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A Novel Exogenous Retrovirus Sequence Identified in Humans

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A 932-bp retrovirus sequence was cloned by reverse transcriptase PCR from salivary gland tissue of a patient with Sjögren's syndrome. The sequence is related to that of type B and type D retroviruses and was present in a sucrose density gradient fraction corresponding to that of an enveloped retrovirus particle. Sequences amplified from tissues of eight individuals with or without Sjögren's syndrome had over 90% similarity and were present at a level of less than one copy per 10^3 cells. The sequence was not detectable in human genomic DNA by PCR or by Southern hybridization. These data indicate that the sequence represents an infectiously acquired genome, provisionally called human retrovirus 5.

Circumstantial evidence has suggested that retroviruses are candidates for the initiation or maintenance of autoimmunity in Sjögren's syndrome (SS) (17, 25, 37). This is a group of diseases characterized by the destruction of exocrine glands, particularly salivary and lachrymal glands. Such evidence includes the presence in SS patients of antibodies which are cross-reactive with retroviral Gag proteins (6, 52), the detection of retrovirus antigens in SS patients (6, 45), and the occurrence of SS-like conditions in patients having confirmed infections with known retroviruses (21, 56). Furthermore, intracisternal A-type retrovirus particles have been described in the H9 subclone of the human T-cell line HUT 78 following coculture with salivary gland tissue from SS patients (16). These particles were purified on a sucrose gradient at 1.22 g ml^{-1}, a density typical of retrovirus cores. The same fractions contained reverse transcriptase (RT) activity and material which reacted with an anti-human immunodeficiency virus p24 monoclonal antibody, but in this study no sequence data were reported.

In light of these reports, we used a PCR-based strategy to investigate the expression of retrovirus sequences in SS. We detected novel sequences spanning parts of the protease and RT open reading frames of a retrovirus in eight individuals. This virus cannot clearly be linked to SS. However, it appears to be exogenous, thus representing part of an infectiously transmitted human retrovirus.

MATERIALS AND METHODS

Cell culture. All adherent cell lines were grown in Dulbecco's modified Eagle's medium (Gibco) containing 5 to 10% fetal calf serum (FCS; Biological Industries) except for A253 cells, which were grown in McCoy's 5A medium supplemented with 10% FCS. Suspension cells were grown in RPMI 1640 medium containing 10% FCS. FCS was heat inactivated by incubation at 56°C for 1 h prior to use. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll gradient and cultured for 72 h in growth medium (RPMI 1640 medium [Gibco] with 20% FCS) supplemented with phytohemagglutinin (1 μg/ml). Cultures were then maintained in growth medium containing 10% human interleukin-2 (from human lymphocytes, at 200 U/ml; Boehringer). In initial experiments, a homogenate of a minor salivary gland biopsy specimen from a patient with primary SS was cocultured with H9 cells exactly as described by Garry et al. (16). Subsequent cocultures of human tissue biopsy samples and cell lines were performed in essentially the same way. For cocultures with PBMCs, homogenized human tissue was added 24 to 48 h after the addition of interleukin-2.

Virus preparation. Culture homogenates were prepared after 14 and 28 days by suspension of cells in homogenization buffer (50 mM Tris-HCl [pH 7.9], 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20% [vol/vol] glycerol) at a concentration of 10^6 cells ml^{-1} and homogenized on ice with an Ultra-Turrax T25 tissue grinder (IKA Labortechnik) at maximum speed. Cellular debris was removed by centrifugation at 3,800 × *g* for 10 min at 4°C. The supernatant was then recentrifuged at 14,000 × *g* for 20 min at 4°C to remove mitochondria and other subcellular organelles. The supernatant from this second spin was layered onto a 20 to 65% (wt/wt) linear sucrose gradient. Sucrose gradients were prepared and run in a Beckman SW28 rotor as previously described (5). Tissue samples were processed on ice by rapid dissection into small (approximately 1-mm^3) pieces. Homogenization buffer was then added to the dissected tissue (4 ml/g of tissue), and the sample was further homogenized on ice with the Ultra-Turrax before processing further as described above for cultured cells.

RNA extractions. RNA extractions were performed by the acidified guanidinium isothiocyanate procedure (7) with the RNAzol B reagent (Biotecx Laboratories Inc.). RNA was extracted from cells in culture by using RNAzol B exactly as recommended by the manufacturer. Tissue fragments were first crushed to a powder in liquid nitrogen before being resuspended in RNAzol B (2 ml per 100 mg of tissue). RNAzol B (750 μl) was mixed with 250 μl of each sucrose gradient fraction, and this was followed by addition of 125 μl of chloroform. Ten micrograms of tRNA (from *Saccharomyces cerevisiae*; Sigma) or 20 μg of glycogen (from mussels; Boehringer) was added as a carrier. This mixture was vortexed briefly, incubated on ice for 5 min, and centrifuged at 14,000 × *g* and 4°C for 15 min. An equal volume of isopropanol was then added to the aqueous phase, after which the mixture was incubated on ice for 15 min. Precipitated RNA was pelleted by centrifugation (14,000 × *g* and 4°C for 15 min), and the pellets were washed in ice-cold 75% ethanol. The RNA pellet was resuspended in 20 μl of water.

