, *Kpn*I, and *Sca*I. These *ex*JSRVs could be classified into two genotypes according to U3 sequences and restriction profiles. U3 sequences of *ex*JSRV proviruses in tumors strongly resembled those of JSRV but differed from those of ESRVs, suggesting that *ex*JSRVs, rather than ESRVs, are primarily associated with oncogenesis in OPC.

Ovine pulmonary carcinoma (OPC) (also called sheep pulmonary adenomatosis and jaagsiekte) is a contagious lung cancer (7, 8, 25, 36, 40, 41) that is presumed to be caused by jaagsiekte sheep retrovirus (JSRV), an exogenous retrovirus isolated from the lung fluid of a diseased sheep (44, 45). The disease has been classified pathologically as a low-grade bronchioloalveolar carcinoma with remarkable histologic similarity to human bronchioloalveolar carcinoma, a pulmonary neoplasm that is associated with a family history of lung diseases after adjusting for smoking, suggesting a role for infectious or genetic factors in the etiology of the human disease (43). As a retrovirus-associated lung cancer of epithelial cell origin, OPC has been suggested as a model for the study of the oncogenesis of pulmonary carcinomas (27).

There are three major problems that hamper progress in OPC research: (i) a permissive cell culture system for JSRV has not been identified, (ii) serum antibodies to JSRV proteins have not been definitively detected in affected sheep, and (iii) the genomic DNA of all sheep tested has 15 to 20 bands that hybridize to JSRV DNA probes under high-stringency conditions (16, 17, 45). We have designated the hybridizing DNA sequences as endogenous sheep retroviruses (ESRVs). Although an exogenous JSRV-like retrovirus (*ex*JSRV) has been consistently isolated from lung fluids of lambs with OPC (8, 17, 20, 25, 40, 45), the etiologic significance, if any, of *ex*JSRV, as well as that of ESRV, is unknown. To clarify this issue, we sought to characterize ESRV proviral sequences and to determine whether they differed in nucleotide sequences from JSRV.

Since ESRV sequences hybridize to JSRV DNA probes (17, 45), we assumed that DNA primers derived from JSRV sequences would amplify ESRV in PCR. Consequently, the molecular characterization of ESRV PCR products would provide information about sequence relationships between ESRV and *ex*JSRV proviruses present in the same tumor cell. The present study has revealed that ESRVs are significantly different from *ex*JSRVs in restriction enzyme profiles. Unique U3 sequences are the hallmark for all *ex*JSRV proviruses that are detectable in OPC lung tumor DNA.

MATERIALS AND METHODS

Genomic DNA. Genomic DNA from lung tumors and nontumor tissues was prepared as described previously (33). DNA sources are described in Table 1.

Oligonucleotide primers. Primers (5′ to 3′), used in PCR for amplifying endogenous proviral sequences, were designed on the basis of published JSRV sequences (see Fig. 1a) (17, 45) and are as follows: JB1 (nucleotides [nt] 16 to 48, in U3), CGTGAAGGGTTAAGTCCTGGGAGCTCTTTGGCA; JB2 (nt 355 to 386, reverse in U5), AGCACAAACAAGAGTCGCACCTGCACAGGGAG; JB3 (nt 1674 to 1706, located in the first *Sca*I site in *gag*), ACAGGCATGGA AAAAACTTCCTAGCTCCAGTAC; and JB4 (nt 1998 to 2029, reverse, located in the second *Sca*I site in *gag*), TCTTGTTCCGGGCTTGCTGCTGGAAAAGT ACT. JB1 and JB4 covered 2 kb of the JSRV genome from the 5′ long terminal repeat (LTR) to the second *Sca*I site in *gag*; JB2 and JB3 covered 6 kb of the viral genome from the second *Sca*I site in *gag* to the 3′ end of the 3′ LTR (see Fig. 1a). The putative 2- and 6-kb fragments overlapped each other in a 296-bp region between the two *Sca*I sites that facilitated the reconstruction of the full-length endogenous proviral genome. JB5 (nt 406 to 437), CAACGTGGGGCTCGAG CTCGACAGTTTTCTTC, was located in the tRNA primer binding site. JB5 and JB2 covered the entire proviral genome except for the 5′ LTR. JB16 (nt 2077 to 2106, reverse), TTGAGGGCATACTGCAGCTCGATGGCCAGG, was located 47 bp downstream from JB4. JB20 (nt 5458 to 5490), AACTTTAGACACAG AAGGCAATTCAGCAGCCCA, was located in the intergenic region between *pol* and *env*. The seven primers described above were common specific primers that had an annealing ability similar to that of both exogenous and endogenous JSRV-like DNA sequences. In order to synthesize *ex*JSRV proviral DNA by

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TABLE 1. DNA samples and sources

DNA sample source and sheep designation	Sheep breed	Geographic source
Lung tumors of naturally occurring OPC		
83RS28	Warhill	Wyoming (1983)
84RS28	Warhill	Wyoming (1984)
90RP2	Junin	Peru (1990)
90RP5	Junin	Peru (1990)
92K3	Merino	Kenya (1992)
92K8	Merino	Kenya (1992)
92K9	Merino	Kenya (1992)
JS7 (lung tumor cell line)	Scottish Blackface	Scotland (1985)
Lambs with experimentally induced OPC (tumor; nontumor DNA)		
86RS44;[a] 86RS44L (pulmonary lymph node)	Mixed breed	Colorado (1986)
91RS13[b]; 91RS13S (spleen)	Mixed breed	Colorado (1991)
805; 805K (kidney)	Scottish Blackface	Scotland (1994)
968; 968K (kidney)	Scottish Blackface	Scotland (1994)
970; 970K (kidney)	Scottish Blackface	Scotland (1994)
Lungs or blood of clinically healthy sheep		
973 (lung)	Scottish Blackface	Scotland (1994)
93RS54 (blood)	Rambouillet	Texas (1993)
1101 (blood)	Suffolk	Texas (1993)

[a] The animal was infected with a viral stock prepared from the lung wash of a sheep with naturally occurring OPC, 83RS28.
[b] The animal was infected with a viral stock prepared from a sheep with naturally occurring OPC, 84RS28.

PCR from lung tumor, the common specific primers (JB4, JB16, and JB20) were combined, respectively, with various exogenous-virus U3-specific primers (JB8, JB9, JB10, JB11, JB15, and JB21) described in Results.

Thermostable DNA polymerases. *Klentaq1* was purchased with 10× buffer (PC2) from Ab Peptide (St. Louis, Mo.). Native *Pfu* DNA polymerase was purchased from Stratagene Corporation (La Jolla, Calif.). An Expand Long Template PCR System (ET System) was purchased with three 10× buffers (buffers 1, 2, and 3) from Boehringer Mannheim Corporation (Indianapolis, Ind.).

PCR procedure. The procedure for long-range PCR amplification of endogenous sequences was based on a previous report (1). The cumulative error rate of this PCR system is 0.14% in terms of point mutations (32). Reaction mixtures were prepared in 100 μl of 1× PC2 buffer (pH 9.2) under mineral oil in the presence of 250 μM each deoxynucleoside triphosphate (dNTP), 20 pmol of each primer, 25 U of *Klentaq1*, and 0.01 U of native *Pfu* plus 200 to 500 ng of sheep genomic DNA. Optimal cycle conditions were carried out in a DNA thermal cycler (Perkin-Elmer Cetus), programmed for 25 cycles of 20 s at 98°C, 30 s at 65°C, and 3 to 11 min at 68°C, depending upon the target length. When the ET System was used, the following conditions were applied. Reaction mixtures were prepared in 50 μl of 1× buffer in the presence of 350 μM each dNTP, 30 pmol of each primer, and 0.75 U of enzyme mixture plus 200 to 500 ng of genomic DNA. Tubes were heated for 2 min at 92°C before cycling and then programmed to 10 s at 92°C, 30 s at 61 to 65°C (depending on the primers used), and 3 min at 68°C for the first 10 cycles. Extension was performed at 68°C for 5 min for the next 10 cycles, followed by a 7-min extension for the final 15 cycles.

