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PCR-Based Detection and Partial Characterization of a Retrovirus Associated with Contagious Intranasal Tumors of Sheep and Goats

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A type D-related retrovirus has been demonstrated in enzootic nasal tumors (ENTs) of sheep and goats. This retrovirus, ENT virus (ENTV), has antigenic cross-reactivity with the jaagsiekte sheep retrovirus (JSRV), which is associated with a contagious lung tumor of sheep (sheep pulmonary adenomatosis). Here, we present the first report of nucleic acid sequence from ENTV which confirms, at the nucleic acid level, that this retrovirus is related to JSRV yet apparently distinct from it. Reverse transcription-PCR followed by restriction enzyme digestion specifically identified ENTV. By this technique, ENTV was demonstrated exclusively in tumor tissues and exudates of animals with ENT. Thus, there is a unique and consistent association between ENT and the retrovirus, just as there is between JSRV and sheep pulmonary adenomatosis. This gives further weight to the hypothesis that these retroviruses are the etiologic agents of the tumors.

Enzootic intranasal tumors (ENTs) of sheep and goats are contagious adenocarcinomas which can also be transmitted experimentally (3, 6). ENTs have similarities with another contagious tumor of secretory epithelial cells, known as sheep pulmonary adenomatosis (SPA, or jaagsiekte ovine pulmonary carcinoma). Both tumors are associated with retroviruses which cannot be propagated *in vitro*. Although no studies have previously been reported to determine whether ENT and SPA are different manifestations of disease caused by the same agent, studies of ENT cases have not found any tumors in the lungs (7, 19), nor have nasal tumors in SPA-affected animals been reported.

In the case of SPA, an association with a type B/D retrovirus, known as jaagsiekte sheep retrovirus (JSRV), is well established (15, 17), and the complete nucleotide sequence of the retrovirus has been determined (22). Recent reports have demonstrated that JSRV is related to sheep endogenous retroviruses (SERVs), of which there are 10 to 15 copies in the sheep genome (1, 12, 15). However, reverse transcription-PCR (RT-PCR)-based techniques, which differentiate JSRV from SERVs, showed that JSRV is a distinct exogenous virus that is associated exclusively with SPA tumors (15).

Although studies are at a less advanced stage than for SPA and JSRV, current evidence points to the association of a type D-like retrovirus, ENT virus (ENTV), with ENT. The presence of retrovirus-like particles in ENT of sheep (14, 21) and goats (7, 19) has been demonstrated by electron microscopy. The detection of RT activity in fractions from isopycnic gradients has shown that the retrovirus found in nasal fluid of ENT-affected goats has the same buoyant density as JSRV (9). In addition, antigenic cross-reactivity with Mason-Pfizer monkey virus p27 (8) and recombinant JSRV capsid protein (unpub-

lished observation) indicates that ENTV has a type D retroviral capsid.

The aim of our studies was to develop a PCR-based detection technique for ENTV, to confirm its association with ENT, and to determine the relationship between ENTV and JSRV.

MATERIALS AND METHODS

Source of material. Nasal fluids from four sheep and two goats with naturally occurring ENT were collected, clarified, and concentrated as described previously (8). The resultant pellets were resuspended as a 100-fold concentrate in TNE buffer (0.01 M Tris-Cl [pH 7.5], 0.1 M NaCl, 0.001 M EDTA). Samples of ENT and of kidney tissues from the same animals were collected at necropsy and snap frozen in liquid nitrogen. The ENTs were all low-grade adenocarcinomas, which were locally invasive but not metastatic, and were from 3- to 4-year-old animals. Lung fluids from SPA-affected animals were collected as described previously (18). All samples were stored at -70°C . The ENT-affected sheep were verified to be negative for SPA by macroscopic and microscopic pathology at necropsy. The ENT-affected and SPA-affected cases used in this study were from the same region of Spain. The negative-control animals were from the United Kingdom, where ENT has never been reported.