Southern analysis. High-molecular-weight DNA was extracted from cultured cells or homogenized tissue samples and digested to completion with *Eco*RI (Boehringer) according to standard protocols (41). Digested DNA (10 to 12 μg) was electrophoresed on a 1% agarose gel and transferred to a nylon membrane (Genescreen Plus; New England Nuclear) by the alkaline transfer procedure as recommended by the manufacturer. [^{32}P]dCTP-radiolabelled probes were prepared by using a Multiprime kit (Amersham) as recommended by the manufacturer. The blot was first hybridized with a 335-bp fragment of human retrovirus 5 (HRV-5) (corresponding to nucleotides 94 to 428; see Fig. 1) under conditions of high stringency (the final wash was performed in 2× SSC (20× SSC is 3.0 M sodium chloride plus 300 mM trisodium citrate)–1% sodium dodecyl sulfate at 65°C). The blot was then stripped and hybridized with a 404-bp fragment derived from the envelope region of ERV-3 (corresponding to nucleotides 2099 to 2502 of the sequence reported by Cohen et al. [9]) and washed under very stringent conditions (0.1× SSC–1% sodium dodecyl sulfate, 68°C).

Cloning of HRV-5 by RT-PCR. A 126-bp fragment of HRV-5 was cloned by RT-PCR with degenerate oligonucleotide primers derived from a conserved region of RT (46). Eight microliters of RNA from each gradient fraction was denatured at 90°C for 3 min in the presence of 100 ng of the reverse PCR primer (TGGAAAGTGYTRCCMCARGG, where Y = C or T, R = A or G, and M = A or C) in a 10-μl reaction mixture also containing 10 mM HEPES (pH 6.9) and 200 μM EDTA. Samples were then chilled on ice (5 min), and reverse transcription was performed upon addition of 4 μl of 5× RT buffer (250 mM Tris-HCl [pH 7.5], 375 mM KCl, 15 mM $MgCl_2$, 50 mM dithiothreitol), 500 μM each deoxynucleoside triphosphate, and 20 U of Moloney murine leukemia virus RT

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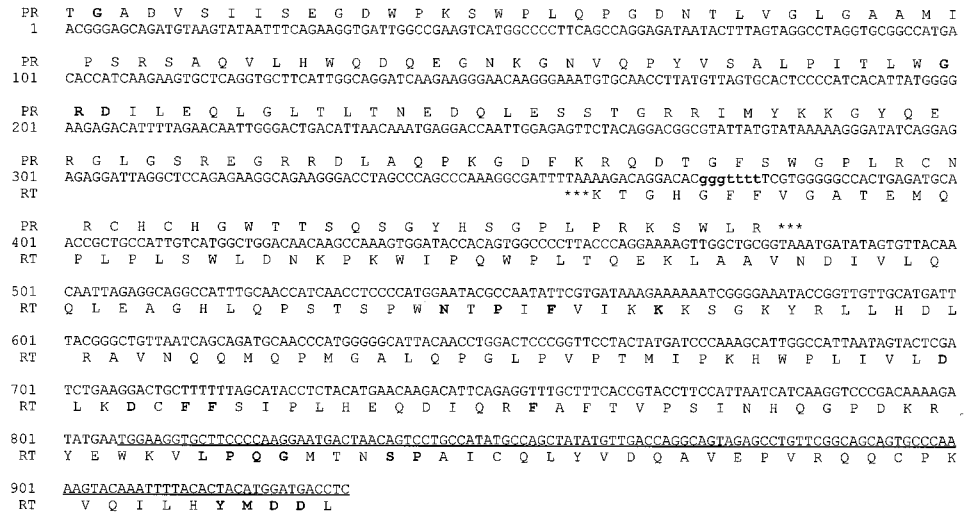

FIG. 1. The HRV-5 nucleotide sequence with its two open reading frames. The protease (PR) open reading frame is indicated above the nucleotide sequence, and the RT open reading frame is shown below the nucleotide sequence. Termination codons are indicated by asterisks. Amino acid residues conserved in retroviral *pol* genes (12) are marked in boldface. A heptanucleotide slippery motif denoting a potential frameshifting site (22) is shown in lowercase letters. Note that nucleotides 1 to 14 and 918 to 932 are derived from the degenerate primers used to clone this fragment of HRV-5 and therefore may not represent the genuine sequence of this element in those regions. The initial 126-bp fragment cloned with the primers of Shih et al. (46) is underlined.

(Gibco). The reaction mixtures were each brought up to a final volume of 20 μl by the addition of water and incubated at 37°C for 90 min. Five microliters of each of these reaction products was then amplified in 50-μl reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 2.5 U of Amplitaq DNA polymerase (Perkin-Elmer), and 20 pmol of each oligonucleotide primer (forward primer, GGMG GCCAGCAGSAKGTCATCCAYGTA, where S = C or G and K = G or T) by using 40 cycles of 94°C for 30 s, 37°C for 30 s, and 72°C for 30 s and then a final extension at 72°C for 5 min. The products were analyzed by electrophoresis through a 3% agarose gel and ethidium bromide (0.1 μg ml^{-1}) staining, and the bands were purified and subcloned into *Eco*RV-digested pBluescript KS(−) (Stratagene) by standard methods (41).