Molecular cloning of PCR products. DNA fragments, synthesized from genomic DNA by PCR with primers treated by T4 DNA polynucleotide kinase, were gel purified and ligated into the *Sma*I site of pBluescript II KS(−). Recombinants were identified as white colonies on ampicillin agar plates in the presence of X-Gal–IPTG (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside–isopropyl-β-D-thiogalactopyranoside) by using the XL-1 Blue host strain.

Southern blotting of PCR products and DNA clones. DNA of PCR products or recombinant plasmids was blotted as described by Southern (39) and hybridized with [α-^{32}P]dCTP-labeled JSRV DNA or [γ-^{32}P]ATP-labeled oligonucleotide primers by using a protocol similar to that described by Dunwiddie and Faras (11), prior to autoradiography. The preparation of the DNA probes is described in the relevant figure legends.

Sequence determination. Dideoxy-chain termination DNA sequencing was performed (34) with a Sequenase II kit (United States Biochemical Co., Cleveland, Ohio) by using M13 universal and specific primers. Extension products labeled with α-^{35}S-dATP were analyzed by polyacrylamide gel electrophoresis and autoradiography.

Computer analysis of sequence data. Sequence data were read with a GrafBar sonic digitizer (International Biotechnologies, Inc.) and assembled by using the program AssemblyLIGN on a Macintosh computer. Database searches were performed with FASTA, and sequences were analyzed with programs of the GCG package (Sequence Analysis Software Package version 7.2; Genetics Computer Group, Madison, Wis.) (10), DNA strider version 1.2 (24), CLUSTAL (18), and PAUP (Illinois Natural History Survey, 1988).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the GenBank and EMBL nucleotide sequence databases under the following accession numbers: X95445, X95446, X95447, X95448, X95449, X95450, X95451, and X95452.

RESULTS

Amplification of ESRV by PCR. In order to assess sequence differences between ESRV loci and their exogenous counterpart, JSRV (45), a long-range PCR was conducted to synthesize full-length ESRV proviral DNA by using primers (Fig. 1a) derived from the JSRV sequence. DNA isolated from lung tumors of sheep 86RS44 and 91RS13 (Table 1) was used as the

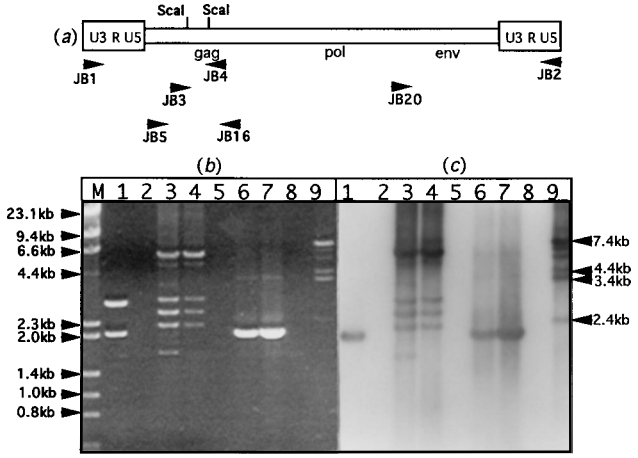

FIG. 1. Amplification of ESRV sequences. (a) The relative locations of oligonucleotide primers in the JSRV genome (45). (b) PCR was performed by using primers JB1 and JB4 (lanes 5, 6, and 7), JB3 and JB2 (lanes 2, 3, and 4), and JB5 and JB2 (lanes 8 and 9). PCR products were analyzed on a 0.7% agarose gel stained with ethidium bromide. (c) DNA was transferred from the agarose gel to a nitrocellulose membrane and probed with [α-^{32}P]dCTP-labeled JSRV DNA prepared as follows. pJSRV382 plasmid containing JSRV 3′ *gag* and *pro* sequences (45) was digested with *Eco*RI to release a 2-kb DNA insert. The digest was separated on an agarose gel. The 2-kb DNA segment was gel purified and labeled with [α-^{32}P]dCTP by nick translation. Lane 1, *Eco*RI-digested pJSRV382 (45) was used as a positive control; note the 2-kb hybridizing band. Lanes 2, 5, and 8, PCR products from no-DNA controls. Lanes 3, 6, and 9, sheep 86RS44 DNA templates. Lanes 4 and 7, sheep 91RS13 DNA templates.

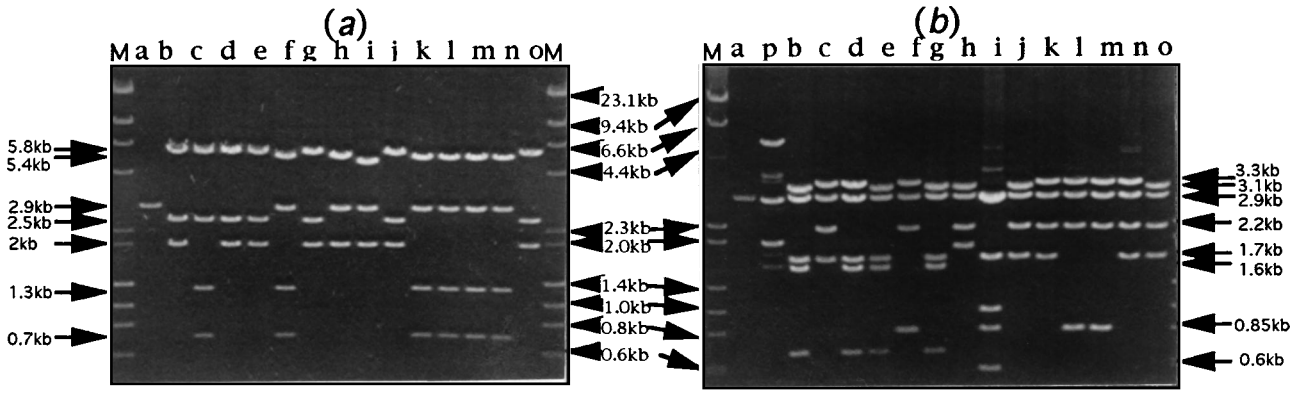

FIG. 2. Characterization of endogenous DNA clones from 7.4-kb inserts. DNA inserts were cloned into the *Sma*I site of pBluescript II KS(−) as described in Materials and Methods. *Eco*RI-digested (a) and *Pst*I-*Xba*I-digested (b) plasmid DNA was separated on a 0.7% agarose gel stained with ethidium bromide. The order of DNA samples in panels a and b is the same. Lane a, *Eco*RI-linearized pBluescript II KS(−). Lanes b to o, DNA clones pKE7.4k-27, -26, -25, -24, -23, -22, -21, -19, -18, -17, -13, -5, -2, and -1. Lane p (panel b), an irrelevant DNA sample. DNA clones in lanes f, h, i, and k to n contained DNA inserts in a sense orientation to the *lacZ* gene in the vector, while the remaining clones contained DNA inserts in an antisense orientation to the *lacZ* gene. (a) *Eco*RI digestion gave rise to 2.9- or 5.8- and 2- and 2.5- or 5.4-kb fragments in order (5′ to 3′). The 5.8-kb (lanes b to e, g, j, and o) and 5.4-kb (lanes f, h, and k to n) fragments contain a 2.9-kb vector sequence. The 2-kb fragments in lanes b, d, e, g to j, and o correspond to the 1.3- and 0.7-kb fragments in lanes c, f, and k to n. (b) *Xba*I did not cut the DNA inserts. *Pst*I-*Xba*I double digestion released a 2.9-kb vector sequence and gave rise to 1.7-, 2.2-, and 3.3- or 3.1-kb fragments from the inserts in order (5′ to 3′). The 1.7-kb fragment in lanes b to e, g, i to k, n, and o correspond to the 0.85-kb fragments in lanes f, l, and m. The 0.85-kb bands show double intensity in the gel. The 2.2-kb fragments in lanes c, f, h, and j to o correspond to the 1.6- and 0.6-kb fragments in lanes b, d, e, and g. The sizes of DNA segments in the gels are labeled on both sides of the figure.