Density gradient fractionation. Pellets from nasal fluids of four sheep and two goats were analyzed by isopycnic centrifugation on 20 to 55% (wt/wt) sucrose gradients (13). Fractions (0.5 ml) were collected, and those with densities between 1.17 and 1.19 g/ml (i.e., those fractions shown to contain peak RT activity [9]) were pooled, diluted in TNE buffer, and centrifuged at $100,000 \times g$ for 1 h at 4°C . The pellet was resuspended in 0.5 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl [wt/vol], 0.1 M 2-mercaptoethanol) for RNA preparation.

RNA preparation and RT. Total RNA was extracted from pooled sucrose gradient fractions, from 100 μl of concentrated nasal fluid, from 100 μl of SPA lung fluid, or from 0.2 to 0.5 g of tissue by the acid guanidinium thiocyanate-phenol-chloroform method (2). cDNA was synthesized by random-primed RT as described previously (15).

gag PCR. The primers and conditions used for PCR of cDNA or of genomic DNA were exactly as described previously (15): P1 (sense), 5'-GCTGCTTTRA GACCTTATCGAAA-3'; and P2 (antisense), 5'-ATACTGCAGCYCATGGC CAG-3'. PCR products were detected by electrophoresis through a 2% agarose gel in $1 \times$ Tris-borate-EDTA buffer in the presence of 0.5 μg of ethidium bromide per ml.

Cloning of ENTV gag fragments. cDNA from nasal fluid from each of three ovine ENT cases was amplified by using primers P1 and P2. The products of four independent PCRs for each animal were pooled, gel purified, and cloned into pGEMT (Promega) according to the manufacturer's protocol. Several clones selected from each ENT case were sequenced on both strands by using the T7 sequencing kit (Pharmacia), with universal and reverse sequencing primers (Pharmacia), as recommended by the manufacturer. The sequences obtained

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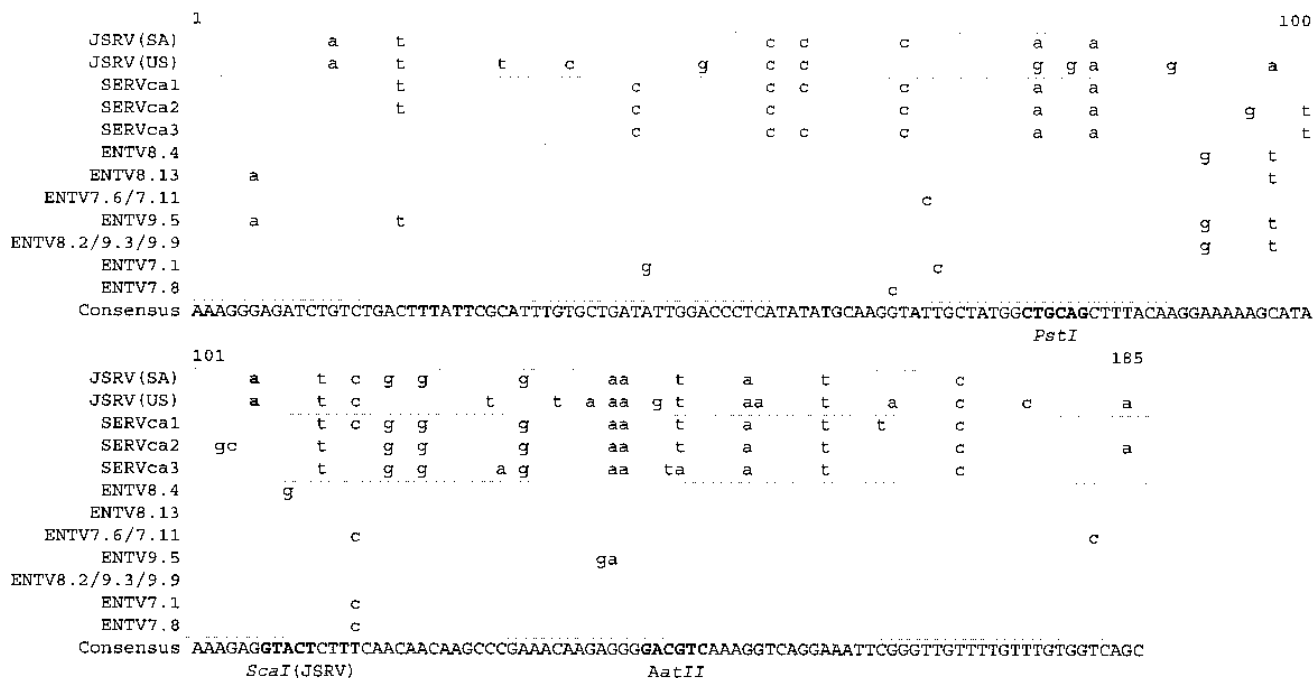