A larger fragment of viral RNA was subsequently amplified by nested RT-PCR from sucrose gradient fractions of a fresh SS salivary gland biopsy obtained from an SS patient. cDNA synthesis was primed with 100 ng of outer reverse primer (GAGGTCATCCATGTAGTGTAAAATTTG), and then PCR was performed with the outer-primer pair (outer forward primer, IIIITAGAYACWG GRGCMGA, where I = inosine and W = A or T) for 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min 30 s. One microliter of each of the products was transferred to a second PCR reaction mixture containing forward (TAGA YACKGGAGCWGATGT) and reverse (TAAAATTTGTACTTTTGGGCACT GCTG) inner primers. This PCR consisted of 25 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min 30 s and a final extension at 72°C for 5 min. Products were then subcloned as described above.

Nested PCR assay for HRV-5. Human genomic DNA (100 to 500 ng) was tested for the presence of HRV-5 by a nested PCR assay. First-stage PCR conditions were 25 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s (forward primer, TCAGGTGCTTCATTGGCAGGATCA; reverse primer, TAAAATTTGTACTTTTGGGCACTGCTG). One microliter of each of the first-stage products was transferred to a second PCR reaction and further amplified for 25 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s (forward primer, TGCAACCTTATGTTAGTGCACTCC; reverse primer, TACTGCCT GGTCAACATATAG). Total RNA (1 μg) and gradient-purified RNA (5 μl) were studied by RT-PCR, with the outer reverse PCR primer being used to initiate cDNA synthesis. Five microliters of each of the cDNA products was amplified as described for genomic DNA. To verify the quality of the genomic DNA samples, the well-characterized endogenous sequence ERV-3 (9, 30, 55) was used as a single-copy control. ERV-3 PCR conditions were 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s (forward primer, GAGGCATAACTA TAGGAGATTGG; reverse primer, CCTTTCCAAGTCTGAACTG), and a product of 404 bp resulted. For RNA samples, template quality was assessed by RT-PCR with primers specific for glyceraldehyde-3-phosphate dehydrogenase (forward primer, TGGATATTGTTGCCATCAATGACC; reverse primer, GAT GGCATGGACTGTGGTCATG) for 40 cycles of 94°C for 30 s, 65°C for 45 s, and 72°C for 30 s, and a product of 460 bp resulted. Control reactions were performed in which RT was omitted in order to check for DNA contamination of the RNA preparations.

Reverse transcription assay. RT activity was detected by the assay of Silver et al. (49) with minor modifications as previously described (34, 50).

DNA sequencing and phylogenetic analysis. PCR products were subcloned into *Eco*RV-digested pBluescript KS(−) by standard methods (41), and six independent plasmid clones were sequenced in both directions with an Applied Biosystems 373A automated DNA sequencer. Computer-aided analysis of protein and nucleotide sequences was performed with the Genetics Computer Group suite of programs (18).

Phylogenetic trees were reconstructed with the PHYLIP package (13) based on a CLUSTALW multiple sequence alignment (54) of retrovirus *pol* genes. The tree (see Fig. 3) was constructed by the neighbor joining method with the NEIGHBOR program of the PHYLIP package, using a Dayhoff PAM 250 distance matrix (produced with PROTDIST from PHYLIP). Bootstrap analysis (1,000 replications) was performed with the SEQBOOT and CONSENSE programs of PHYLIP. Trees were also constructed by parsimony methods (using PROTPARS from PHYLIP) and least-squares-distance matrix methods (using FITCH from PHYLIP).

Nucleotide sequence accession numbers. The following sequences have been deposited with the GenBank and EMBL databases: HRV-5 (patient A), gb_vi: u46939; patient C variant, gb_vi:u46940.

RESULTS

Amplification of a novel retrovirus element from human salivary gland tissue. We used an RT-PCR approach to study expression of retrovirus sequences in SS patient tissues. A homogenate of a minor salivary gland biopsy specimen from a patient with primary SS was cocultured with H9 cells as described by Garry et al. (16). After 14 days, culture homogenates were fractionated by sucrose gradient centrifugation and RNA was extracted from gradient fractions. RT-PCR was then performed with degenerate primers derived from the most conserved regions of the *pol* genes of known retroviruses (46). These primers were originally used to amplify endogenous retrovirus sequences from human genomic DNA (46), and so the physical separation of virion-bound RNA from soluble, cellular sequences by density gradient centrifugation is necessary to avoid amplification of unpackaged endogenous sequences. A 126-bp sequence was amplified from a gradient fraction with a density of 1.16 g ml^{-1} and found to consist of a previously undescribed fragment of *pol* sequence. This sequence was not detected in control H9 cells or in gradient fractions of other densities. We found that the coculture with H9 cells resulted in the loss of this viral sequence after 4 weeks, suggesting that the H9 cells did not become productively or