substrate. Oligonucleotide primers JB1 and JB4 synthesized a 2-kb DNA fragment (Fig. 1b, lanes 6 and 7) from each DNA, while JB3 and JB2 yielded five different-sized DNA fragments (6, 3, 2.5, 2.1, and 1.7 kb; Fig. 1b, lanes 3 and 4). The 6-kb fragment was the size expected for the two primers. JB5 and JB2 synthesized six DNA fragments (2.1 to 7.4 kb) (Fig. 1b, lane 9), a 7.4-kb DNA band being the size expected for these two primers. All DNA fragments in the PCR products hybridized to JSRV *gag* (Fig. 1c, lanes 3, 4, 6, 7, and 9) and LTR probes under stringent conditions (data not shown). Similar results were obtained by using the same primers with samples from sheep 86RS44L and 91RS13S and DNA samples from sheep of four other breeds (92K3, 973, 93RS54, and 1101; Kenyan Merino, Scottish Blackface, U.S. Rambouillet, and U.S. Suffolk, respectively [Table 1]) (data not shown). Subsequently, the 2-, 6-, and 7.4-kb DNA bands (Fig. 1b, lanes 6 and 7, 3 and 4, and 9, respectively) were gel purified from sheep 86RS44 and 91RS13 PCR products and analyzed on an agarose gel following digestion with *Hin*dIII (for the 2-kb band) and *Eco*RI (for the 6- and 7.4-kb bands). We found that the sum of the sizes of the fragments for each target DNA fragment following digestion was larger than the expected size (data not shown), implying that the DNA samples used as noted above consisted of heterogeneous DNA molecules that might be synthesized from different ESRV loci.

Characterization of ESRV DNA clones. DNA fragments (7.4, 4.4, and 3.4 kb; Fig. 1c, lane 9), purified from sheep 86RS44 PCR products amplified with primers JB5 and JB2, were ligated into the *Sma*I site in pBluescript II KS(−), and clones were screened by Southern hybridization using the same JSRV *gag* probe as that used for Fig. 1c. The 2- and 6-kb DNA fragments (Fig. 1b, lanes 6 and 3, respectively) synthesized by PCR from the same DNA substrate were also cloned this way, resulting in 24 positive clones for the 2-kb insert and 13 positive clones for the 6-kb insert. After digestion with *Eco*RI, 12 of 14 positive clones isolated from the 7.4-kb insert were segregated into two groups (groups I and II; Fig. 2a). Clones pKE7.4k-21 and -19 (lanes h and i, respectively) were not used for detailed analysis because of the presence of rearranged DNA inserts. Compared with the clones in group II (pKE7.4k-27, -24, -22, -18, and -1; Fig. 2a, lanes b, e, g, j, and o), those in group I (pKE7.4k-26, -23, -17, -13, -5, and -2; Fig. 2a, lanes c, f, and k through n) had a novel *Eco*RI site in a 2-kb fragment. The 2-kb bands in lanes b, d, e, g, j, and o (Fig. 2a) converted into 1.3- and 0.7-kb DNA bands in lanes c, f, and k through n (Fig. 2a). Digestion with *Pst*I permitted further separation into two subgroups within each group (Fig. 2b). They were arbitrarily referred to as ESRV subgroups 1 to 4. Subgroup 1 (pKE7.4k-26, -17, and -2; Fig. 2b, lanes c, k, and n) could be differentiated from subgroup 2 (pKE7.4k-23, -13, and -5; Fig. 2b, lanes f, l, and m) by a *Pst*I site in the middle of the 1.7-kb fragment (see the 0.85-kb segment with double intensity in Fig. 2b, lanes f, l, and m). Subgroup 3 (pKE7.4k-18 and -1; Fig. 2b, lanes j and o) could be distinguished from subgroup 4 (pKE7.4k-27, -24, and -22; Fig. 2b, lanes b, e, and g) by a *Pst*I site in the 2.2-kb fragment (see the 1.6- and 0.6-kb segments in Fig. 1b, lanes b, e, and g). Group I clones had a 3.3-kb *Pst*I fragment (located at the 3′ ESRV proviral genome) that was larger than the corresponding fragment (3.1 kb) in group II clones (Fig. 2b). Clone pKE7.4k-25 (Fig. 2, lanes d) belonged to group II, but its *Pst*I profile resembled those of subgroup 4 clones. However, the latter had a 3.1-kb, but not a 3.3-kb, *Pst*I segment. We concluded that pKE7.4k-25 did not belong to any of the four subgroups. It probably represented another population of ESRV sequences. The analysis of 13 positive clones derived from the 6-kb PCR products of sheep 86RS44 DNA (Fig. 1b, lane 3) in the same way also yielded similar results. Clones from the 3.4- and 4.4-kb fragments (Fig. 1b, lane 9) showed distinct deletions and unique restriction maps and LTR sequences, respectively (data not shown). These were referred to as ESRV groups III and IV.

Restriction sites specific for JSRV or ESRVs. All of the clones from ESRV subgroups 1 to 4 were analyzed by using restriction enzyme digestion and Southern hybridization with probes of various regions of the JSRV sequence (45) (data not shown). Restriction maps were constructed and aligned with those of JSRV (Fig. 3). The results were also confirmed by analyzing 24 clones from the 2-kb ESRV DNA insert and 13 clones from the 6-kb ESRV DNA insert mentioned above (data not shown). DNA clones of ESRV subgroups 1 to 4 were