FIG. 1. Alignment of the sequences of RT-PCR gag products from ovine ENTV with SERV sequences (15) and JSRV sequences from South Africa [JSRV(SA)] (22) and the United States [JSRV(US)] (12). Only the sequence between the PCR primers is shown. Base differences in comparison with the ENTV consensus (letters) and restriction sites specific to ENTV or JSRV (boldface) are shown.

were aligned by using the Genetics Computer Group "pileup" program (11). The phylogenetic tree was constructed by using the DNAML program from version 4.0 of the PHYLIP package (10).

Restriction endonuclease digestion of RT-PCR products. A 3- μ l volume of 10 \times restriction buffer (Boehringer) and 1 μ l (10 U) of restriction endonuclease (Boehringer) were added to 26 μ l of PCR product, and the mixture was incubated at 37°C overnight. Digestion products were detected by electrophoresis through a 2% agarose gel in 1 \times Tris-borate-EDTA buffer in the presence of 0.5 μ g of ethidium bromide per ml.

DNA extraction. High-molecular-weight genomic DNA was extracted from nasal tumor and kidney samples of the same naturally affected animals or from lung and kidney samples of unaffected controls by cell lysis followed by precipitation of DNA in tissue culture plates, as described by Wu et al. (20).

Nucleotide sequence accession numbers. The nucleotide sequence of one of the ENTV gag fragment PCR clones, pENTV7.6, is deposited in GenBank under accession no. Z71758.

RESULTS

Development of a technique for specific detection of ENTV. Primers P1 and P2, which amplify a 229-bp region internal to the gag gene of JSRV and of SERVs (15), amplified products of the same size from cDNAs derived from clarified nasal fluids of four sheep and two goats affected by ENT and from density gradient-purified virus from the same sources. The gag RT-PCR product from JSRV isolates cut with ScaI, while that from SERVs or ENTV did not (data not shown). To look for an alternative restriction site by which to distinguish ENTV from SERVs, gag RT-PCR products of three ovine ENT cases, from different farms, were cloned and sequenced. Alignment of the sequences (Fig. 1) with the published sequences of JSRV and SERVs showed a number of bases at which there appear to be conserved differences between ENTV and JSRV or SERV. Of particular interest were the presence of a PstI site (positions 75 to 80) and an AatII site (positions 141 to 146) unique to the ENTV gag sequences and the lack of the ScaI site (positions 106 to 111) which has been shown to be present in all JSRV isolates tested to date (15).

Association of ENTV with ENT. To confirm the association of the virus with ENT and the conservation of the AatII and PstI restriction sites, gag RT-PCR was done with 26 different samples (typical results are shown in Fig. 2). The product from nontumor (kidney) tissues of four of the ENT-affected animals or of four unaffected controls did not cut, as was expected for endogenously derived amplimers. The product from JSRV, in lung fluid or tumor samples from SPA-affected sheep, also did not cut with AatII or PstI. However, the RT-PCR gag products