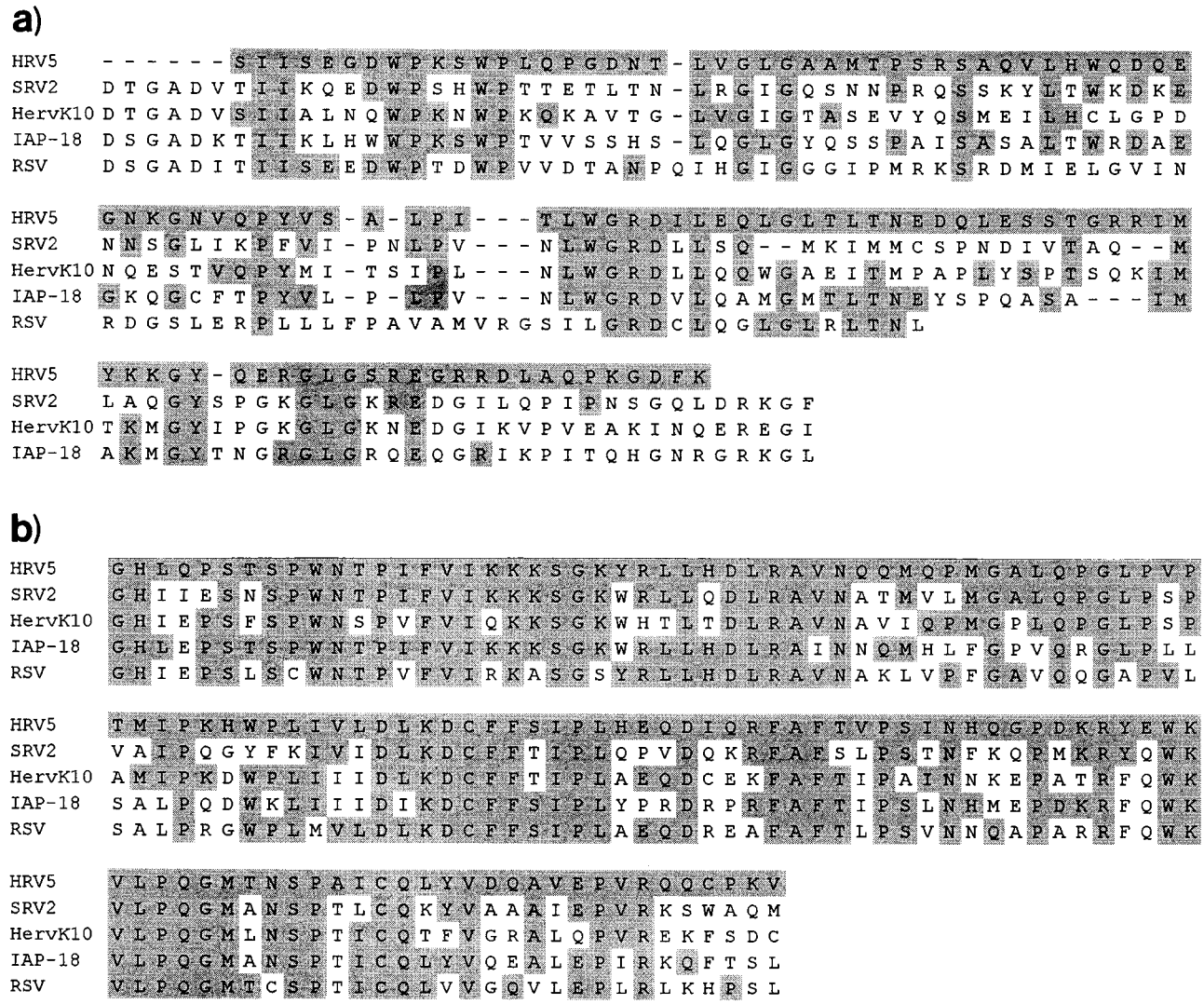

FIG. 2. Multiple sequence alignments of the cloned HRV-5 element and *pol* genes of other retroviruses. The deduced protein sequences of the HRV-5 protease (a) and RT (b) are shown aligned with those of related retroviruses. Residues identical to those of HRV-5 are shaded. Dashes indicate gaps introduced to improve the alignments. The regions of HRV-5 derived from the degenerate PCR primers and from the region of overlap of the protease and RT open reading frames are excluded from these alignments. SRV2, simian type D retrovirus, serotype 2 (53); HervK10, human endogenous retrovirus K10 (33); IAP-18, Syrian hamster IAP gene (32); RSV, Rous sarcoma virus (42).

stably infected by this procedure. Subsequent experiments omitted coculture with H9 cells.

Those gradient fractions which contained the novel *pol* sequence were analyzed for RT activity by an assay based on that described by Silver et al. (49). Although a positive signal was obtained (data not shown), this assay is not sufficient to characterize the substrate specificity or cation preference of this enzyme. Furthermore, the possibility that the observed RT activity is due to the presence of endogenous cellular RT activity in these gradient fractions cannot be excluded.