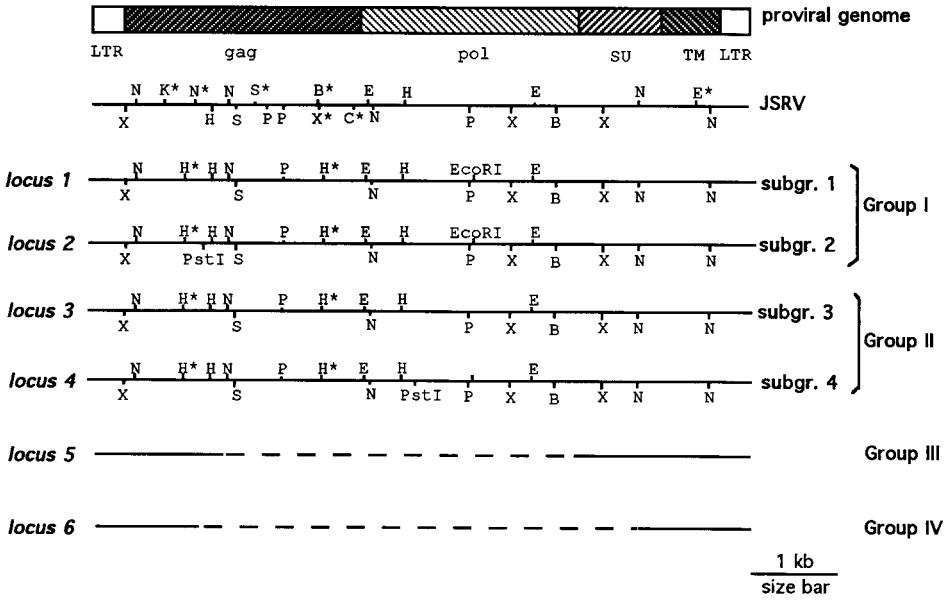

FIG. 3. Comparison of restriction profiles of ESRVs and JSRV. The JSRV restriction map was derived from an assembled full-length cDNA sequence published previously (45). Restriction profiles of endogenous sequences are aligned with those of JSRV. The sites present only in JSRV or endogenous sequences are highlighted with asterisks. Those sites that were used to distinguish the four subgroups (loci 1 to 4) are indicated by full enzyme names, *Eco*RI and *Pst*I. The restriction maps for ESRV loci 5 and 6 and the deletion boundaries in the proviral genomes have not been determined. Dashed lines indicate the gross locations of deletions. Capital letters represent restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I; P, *Pst*I; S, *Sca*I; X, *Xho*I. Restriction sites for *Sma*I, *Sac*I, and *Pvu*II are not shown. *Xba*I, *Sal*I, and *Cla*I did not cut the proviral genomes in loci 1 to 4.

probably derived from some intact proviral loci, while clones of ESRV groups III and IV had large deletions in *pol* and adjacent sequences (Fig. 3). DNA clones in the same subgroup had identical restriction profiles for 14 different restriction enzymes (Fig. 3) and identical DNA sequences in the regions sequenced (including the LTR, part of the *gag* region, and the entire *env* transmembrane domain [TM], described in sections below). Subgroups could be distinguished from one another on the basis of *Eco*RI and *Pst*I profiles (Fig. 3; also refer to Fig. 2), as well as DNA sequences. Thus, we designated the four subgroups ESRV loci 1 to 4 for the convenience of description (Fig. 3). Groups III and IV were assigned to ESRV loci 5 and 6. Although potentially full-length ESRV sequences were homologous to JSRV along the entire genome, they were not identical. Putative molecular markers that could be used to distinguish JSRV from its endogenous counterparts were identified. These included *Kpn*I, *Nde*I, *Hin*dIII, *Sca*I, *Bam*HI, and *Xho*I sites in *gag*, a *Cla*I site in the *gag-pol* junction, and an *Eco*RI site in the *env* TM (see asterisks in Fig. 3).

Sequence analysis of ESRV proviral DNAs. Nucleotide sequences of the entire LTR, the TM domain of *env*, the 5′ untranslated leader region, and part of *gag* were determined for 11 DNA clones from ESRV loci 1 to 4 and for 4 clones from ESRV loci 5 and 6 (Fig. 4). The results were confirmed by sequencing 4 clones from the 2-kb insert and 13 clones from the 6-kb insert (data not shown). Generally, ESRV proviruses had more than 97% nucleotide identity with each other in the regions sequenced and 95% identity to JSRV in the 5′ untranslated leader region and the *gag* region. Full-length ESRVs could be distinguished from each other according to sequence variations in *gag*, *env* TM (data not shown), and the LTR (Fig. 4d) as well as by restriction enzyme polymorphism in the proviral genome (Fig. 3). Sequence analysis of ESRV group III and IV DNA clones indicated that the 3.4- and 4.4-kb fragments did not result from internal primer (JB5 and JB3) misannealing on the full-length ESRV proviral genome. Taken together, these results support the isolation of six ESRV proviral sequences (ESRV loci 1 to 6; Fig. 3).

An ATG start codon followed by a *gag* open reading frame that was identical to that in JSRV (45) was found in 15 sequenced clones of ESRV loci 1 to 6 (data not shown). Nucleotide sequences in the sequenced region were 98% identical among ESRV loci 1 to 6. The amino acid sequence deduced from a consensus sequence of 15 clones in the 5′ 220-bp region was 95% identical to that of JSRV (Fig. 4b). All 11 clones from the four potentially full-length ESRVs contained an open reading frame similar to that of JSRV in the *env* TM domain (713 bp, nt 6756 to 7469). Nucleotide sequences of the *env* TM domains of ESRV loci 1 to 4 were about 98% identical with each other and 96% identical to JSRV in the 5′ 500-bp region but only 62% identical in a 130-bp region (Fig. 4c) at the 3′ end of *env* with a 12-bp deletion. The deduced amino acid sequences from the 130-bp region of 11 clones of ESRV loci 1 to 4 were only 30% identical to that of JSRV (Fig. 4c).

Unique U3 sequences in JSRV LTR. Endogenous LTR sequences (Fig. 4d) were about 94 to 98% identical among ESRVs and among sheep of different breeds (including Rambouillet, Suffolk, Merino, and Scottish Blackface) and were closely related to those of JSRV (45), particularly in R and U5. However, they had significant divergence in U3 except for a 40-bp region located at the extreme 5′ end of ESRV proviral genomes (Fig. 4d). The R region of ESRV loci was nearly identical to that of JSRV. JSRV U5 showed 90% sequence identity with ESRV loci (Fig. 4d). Like JSRV, ESRV loci 1 to 6 also had a TATA box and a polyadenylation signal believed to be necessary for viral gene expression [poly(A); Fig. 4d].

The most remarkable difference between the two types of sequences was that the endogenous U3 region was 47 bp longer than that of JSRV (45). The latter had an average of 78% sequence identity with ESRV loci 1 to 6 in the entire U3. However, the 3′ 173-bp region of JSRV (nt 98 to 271) showed only 56% sequence identity with ESRV counterparts because