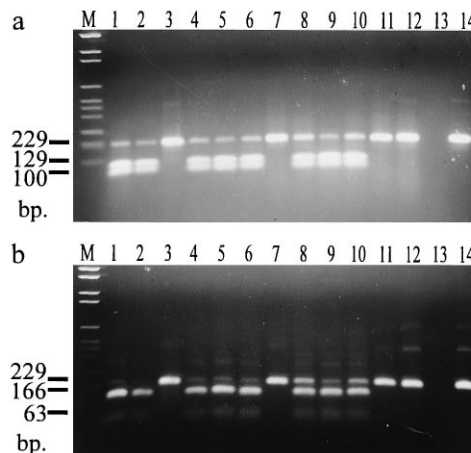


FIG. 2. Detection of ENTV transcripts. gag RT-PCR products after incubation with PstI (a) or AatII (b) are shown. Lanes 1, 2, 4, and 5, nasal fluids from ENT-affected sheep; lanes 6, 8, 9, and 10, tumor tissues from ENT-affected animals; lanes 5 to 7, nasal fluid, tumor, and kidney samples, respectively, from the same ENT-affected sheep; lanes 3, 11, and 12, lung fluid, tumor, and kidney specimens, respectively, from the same SPA-affected animal; lane 13, negative control; lane 14, pJS382 (JSRV gag clone); lane M, Boehringer marker VI.

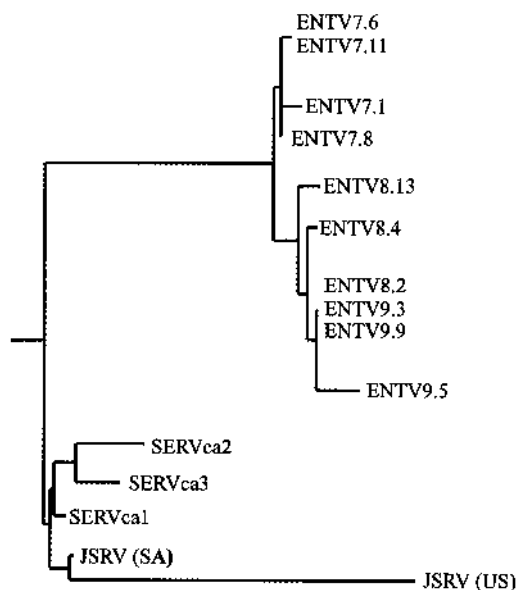


FIG. 3. Phylogenetic tree constructed from the sequences shown in Fig. 1.

from six ENT nasal fluid specimens (from four sheep and two goats) and six ENT tissue specimens (from four sheep and two goats) cut with both enzymes, demonstrating the presence of exogenous ENTV. Amplimers from isopycnic gradient-purified fractions were expected to cut completely, as they should contain no endogenous sequences. However, restriction enzyme digestion was incomplete. The efficiency of the restriction enzymes was checked by digestion of PCR product amplified from plasmid pENTV7.6 (sequence shown in Fig. 1) and was found to be less than expected. Nevertheless, the results show that *AatII*- and *PstI*-sensitive product was detectable in all tumor and fluid samples from animals with ENT.

DNAs from tumor and kidney tissues of two sheep and two goats affected by ENT were used as templates for *gag* PCR followed by *PstI* or *AatII* digestion to determine whether these restriction sites were present in any of the related endogenous sequences. No *AatII*- or *PstI*-sensitive product was detected (results not shown).

Relationship between ENTV, JSRV, and SERVs. Comparison of the 10 ENTV sequences with the published sequences of JSRV and SERVs showed that the ENTV sequences were 95 to 100% similar to each other, 83 to 92% similar to JSRV sequences, and 83 to 92% similar to SERV sequences. Figure 3 shows a phylogenetic tree constructed from the *gag* sequences. The long branch linking the ENTV sequence cluster to the rest (SERV and JSRV) was significantly greater than 0 (length, 0.09676 nucleotide substitutions per position, i.e., about 10% sequence divergence or 22 nucleotide substitutions in 229 bp). The ordering of the *gag* fragments within the ENTV cluster and within the SERV-JSRV cluster was not significant. The optimum Ts/Tv value was 7.0, suggesting that transitions are much more common than transversions (e.g., A→G is 14 times more likely than A→T). The relative rates at the three codon positions were 1:1:10, confirming that the first two codon positions are constrained relative to the third position (which is likely to be evolving at close to the neutral rate) and suggesting that the part of the CA protein encoded by this region of *gag* does not evolve rapidly.