We then cloned a further 800 bp of viral sequence by nested PCR with gradient-purified RNA from a fresh salivary gland biopsy specimen obtained from an SS patient. Two primer pairs were used, each consisting of a conserved sequence derived from retrovirus protease genes and a specific primer derived from the 3′ end of the 126-bp sequence. By this means, we extended the sequence to 932 bp, thereby encompassing most of the protease gene and approximately 50% of the polymerase domain of the RT gene (Fig. 1). The sequence was provisionally termed HRV-5.

HRV-5 shows a high degree of sequence similarity to the simian type D retroviruses (15, 27), the type B retrovirus mouse mammary tumor virus (24), and intracisternal A particle (IAP) genes of rodents (26) (Fig. 2). It has little similarity to the known human retroviruses human immunodeficiency virus and human T-cell leukemia virus. In addition to the sequence similarity, the region which has been sequenced has attributes which are common to the *pol* genes of other retroviruses. In particular, two open reading frames are present which correspond to the 3′ end of protease and the 5′ end of RT. The open reading frames overlap in a manner characteristic of several retroviruses, including type B and type D retroviruses. As with these viruses, the region of overlap of the two open reading frames contains a heptanucleotide "slippery" sequence motif in which ribosomal frameshifting could potentially occur (19, 22). HRV-5 also has several other sequence

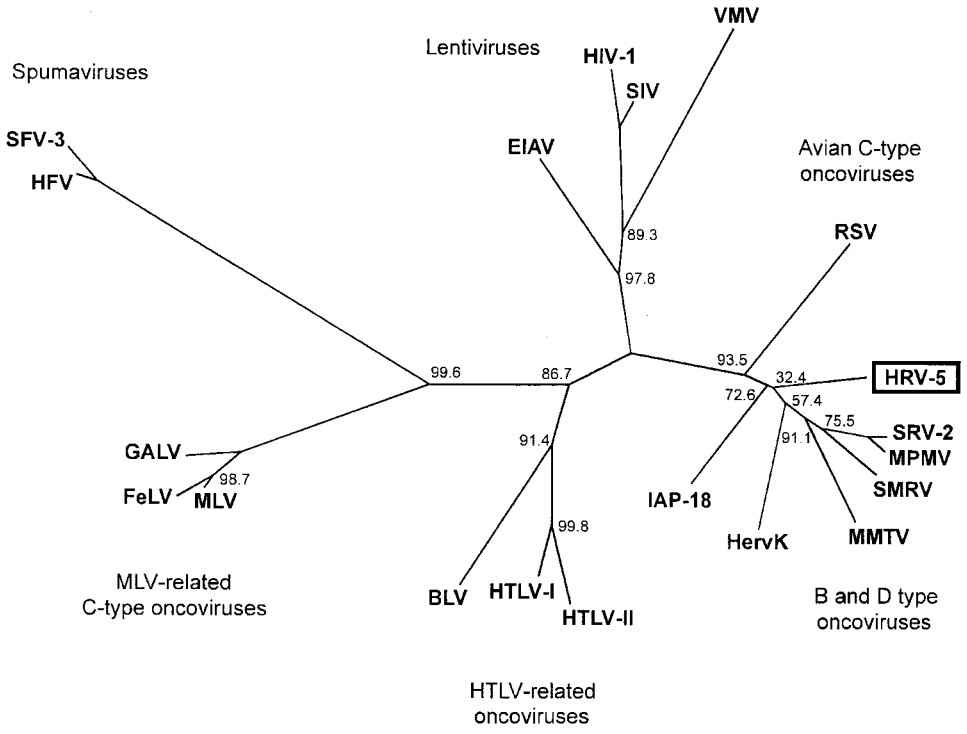

FIG. 3. Phylogenetic analysis of HRV-5. An unrooted neighbor-joining phylogenetic tree is shown based on the alignments of Fig. 2 and extended to include other retrovirus sequences. HRV-5 is boxed. Branch lengths are proportional to evolutionary distances measured as percentages of divergence. The number at each node indicates the percent frequency of this particular grouping in 1,000 trials of bootstrap resampling. Nodes for which no values are given had a value of 100%. BLV, bovine leukemia virus (40); EIAV, equine infectious anemia virus (35); FeLV, feline leukemia virus (11); GALV, gibbon ape leukemia virus (10); HervK, human endogenous retrovirus K10 (33); HIV-1, human immunodeficiency virus type 1 (57); HTLV-I, human T-cell leukemia virus type 1 (44); HTLV-II, human T-cell leukemia virus type 2 (47); HFV, human foamy virus (28); IAP-18, Syrian hamster IAP gene (32); MLV, Moloney murine leukemia virus (48); MMTV, mouse mammary tumor virus (29); MPMV, Mason-Pfizer monkey virus (51); RSV, Rous sarcoma virus (42); SFV-3, simian foamy virus 3 (39); SIV, simian immunodeficiency virus (14); SMRV, squirrel monkey retrovirus (31); SRV-2, simian type D retrovirus, serotype 2 (53); VMV, visna/maedi virus (38).

motifs which are conserved among retrovirus groups (12), including a glycine-arginine-aspartate (GRD) motif in the protease reading frame and a leucine-proline-glutamine-glycine (LPQG) motif in the RT sequence (Fig. 1). Taken together, these features provide strong evidence that HRV-5 is a fragment of the genome of a novel retrovirus.