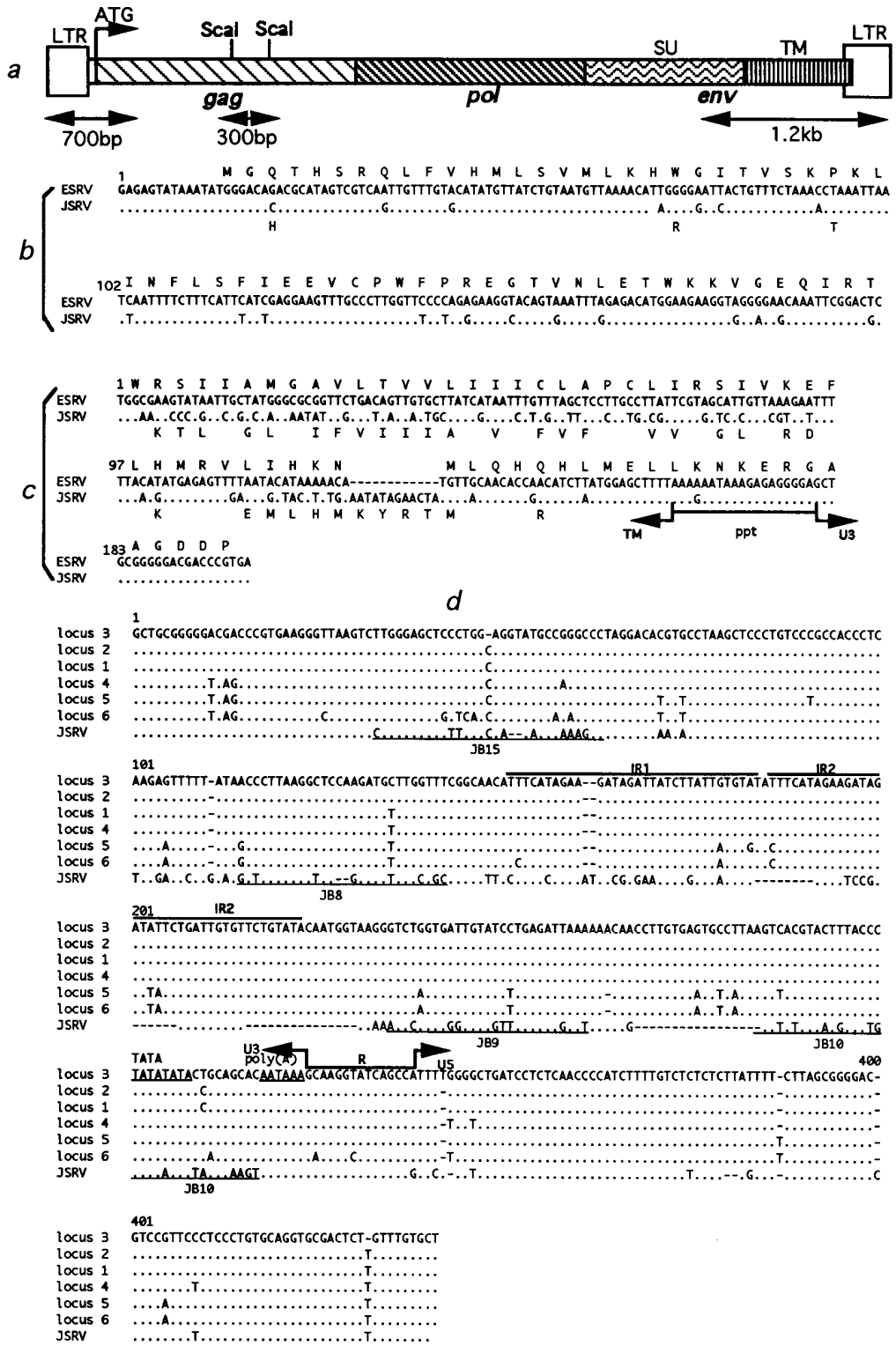

FIG. 4. Comparison of sequences of ESRVs and JSRV. Amino acid and nucleotide sequences of JSRV (b, c, and d) were published previously (45). Dots refer to identical sequences (b, c, and d) and dashes refer to deletions (c and d). (a) Schematic representation of proviral genome. Double arrowheads indicate the regions that have been sequenced. (b) Comparison of JSRV and endogenous *gag* sequences. A consensus sequence from 15 clones of ESRV loci 1 to 6 was used for comparison. Shown is the ESRV 5′ endogenous *gag* sequence corresponding to nt 523 to 727 in the JSRV sequence (45). The deduced endogenous amino acid sequence is shown above the nucleotide sequence. Amino acid differences in the JSRV sequence (45) are shown below the nucleotide sequence. (c) Comparison of JSRV and ESRV *env* TM sequences. Shown is the ESRV 3′ endogenous TM sequence corresponding to nt 7259 to 7469 in the JSRV sequence. The entire TM regions of 11 clones of ESRV loci 1 to 4 were sequenced, but only the 3′ part of a consensus nucleotide sequence from the 11 clones is presented. The deduced endogenous amino acid sequence is shown above the nucleotide sequence. Amino acid differences in the JSRV sequence (45) are shown below the nucleotide sequence. ppt, polypurine tract. (d) Comparison of ESRV and JSRV LTR sequences. Loci 1 to 6 refer to six independent consensus LTR sequences derived from 15 DNA clones of ESRV loci 1 to 6 (Fig. 3) and 13 clones from the 6-kb PCR products amplified by using primers JB3 and JB2 (Fig. 1a, lane 3; also see the text). These endogenous sequences correspond to nt 1 to 398 in the JSRV sequence (45). Endogenous LTR sequences derived from 12 clones of the Suffolk (sheep 1101), Scottish Blackface (sheep 973), and Rambouillet (sheep 93RS54) breeds (see Table 1 for DNA source) are not presented because of space limitations. IR, imperfect direct repeats. The locations of exogenous-virus U3-specific primers (JB8, JB9, JB10, and JB15, in a sense orientation) are indicated by underlining in the JSRV sequence.

of deletions and many point mutations. Thus, ESRVs had sequence features that were not shared by JSRV (Fig. 4d). On the basis of this analysis, four putative exogenous-virus U3-specific oligonucleotide primers (JB8, JB9, JB10, and JB15) were designed (Fig. 4d) for use in PCR to synthesize *ex*JSRV proviral sequences from tumor DNA (described below).

***ex*JSRV proviral sequences were only present in lung tumor.** Given that OPC is uniquely associated with *ex*JSRVs but not with ESRVs, the putative exogenous-virus U3-specific DNA primers designed as described above would be expected to amplify DNA fragments from lung tumors but not from tissues of healthy, presumed uninfected sheep. Restriction profiles of the DNA fragments amplified by these exogenous-virus primers from lung tumors would be expected to differ from those of ESRVs presented in Fig. 3 but resemble those of JSRV unless viral sequences are significantly varied. To test this hypothesis, putative exogenous-virus U3-specific primers JB8, JB9, JB10, and JB15 (Fig. 4d) were each paired with each of the common specific downstream primers, JB4 and JB16, to perform PCR under various conditions by using *Klentaq1*. The *gag* region was chosen initially, as there were six potential restriction site differences between JSRV and full-length ESRVs (Fig. 3). Of 21 DNA samples (including 13 tumor and 8 nontumor tissues; Table 1), only samples from sheep 83RS28 and 84RS28 produced positive responses with JB8-JB4, JB9-JB4, JB8-JB16, and JB9-JB16 when PCR products were detected by Southern hybridization with the [α-^{32}P]dCTP-labeled JSRV *gag* probe (data not shown). These JSRV primers, derived from the South African JSRV sequence, apparently did not match well with their target sequences in lung tumor DNAs of sheep from other countries. To overcome this problem, an enzyme mixture containing a native thermostable DNA polymerase was employed. By using the same primers, the specificity and sensitivity of the *Klentaq1-Pfu* mixture and the ET System in the synthesis of *ex*JSRV proviral sequences from lung tumor DNA were tested. The ET System yielded better results (data not shown) in the presence of dimethyl sulfoxide and Tween 20 (ET System buffer 3). A 2-kb DNA fragment (spanning the 5′ LTR and *gag*) was amplified by using primers JB15 and JB16 at 62°C for annealing only from DNAs of lung tumors, including those from 4 of 5 experimentally infected lambs (Fig. 5a, lanes 6, 16, 17, and 19), 3 of 7 sheep with naturally occurring OPC (Fig. 5a, lanes 7, 8, and 13), and the JS7 cell line (Fig. 5a, lane 3). These products were not seen with DNA templates from the spleens (Fig. 5a, lane 2), lymph nodes (lane 9), or kidneys (lanes 10 and 11) of affected animals or from the lungs or blood of three healthy sheep (data not shown). However, a 1.3-kb DNA fragment was seen with DNAs from both tumor and nontumor tissues (Fig. 5a, lanes 2, 3, 6 to 11, 13, 15 to 17, and 19). The 2-kb, but not the 1.3-kb, DNA bands hybridized to the JSRV *gag* probe (Fig. 5b), suggesting that the latter were not retroviral sequences. Tumor DNA (from sheep 90RP5, 90RP2, 92K8, and 968; Fig. 5a and b, lanes 4, 5, 14, and 18, respectively) and nontumor DNA (from sheep 805K; Fig. 5a and b, lanes 12) did not produce any visible DNA bands with primers JB15 and JB16 and did not produce endogenous PCR products with common specific primers (JB1-JB4 or JB1-JB16) (data not shown), indicating that DNA in these samples was degraded.

