

## Detection of HERV-K(HML-2) Viral RNA in Plasma of HIV Type 1-Infected Individuals

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### ABSTRACT

Approximately 8% of the human genome sequence is composed by human endogenous retroviruses (HERVs), most of which are defective. HERV-K(HML-2) is the youngest and most active family and has maintained some proviruses with intact open reading frames (ORFs) that code for viral proteins that may assemble into viral particles. Many HERV-K(HML-2) sequences are polymorphic in humans (present in some individuals but not in others) and probably many others may be unfixed (not inserted permanently in a specific chromosomal location of the human genome). In the present study HIV-1 and HCV-1-positive plasma samples were screened for the presence of HERV-K(HML-2) RNA in an RT-PCR using HERV-K *pol* specific primers. HERV-K(HML-2) viral RNA sequences were found almost universally in HIV-1<sup>+</sup> plasma samples (95.33%) but were rarely detected in HCV-1 patients (5.2%) or control subjects (7.69%). Other HERV-K(HML-2) viral segments of the RNA genome including *gag*, *prt*, and both *env* regions, surface (*su*), and transmembrane (*tm*) were amplified from HERV-K *pol*-positive plasma of HIV-1 patients. Type 1 and type 2 HERV-K(HML-2) viral RNA genomes were found to coexist in the same plasma of HIV-1 patients. These results suggest the HERV-K(HML-2) viral particles are induced in HIV-1-infected individuals.

THE HUMAN GENOME HARBORS NUMEROUS RETROVIRAL SEQUENCES that comprise up to 8% of the host genome, many of which have accumulated lethal mutations that have impaired their ability to replicate.<sup>1–3</sup> The human endogenous retrovirus type-K (HERV-K.HML-2) family is represented by many proviruses, some of which possess intact open reading frames (ORFs) for *gag*, *prt*, *pol*, and *env* genes.<sup>4,5</sup> To date, HERV-K(HML-2) is the only endogenous retroviral subfamily with the ability to produce viral particles, apparently not infectious.<sup>6–9</sup> However, an intact HERV-K proviral sequence (K113) and perhaps other unidentified unfixed elements might code for replication-competent viruses.<sup>10–12</sup>

Lower *et al.* reported the detection of anti-HERV-K antibodies in the plasma of 70% of HIV-1 patients compared to only 3% of healthy blood donors.<sup>13</sup> Antibodies to HERV-K were also detectable in drug users, but only after HIV-1 seroconversion.<sup>14</sup> However, others have found an association between anti-HERV-K and seminoma, testicular cancer, and teratocarcinoma but not with the HIV-1 infection.<sup>15</sup> Anti-

HERV-K antibodies formed during the course of HIV-1 infection suggest that HERV-K viral-associated proteins are produced in HIV-1-infected individuals as has been observed for other tumor malignancies.<sup>9,15</sup> If HERV-K viral particles are made, it might be expected that they could be protected by viral envelopes in plasma of HIV-1-infected individuals. The RNA genome might be directly amplified from viral RNA extractions of plasma. We selected plasma-derived viral RNA samples from patients infected with HIV-1, HIV-1/HCV-1, and HCV-1 and seronegative control subjects. We screened for plasma-associated HERV-K RNA using HERV-K *pol*-specific primers. We further confirmed the presence of HERV-K(HML-2) using specific primers (Table 1).<sup>16,17</sup> Reverse transcription polymerase chain reaction (RT-PCR) was performed using the One-Step RT-PCR kit (Qiagen) according to the instructions of the manufacturer. Briefly, 5  $\mu$ l of Viral RNA equivalent to 14  $\mu$ l of plasma was reverse transcribed at 50°C for 30 min. The PCR was performed in 40 cycles, each consisting of 94°C for 1 min; an annealing step

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TABLE 1. PRIMERS USED FOR RT-PCR AMPLIFICATION OF SEVERAL HERV-K REGIONS AND OTHER CONTROLS

Target region	Forward	Reverse	$T_m$ (°C) <sup>a</sup>	Size (bp)
HERV-K <i>gag</i>	G1: 5'-AGAAGGAAAAGGTCAGAAATTA-3'	G2: 5'-AGACTTGTATCTGGCCTCAACT-3'	55	437
HERV-K <i>prt</i>	P1: 5'-GACTATAAAGGCGAAATTC-3'	P2: 5'-AGGTGAGAACGAAAGGCTCAA-3'	58	805
HERV-K <i>pol</i>	P3: 5'-TCCCTTGGAAATACTCCTGTTTTTYGT-3'	P4: 5'-CATTCCCTTGTGGTAAAACTTTCCCA YTG-3'	50	297
HERV-K <i>su</i>	ES1: 5'-AGAAAAGGGCTCCACGGAGATG-3'	ES2: 5'-ACTGCAATTAAGTAAAAATGAA-3'	52	1100
HERV-K <i>tm</i>	ET1: 5'-GCTGTAGCAGGAGTTGCATTG-3'	ET2: 5'-TAATCGATGTACTTCCAATGGTC-3'	50	1392
HERV-K <i>U5-pol</i>	U5: 5'-AAATCTCTCGTCCCACCTTAC-3'	L2: 5'-CATTCCCTTGTGGTAAAACTTTCCAYTG-3'	50	462
HERV-H <i>pol</i>	5'-TTAAGAACCTCTCATTTCCTTTCCATC-3'	5'-CTTGATGTAGGGAAGGGAGG-3'	57	126
$\beta$ -Actin RNA	5'-GCCGGCTACAGCTTCA-3'	5'-TCTCCTTAATGTCACGCACGAT-3'	58	58

<sup>a</sup>Annealing temperature: The annealing step in the PCR reaction was performed 5–8°C below the lowest  $T_m$  of the subset of primers for each reaction.