Phylogenetic analysis. In order to define further the relationship of HRV-5 to known retroviruses, phylogenetic trees were constructed with the PHYLIP package (13) based on multiple sequence alignments of protease and RT amino acid sequences. Regions encoded by the degenerate PCR primers were omitted from this analysis, as were amino acid sequences derived from the region of overlap of the two open reading frames. Figure 3 shows an unrooted neighbor joining tree based on a composite sequence alignment of protease and RT sequences. Trees constructed by parsimony methods (using PROTPARS from PHYLIP) and least-squares-distance matrix methods (using FITCH from PHYLIP) had similar branching patterns (data not shown). The overall topology of the tree is in general agreement with previous reports (12, 59). On the basis of this analysis, HRV-5 can clearly be placed in a cluster comprising type B and type D retroviruses and rodent IAP genes. However, given the low bootstrap value (32.4%), the exact position of HRV-5 within this cluster cannot be resolved with these data.

Investigation of the distribution of HRV-5. A nested PCR assay specific for HRV-5 was developed in order to investigate the distribution of this viral sequence in a variety of human genomic DNA and RNA samples. The results of these experiments indicated that HRV-5 is present at extremely low levels in human tissues, since proviral DNA could be detected in only one of the more than 100 samples tested, which comprised 54 blood specimens (18 from SS patients), 26 salivary gland specimens (21 SS), 5 lymphoid tissue specimens (2 SS), and 19 human cell lines, including one derived from a salivary gland tumor. This positive DNA sample (patient C) was from the submandibular gland of a patient with rheumatoid arthritis and secondary SS. RNA from that patient was not available. Nested PCR experiments on serial dilutions of the plasmid

TABLE 1. Sources of independent clones of HRV-5[a]

Patient	Patient status	Tissue source
A	Primary SS	Lip biopsy/H9 coculture
B	Primary SS with lymphoma	Spleen
C	Secondary SS with RA	Submandibular gland
D	Non-SS	Submandibular gland
E	Non-SS	Parotid gland
F	Non-SS	Parotid gland
G	Primary SS	Lip biopsy/PBMC coculture
H	SLE with lymphoma	Axillary lymph node

[a] All clones were amplified from RNA extracted from sucrose gradient-fractionated tissue except clones C and G, which were amplified from genomic DNA. Samples negative for HRV-5 following gradient fractionation and RT-PCR were as follows: two from salivary glands of SS patients, three from salivary glands of patients without SS, one from the spleen of a patient without SS, three blood donors PBMCs (uncultured), and one from synovial tissue of a patient with rheumatoid arthritis (RA).