However, the combination of JB8 with JB16 in PCR at 65°C for annealing gave rise to a 2-kb exogenous DNA fragment (Fig. 5c, lanes 5 to 9; note the 2-kb faint bands in lanes 8 and 9) that hybridized with the JSRV *gag* probe (Fig. 5d, lanes 5 to 9) from five representative lung tumor DNAs from sheep 92K3 (Kenya), 83RS28 and 86RS44 (Wyoming), and 805 and JS7

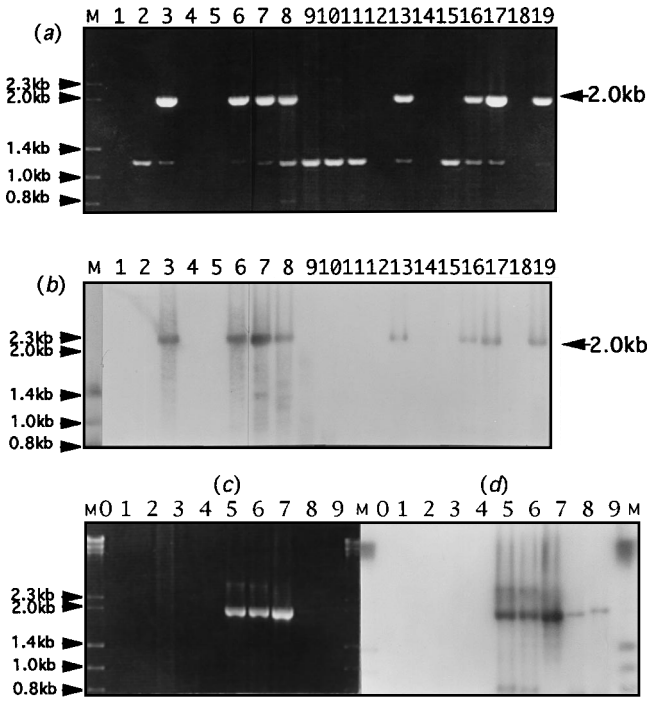

FIG. 5. Amplification of *ex*JSRV proviral sequences from lung tumor DNA. (a) PCR was conducted at an annealing temperature of 62°C with primers JB15 and JB16. PCR products were analyzed on an agarose gel stained with ethidium bromide. (b) This was followed by Southern hybridization with the JSRV *gag* probe described in the legend to Fig. 1c. Lane 1, PCR products from a reaction without a DNA template. Tumor DNA (cell line or sheep designation and lanes are given): JS7 (lane 3), 90RP5 (lane 4), 90RP2 (lane 5), 91RS13 (lane 6), 84RS28 (lane 7), 83RS28 (lane 8), 92K3 (lane 13), 92K8 (lane 14), 92K9 (lane 15), 86RS44 (lane 16), 970 (lane 17), 968 (lane 18), 805 (lane 19). Nontumor DNA: 91RS13S (lane 2), 86RS44L (lane 9), 970K (lane 10), 968K (lane 11), 805K (lane 12). (c) PCR was conducted at an annealing temperature of 65°C with primers JB8 and JB16. PCR products were analyzed on an agarose gel. (d) This was followed by Southern hybridization as described above. Lane 0, PCR products from a reaction without a DNA template. Nontumor DNA: 93RS54 (lane 1), 973 (lane 2), 86RS44L (lane 3), 805K (lane 4). Tumor DNA: JS7 (lane 5), 83RS28 (lane 6), 86RS44 (lane 7), 92K3 (lane 8), 805 (lane 9).

(Scotland) without obvious background. No visible bands could be seen with control DNAs (Fig. 5c and d, lanes 1 to 4).

Nontumor DNAs did not produce the 2-kb fragments with primers JB8-JB16 and JB15-JB16 but still produced 2- or 2.1-kb endogenous DNA fragments with common specific primers JB1-JB4 or JB1-JB16 (data not shown), indicating that the failure in the amplification of *ex*JSRV proviral sequences from nontumor DNAs with primers unique for exogenous-virus U3 was specific and not due to artifacts in PCR or poor-quality DNA templates.

Restriction polymorphism of *ex*JSRV proviral sequences from lung tumor. Figure 6 shows that *ex*JSRVs of various tumor sources were different from ESRVs in *Hin*dIII, *Nde*I, *Kpn*I, and *Sca*I restriction patterns. The four enzyme profiles for the 5′ 2-kb proviral genome of ESRVs from different genomic DNAs (Fig. 6) were consistent with the data presented in Fig. 3. *ex*JSRVs of the sheep from Wyoming and Scotland showed identical restriction patterns for the four enzymes but were distinct from those of South African JSRV and Kenyan sheep 92K3 upon digestion with *Hin*dIII (Fig. 6a), *Nde*I (Fig. 6b), and *Kpn*I (Fig. 6d). South African JSRV and the *ex*JSRV of Kenyan sheep 92K3 had a unique *Kpn*I site that did not exist in either ESRVs or *ex*JSRVs of the Wyoming and Scotland sheep (Fig. 6d). However, *ex*JSRVs of the South