DISCUSSION

We have previously reported that the presence of a *ScaI* restriction site within *gag* is a molecular marker for JSRV (15). Here, we report that the restriction sites *AatII* and *PstI* within *gag* act as unique molecular markers for ENTV. *gag* RT-PCR analysis of samples from clinical ENT cases and from unaffected controls demonstrated that the virus transcripts distinguished by the *AatII* and *PstI* restriction sites were present exclusively and consistently in tumor tissues and exudates from ENT cases. ENTV therefore appears to be a distinct virus associated with ENT. By analogy with JSRV, ENTV is likely to be an exogenous virus as opposed to a reactivated endogenous virus. Our findings that no endogenously derived sequences containing *AatII* or *PstI* were detected at either the RNA or the DNA level support this notion but because of the limitations of sensitivity of the present technique are not conclusive. These limitations are highlighted by the fact that we were unable to demonstrate the presence of ENTV provirus in tumor tissue DNA. This observation agrees with our findings for JSRV, for which provirus could not be detected in SPA tumor DNA by using the *gag* PCR-*ScaI* technique because of the high background of endogenous sequences (15). This problem was overcome by the development of primers specific for exogenous JSRV which enabled detection of proviral DNA in tumor tissue. These JSRV primers do not amplify ENTV, and further sequence data for ENTV are necessary in order to design specific primers and develop a more sensitive PCR for detection of ENTV.

The fact that the *AatII* and *PstI* sites were conserved in ENTV whereas the *ScaI* site is conserved in JSRV suggests that ENTV and JSRV are distinct viruses. This notion is supported by the phylogenetic tree, which shows that the JSRV and SERV sequences are more similar to each other than they are to the ENTV sequences. However, such short regions of sequence should not be overinterpreted. It is not possible to extrapolate the conclusion from this small region of sequence to predict differences in other regions of the viral genomes, as it is likely that the *gag* gene may be the region of greatest homology between the two viruses, especially since analysis of the sequences suggests that the *gag* region is not evolving rapidly. In addition, the CA proteins of JSRV and ENTV, encoded by *gag*, are antigenically related (8). However, antigenic relatedness of other viral components has not yet been investigated. PCRs which amplify other regions of JSRV and of SERVs (long terminal repeat, *pol*, and *env*) did not amplify ENTV (data not shown). Caution should be exercised in interpretation of negative PCR results since minor differences between two genomes can prevent efficient primer binding; nevertheless, this result suggests not only that other genes of ENTV are less similar to those of JSRV and SERVs but also that ENTV is less similar to SERVs than is JSRV. This agrees with the phylogenetic tree.

In sheep and goats, ENTs are clinically and pathologically identical (4, 5), but, until now, there have been no data to confirm that the same virus affects the two species. Our studies show that the *AatII* and *PstI* sites are present in the *gag* region of the viruses from ENT samples of both sheep and goats, suggesting that the viruses are highly related. Similar intranasal tumors in other animals including cattle, buffaloes, and pigs have also been reported (16), and the RT-PCR-restriction enzyme technique could be used to determine whether these tumors are also associated with ENTV.

In conclusion, this is the first report of nucleic acid sequence from ENTV, and it confirms, at the nucleic acid level, that this retrovirus is related to JSRV yet apparently distinct from it.

ENTV can be specifically identified by RT-PCR followed by *AatII* or *PstI* digestion, and, by this technique, ENTV was demonstrated exclusively and consistently in nasal tumor tissues and exudates from ENT-affected sheep and goats. The facts that SPA and ENT are both adenocarcinomas of secretory epithelial cells but target different areas of the respiratory tract and that JSRV and ENTV are also similar but distinct add further weight to the hypothesis that these retroviruses are the etiological agents of the tumors.

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