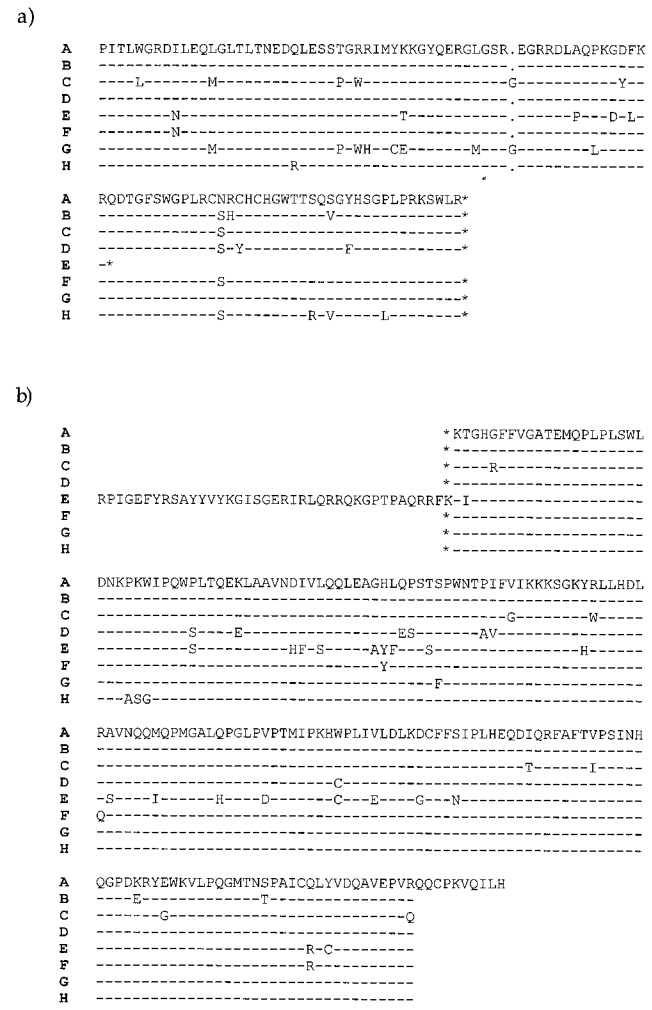

FIG. 4. Multiple sequence alignments of the deduced amino acid sequences of HRV-5 sequences amplified from eight individuals. (a) Alignment of protease reading frame; (b) alignment of RT reading frame. The sequence of each isolate is shown compared to that of the original clone. Dashes indicate identity with the HRV-5 sequence. Asterisks indicate stop codons interrupting the open reading frames. Dots indicate gaps in the alignment to allow for the insertion of glycine residues. Residues representing primer sequences are omitted. Sequences are as follows: A, the original clone from RNA extracted from a gradient-fractionated lip biopsy specimen obtained from a patient with primary SS; B, RT-PCR clone from a gradient-fractionated spleen sample obtained from a patient with primary SS and a B-cell lymphoma; C, DNA amplified from the submandibular gland tissue of a rheumatoid arthritis patient with secondary SS; D, RT-PCR clone from a gradient-fractionated parotid gland specimen obtained from a subject without SS; E, RT-PCR clone from a gradient-fractionated submandibular gland specimen obtained from a subject without SS; F, RT-PCR clone from a gradient-fractionated parotid gland specimen obtained from a subject without SS; G, DNA amplified from a 7-day coculture of a lip biopsy specimen from an SS patient and blood donor PMBCs; and H, RT-PCR clone from a gradient-fractionated axillary lymph node specimen obtained from an SLE patient.

containing the prototype HRV-5 sequence in normal human DNA indicated that the sensitivity of the nested PCR method was sufficient to detect one copy per 10^5 cells (data not shown). These experiments indicated that the abundance of HRV-5 DNA in the positive sample was at least 3 orders of magnitude below that of a single-copy gene.

By RT-PCR, HRV-5 could not be detected in whole-cell RNA or poly(A)$^+$-enriched RNA from 38 blood or salivary gland samples. Nevertheless, samples from several different patients were found to contain sequences closely related to

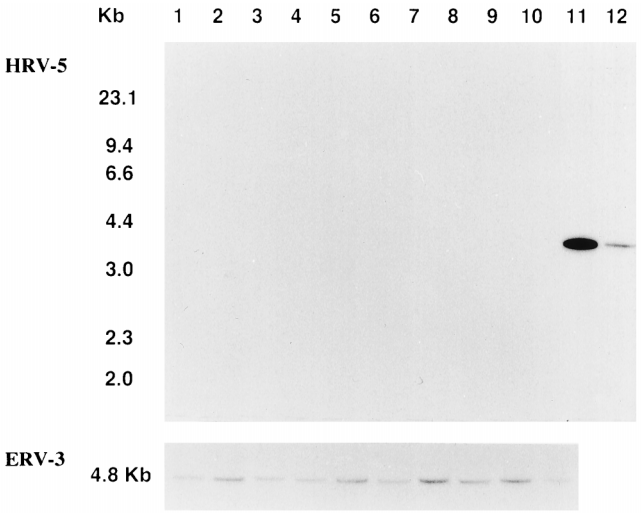

FIG. 5. Southern blot of *Eco*RI-digested human genomic DNA from six tissues and four human cell lines probed with either a 335-bp HRV-5-specific probe (upper panel) or a 404-bp probe derived from the single-copy endogenous retrovirus ERV-3 (9, 30) (lower panel). Lanes 1 and 2, peripheral blood from patients with primary SS; lanes 3 and 4, salivary gland tissue from subjects without SS (patients D and E); lane 5, spleen tissue from a patient with primary SS; (patient B); lane 6, axillary lymph node tissue from an SLE patient with SS (patient H); lane 7, H9 T-lymphoblastoid cell line; lane 8, A253 salivary gland epidermoid carcinoma cell line; lane 9, RD embryonic rhabdomyosarcoma cell line; lane 10, HeLa cervical carcinoma cell line. Lanes 11 and 12 contain 10 and 1 pg, respectively, of cloned HRV-5 sequence. The positions of molecular size markers are indicated on the left.

that of the prototype HRV-5 *pol* by using the sucrose gradient fractionation procedure prior to RT-PCR, suggesting that this step has the effect of concentrating the extremely small amount of viral RNA present. To date, with the latter method, the positive samples include one salivary gland specimen from an SS patient (the original clone), three salivary gland specimens from patients without SS undergoing surgery for head and neck tumors, one spleen specimen from an SS patient, and one lymph node specimen from a patient with systemic lupus erythematosus (SLE) (Table 1). Genomic DNA from these positive samples was negative by nested PCR. The deduced amino acid sequences of eight clones are shown in Fig. 4. Each contains open reading frames with greater than 90% similarity to the prototype sequence. This variation is due mostly to single-base changes, but in two clones an extra codon is present in the protease gene. These differences indicate that our clones are independent isolates and that they do not result from contamination of samples with the prototype sequence. Although the low fidelity of *Taq* polymerase is well documented, the sequences of six independent plasmid clones were determined for each isolate and a consensus was formed. Therefore, it is unlikely that the differences observed are due to PCR errors.