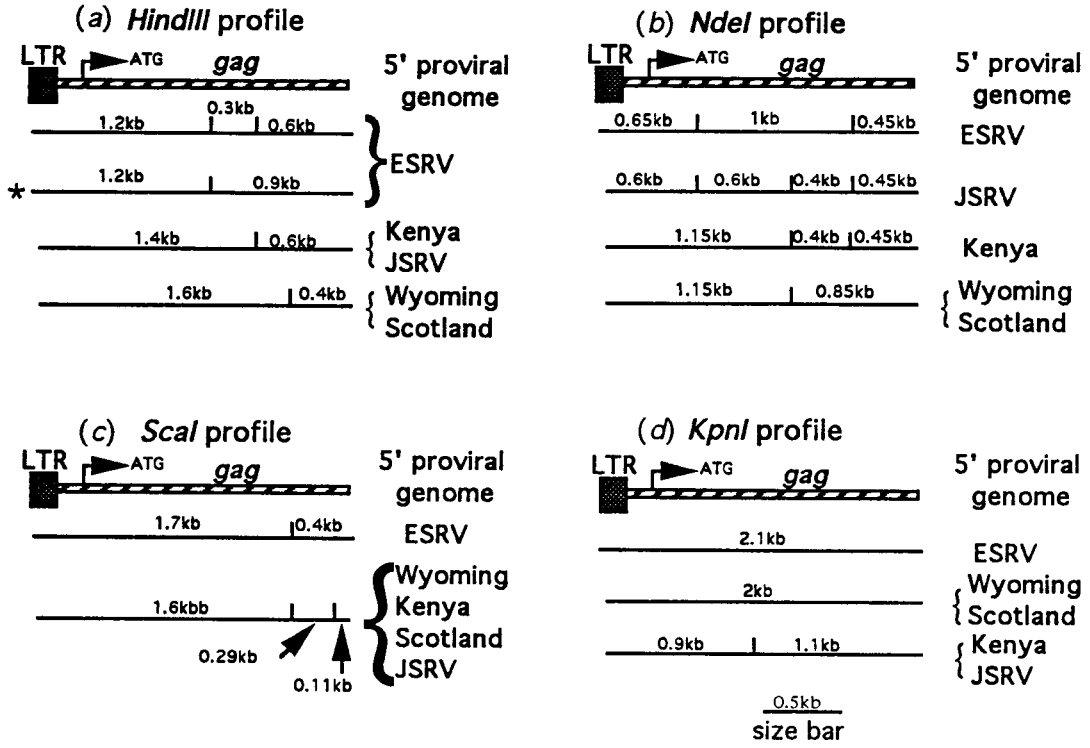

FIG. 6. Restriction polymorphism of *ex*JSRV proviral sequences based upon the analysis of DNA clones from PCR products. ESRV, endogenous sequences. Kenya, Kenyan OPC tumor DNA from sheep 92K3. Wyoming, four tumor DNAs from Wyoming virus strains from sheep 83RS28, 84RS28, 86RS44, and 91RS13. Scotland, three tumor DNAs from Scotland virus strains from cell line or sheep JS7, 805, and 970. JSRV, an OPC virus strain isolated from South Africa. The JSRV map was derived from a published full-length cDNA sequence (45) and used for the alignment of restriction sites. *ex*JSRV proviral sequences amplified with primers JB8 and JB16 were digested with *Hin*dIII (a), *Nde*I (b), *Sca*I (c), and *Kpn*I (d). Endogenous sequences were also amplified from the same DNA samples with common specific primers JB1 and JB16 and were analyzed in the same way. Exogenous proviral sequences that had identical restriction profiles are shown in the same group; otherwise they are shown separately. *, this population of ESRV sequences was identified when primers JB1 and JB4, but not JB5 and JB2, were used.

African, Kenyan, Wyoming, and Scotland sheep had two common *Sca*I sites, while ESRVs only had the first one (Fig. 6c).

U3 sequences of exogenous proviruses from OPC lung tumors were very similar to those of JSRV but divergent from those of ESRVs. U3 sequences of exogenous proviral genomes in tumor DNAs from sheep from Kenya, the United States, and Scotland were well conserved among one another and were also similar to those of JSRV but were significantly divergent from a consensus U3 sequence of ESRV loci 1 to 6 (Fig. 7a). Compared with these endogenous sequences, *ex*JSRV proviruses had eight deletions (for a total of 47 bases) and many point mutations in the U3 of the LTR. As a result, endogenous and exogenous proviruses had their own unique U3 sequence features, such as CArG and CCAAT-like motifs that were not shared by their counterparts.

The U3 from sheep 92K3 had over 99% sequence identity with that of JSRV but had only 88% identity with those of four other sequences (from sheep 83RS28, 84RS28, 970, and JS7). In contrast, the latter four U3 sequences were nearly identical (Fig. 7a). Upon phylogenetic analysis, JSRV and the virus from sheep 92K3 from Africa were segregated from the viral strains from Wyoming and Scotland (Fig. 7b). This was consistent with the restriction profiles shown in Fig. 6.

Characterization of exogenous-virus-specific DNA probes. On the basis of the novel sequence information, *ex*JSRV strain-specific U3 oligonucleotides (Fig. 7a) were labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase for use as probes. Exogenous and endogenous proviral sequences synthesized by PCR from lung tumor tissues were tested (Fig. 7c). The JB21 probe, derived from the U3 sequences of the Wyoming and Scotland viruses (Fig. 7a), hybridized to exogenous PCR products synthesized from tumor DNAs of the same DNA sources (Fig. 7c-c′, lanes 3 to 7 and 9 to 11) but did not hybridize with those from sheep 92K3 (Fig. 7c-c′, lane 8). In contrast, the JB10 and JB11 probes, derived from the U3 sequences of JSRV and from Kenyan 92K3, hybridized only to those from sheep 92K3 (Fig. 7c-b′, lane 8). In no circumstances did endogenous PCR products hybridize to these DNA probes (Fig. 7c-b′ and c′, lanes 1).

Additionally, primer JB21 was paired with common specific primer JB2 for use in PCR, whereupon a 6-kb exogenous proviral sequence (from mid-*gag* to the 3′ end of the LTR) was synthesized from Scotland JS7 and Wyoming sheep 83RS28 and 84RS28 but not from Kenyan sheep 92K3 or DNAs from nontumor tissues (data not shown). In contrast, primers JB11 and JB20 synthesized a 2.3-kb exogenous *env*-LTR region only from Kenyan sheep 92K3 but not from Wyoming tumor DNAs, Scotland JS7, or DNAs from nontumor tissues (data not shown). Like JSRV, the exogenous proviral sequences from Wyoming and Kenyan tumor DNAs had an *Eco*RI site in the *env* TM domain which was not present in the corresponding locations of the 6-kb exogenous JS7 sequences (data not shown) and ESRV loci (refer to Fig. 3).

DISCUSSION

JSRV is proposed to be the agent that causes OPC, but it is related to an endogenous sequence (45). In the present work, we have shown that JSRV could be distinguished from endog-