The failure to amplify HRV-5 from many genomic DNA samples indicates that this retrovirus is not present as an endogenous retrovirus in the human genome. However, in order to eliminate the possibility that the negative PCR data may be due to the presence of a large intron between the PCR primer binding sites, genomic DNA was also analyzed for the presence of HRV-5 DNA by Southern hybridization. HRV-5 was undetectable in four human cell lines and six human DNA preparations by this method, whereas ERV-3 was detectable in all the samples tested (Fig. 5). The HRV-5 probe did not hybridize to the genomic digests under conditions of relatively high stringency (final wash in 2× SSC–1% SDS at 65°C) but did

hybridize to a positive-control plasmid, producing a 3.9-kb band (Fig. 5, upper panel). In contrast, a probe specific for the single-copy element ERV-3 hybridized to all genomic samples under conditions of very high stringency (final wash in 0.1× SSC–1% SDS at 68°C) and produced bands at the expected size of 4.8 kb (30) (Fig. 5, lower panel).

Cocultivation of tissue biopsy specimens with human cell lines. Attempts were made to culture the putative retrovirus encoding HRV-5 by cocultivation of clinical samples with a variety of human cell lines. The clinical specimens studied included six labial salivary glands from SS patients, two tonsil specimens, one spleen, and one axillary lymph node. The cell lines used for cocultivation included the T-cell lines H9, SupT1, CEM, and MOLT4, the B-cell line Raji, the salivary gland epidermoid line A253, the rhabdomyosarcoma line TE671/RD, and the cervical carcinoma line HeLa. In addition, some cocultures were performed with phytohemagglutinin-stimulated PBMCs.

The presence of the HRV-5 sequence in these cocultures was assessed at weekly intervals by nested PCR with specific primers, using DNA, total cellular RNA, and RNA from sucrose gradient-fractionated homogenates of the cultures as templates. The results of these experiments showed that no cell line became consistently positive for HRV-5 by the PCR assay, indicating that the virus, if present, was not transferred to any cell line. Nevertheless, in one case, DNA from a lip biopsy sample obtained from an SS patient (patient G) which had been cocultured with PBMCs was positive for HRV-5 after 7 days in culture but was negative at 14 days and thereafter. RNA from the coculture was negative at all times (up to 28 days), as were DNA and RNA from the donor PBMCs cultured without the lip biopsy tissue. Sucrose gradients were not performed in this case.

DISCUSSION

The low abundance of HRV-5 in tissues, the high level of sequence similarity between different isolates, and the maintenance of open reading frames for two of its enzymes support the hypothesis that HRV-5 is part of an exogenous retrovirus. Minor sequence changes, including the insertion of an extra codon, indicate that our RT-PCR amplifications from RNA in particles with buoyant densities typical of retroviruses represents independent events of detection. Our inability to amplify similar sequences from human cell lines and numerous reagents in our laboratory indicate that the sequences are not laboratory artifacts and therefore are genuinely derived from human biopsy specimens. The rest of the genome remains to be characterized, but thus far RT-PCR with degenerate primers based on other conserved regions of retrovirus genomes has not allowed us to extend the sequence into *gag* or *env*.

Our data show that the 932-bp sequence resembles that of a type B or type D retrovirus *pol* sequence. The high rate of detection by the most sensitive technique, RT-PCR on gradient fractions, suggests that HRV-5 may represent a widespread infection. It will be important to determine whether it has a role in SS or other autoimmune diseases which might develop as a rare consequence of a frequent infection.

The similarity of HRV-5 to rodent IAP genes raises the possibility that HRV-5 may encode the human IAPs reported by Garry et al. (16). However, rodent IAPs are thought to be encoded by endogenous retroviruses with defective envelope genes (26). In addition, extracellular virions were not detected in the cultures studied by Garry et al. (16). Since we have shown that HRV-5 is not endogenous in the human genome, and since this element was originally detected in a sucrose gradient fraction typical of enveloped retroviruses, it seems unlikely that HRV-5 encodes the human IAPs. However, sequencing of the genomes packaged by human IAPs will be required to resolve this issue.

Although there have been many reports of retrovirus particles or RT activity associated with human tissues, cells, and secretions (58), only four retroviruses infecting humans have been characterized at the molecular level. Human T-cell leukemia virus types 1 (36) and 2 (23) were first reported in 1980 and 1982, respectively, and the lentiviruses human immunodeficiency virus types 1 (3) and 2 (8) were first reported in 1983 and 1986, respectively. A foamy (spuma) retrovirus was isolated in 1971 from a nasopharyngeal carcinoma from a Kenyan patient (1); however, recent studies (2, 43) show that foamy retroviruses are not transmitted as infectious agents from human to human but rather occur as rare zoonoses from other primates. A type D retrovirus indistinguishable from Mason-Pfizer monkey virus was reported in a lymphoma from a single patient with AIDS in 1991 (4), but further evidence of human infection by Mason-Pfizer monkey virus has not been forthcoming (20).

Assuming that the viral sequence reported in our study represents a natural human infection, it therefore is part of the fifth exogenous human retrovirus. By analogy to the nomenclature established for type D simian retroviruses (SRV-1 to -5), to human papillomaviruses, and to the three most recently described human herpesviruses (HHV-6 to -8), we have provisionally termed this virus HRV-5. As the acronym HRV has been used to denote human rhinovirus strains, the designation HuRV-5, analogous to FeLV for feline leukemia virus, might be preferred.

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