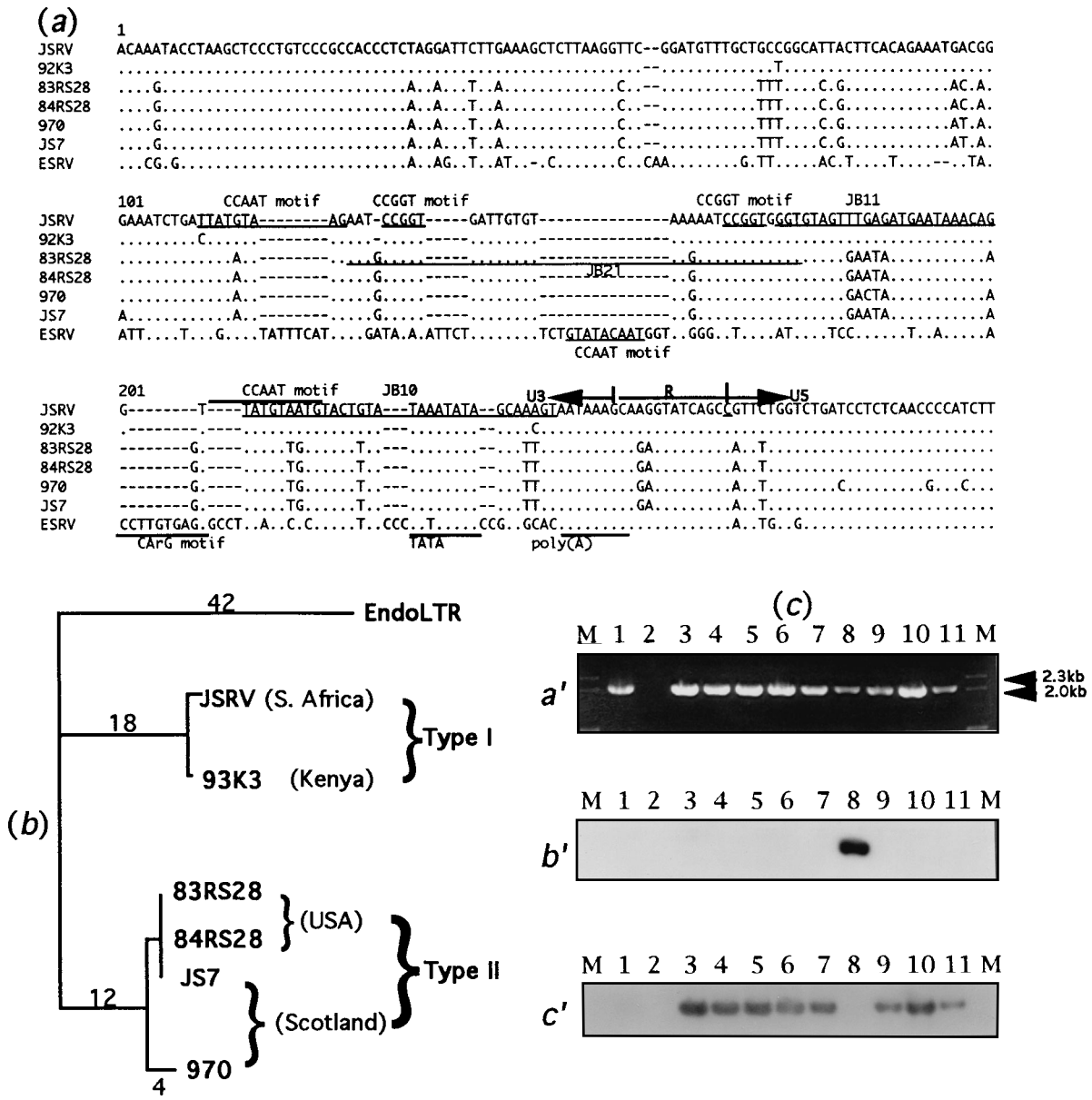

FIG. 7. U3 sequences of *ex*JSRV proviruses. (a) Multiple sequence alignments were performed by using CLUSTAL version 5 (18). Dots refer to identical sequences, and dashes refer to deletions. Potential CArG (3) and CCAAT (30) motifs are indicated. CCGGT is present only in exogenous sequences. The JSRV sequence was published previously (45). JB10, JB11, and JB21 show the sequences of *ex*JSRV strain-specific oligonucleotide primers. (b) Phylogenetic analysis was performed by using the program PAUP (Illinois Natural History Survey, 1988). Arabic numbers show the scores of distance relationship between sequences. (c) Southern analysis of endogenous and exogenous proviral sequences by using *ex*JSRV strain-specific U3 oligonucleotide probes. (a′) Gel-purified 2-kb DNA fragments from PCR products amplified with JB15 and JB16 (lanes 3 to 11, *ex*JSRV proviral sequences) and JB1 and JB16 (lane 1, ESRV proviral sequences) were separated on an agarose gel stained with ethidium bromide. (b′) The filter was probed with [γ-^{32}P]ATP-labeled oligonucleotides JB10 and JB11 derived from JSRV and Kenyan 92K3 virus (Fig. 7a). (c′) After being stripped, the same filter was reprobed with [γ-^{32}P]ATP-labeled oligonucleotide JB21 specific for the viral sequences from Wyoming and Scotland (Fig. 7a). Lane 1, endogenous sequences. Lanes (cell line or sheep designation are given): 2, no-DNA control; 3, JS7; 4, 91RS13; 5, 86RS44; 6, 84RS28; 7, 83RS28; 8, 92K3; 9, a tumor DNA mixture prepared from tumor nodes of sheep 86RS44 and 91RS13; 10, 970; 11, 805.

enous proviral sequences by restriction mapping, especially by exogenous-virus U3-specific oligonucleotide-mediated PCR and Southern blot hybridization. The observation that *ex*JSRV proviral sequences could be detected in lung tumor DNA but not in DNA from lungs or blood of healthy sheep or in non-tumor tissues of affected animals strongly suggests that exogenous viruses, not endogenous loci, are primarily associated with oncogenesis in OPC. The significance of recombination, if any, between ESRV and *ex*JSRV in the oncogenesis of OPC remains to be investigated.

Cultured OPC tumor cell lines JS7 and JS8 of passages 1 to 140 have been shown to cause neoplastic lesions in newborn lambs through transplantation, but viral *gag* proteins could be detected only within the first 10 culture passages (20). Culture supernatants and disrupted cell extracts prepared after that passage level did not efficiently induce OPC in newborn lambs; this is consistent with the inability to detect viral *gag* proteins in the inocula (20). The present study has shown that JS7 DNA from the 140th passage still carried an exogenous proviral genome. This may imply that certain factors necessary for the

viral gene expression in tumor cells in vivo are not present in culture medium or that the virus is defective.

The inhibition of viral gene expression in vitro also may be caused by deletion mutations at the 5′ end of the proviral genome during subculture, which frequently occur in avian leukosis virus-induced lymphomas (2, 5, 6, 12, 29). In the present study, a 2-kb DNA fragment, synthesized from the 5′ end of the proviral genome from tumor DNAs with exogenous-virus U3-specific primers, did not show any significant internal deletions in the LTR and *gag*, indicating that this mechanism may not be significant in the oncogenesis of OPC.

Enhancer elements that control viral transcription, oncogenic potential, and disease specificity are located in the U3 region of the LTR in avian leukosis (5, 6, 14, 23, 28, 42) and murine leukemia (4, 13, 21, 22) viruses. The enhancer elements consist of special motifs for binding by transcriptional factors (35, 37). Sequence variations in the enhancer region affect viral gene expression (5, 6, 15, 31, 42) and oncogenicity (9, 13, 19, 38). The U3 sequences of *ex*JSRV proviruses reported here have eight deletions and many point mutations compared with the endogenous proviruses. As a result, they have acquired novel sequence features, and some, if not all, of these changes may be responsible for the oncogenesis of OPC in vivo. It is likely that the regulation of gene expression in endogenous loci is different from that in *ex*JSRV. The sequence features of the endogenous U3 may contain suppresser elements or confer weak promoter and enhancer functions on ESRV loci. This may be one of the reasons that ESRVs do not cause pulmonary carcinomas spontaneously even though endogenous viral mRNA is expressed (26).

The U3 sequence and restriction profiles of virus from Kenyan sheep 92K3 were nearly identical to those of South African JSRV but were distinct from those of viruses isolated in Scotland and Wyoming, suggesting that there are two types of exogenous retroviral sequences. We designated the viruses isolated from sheep 92K3 and JSRV as type I viruses and the others as type II viruses. The endogenous U3 sequences have greater identity to the type II than to the type I viruses (see Fig. 7a). This may suggest that the two types of viruses have emerged from different genetic events or that they have diverged from the same source through transmission among hosts. The homologous relationship between ESRVs and *ex*JSRVs in genomic organization and nucleotide sequences reported here suggests that *ex*JSRVs might have been derived from some ESRV loci following mutations in U3 of the LTR and other regions of the proviral genome during evolution.

Our previous study (16) indicated that there were many copies of ESRVs in the sheep genome. We have now cloned endogenous sequences that cover the entire proviral genome. In particular, clones from the 7.4-kb PCR products span the region from the 5′ tRNA primer binding site to the 3′ end of U5, including the entire *gag*, *pol*, *env*, and the LTR. Thus, in terms of genomic organization, some ESRV loci seem to resemble the JSRV provirus.

In conclusion, we have cloned ESRV proviral sequences and isolated parts of *ex*JSRV proviral sequences from lung tumor DNAs of eight OPC-affected sheep by using long-range PCR. In particular, the 2- and 6-kb exogenous proviral sequences synthesized from tumor DNAs of two sheep with naturally occurring OPC (83RS28 and 84RS28) overlapped each other and included the complete proviral genome. These DNA fragments can serve for the reconstruction of the full-length proviral genome to recover infectious viral particles by DNA transfection or can be used in further investigation of viral gene expression, identification of oncogenic determinants, and the study of the pathogenesis of *ex*JSRV. The exogenous-virus U3-specific primers and DNA probes reported here will be useful tools to isolate proviral clones from a genomic DNA library and to characterize sites of *ex*JSRV proviral integration in OPC tumors.

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