TABLE 2. DETECTION OF HERV-K RNA IN PLASMA FROM CLINICAL PATIENTS<sup>a</sup>

Source of plasma tested	Number positive	Number tested	Percentage positive
HIV-1-positive patients	184	193	95.33
HIV-1/HCV-positive patients	15	15	100.00
HCV-positive patients	1	19	5.20
Seronegative blood donors	1	13	7.69

<sup>a</sup>HERV-K viral *pol* RNA was amplified by RT-PCR using 5  $\mu$ l of RNA extractions equivalent to 14  $\mu$ l of plasma. Positive results consisted of at least two of three positive PCR replicates.

5–8°C below the  $T_m$  of the primers for 1 min and an extension step of 1 min per 0.5 kb (see Table 1).

HERV-K *pol* was positive by RT-PCR in 95.33% of the HIV-1 cases, but was rarely detected in HCV-1<sup>+</sup> and HIV-1/HCV-1 seronegative control plasma samples (Table 2). The authenticity of the PCR products was confirmed by sequencing. Neighbor-joining phylogenetic analysis of 30 HERV-K *pol* clonal sequences amplified from six different plasma samples confirmed the existence of the subfamily HERV-K(HML-2) (Fig. 1). The subfamily HERV-K(HML-3) was also coamplified in all HIV-1-positive plasma samples studied. All the *pol* sequences amplified corresponding to HERV-K(HML-2) have intact open reading frames (ORFs).

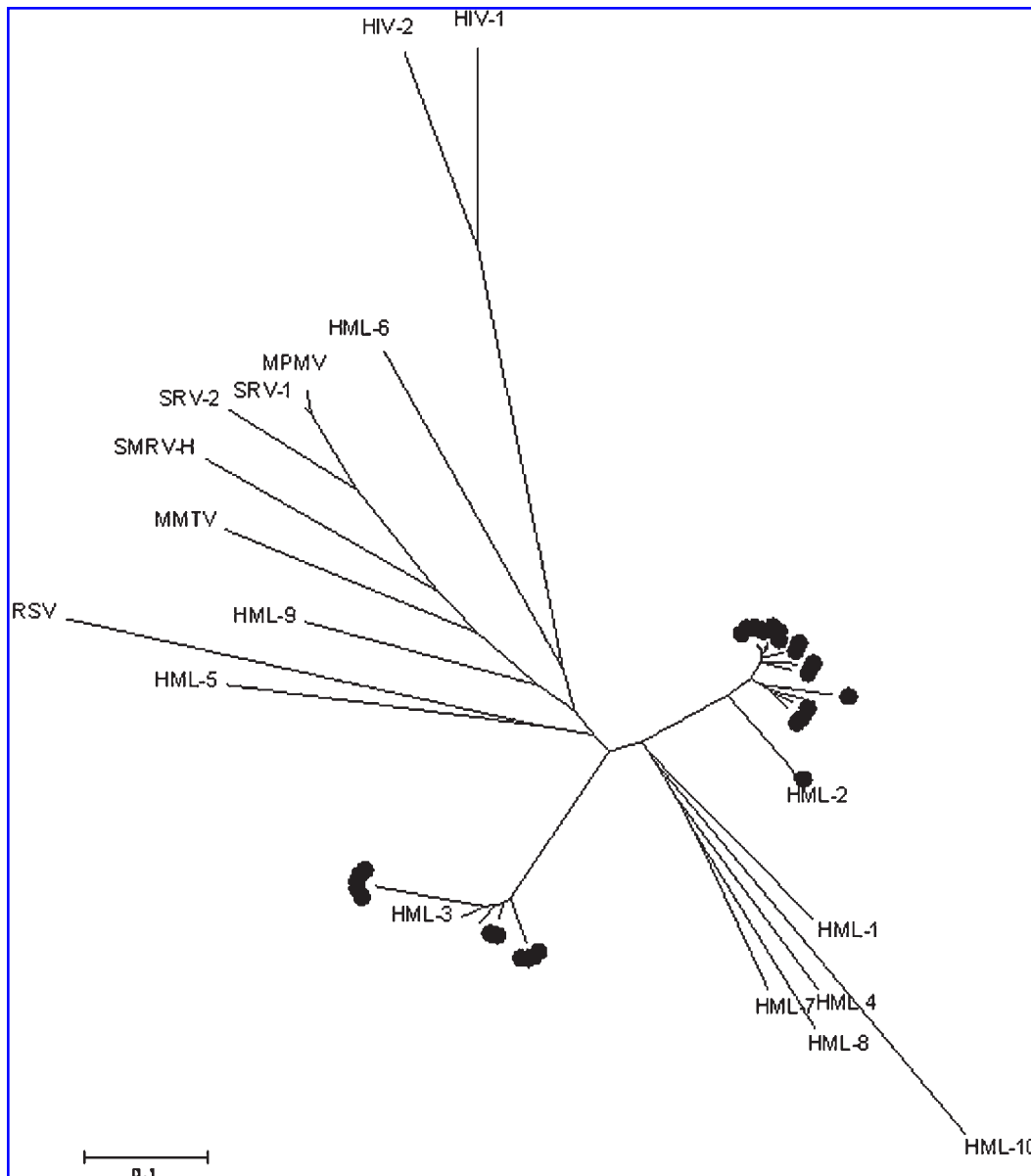
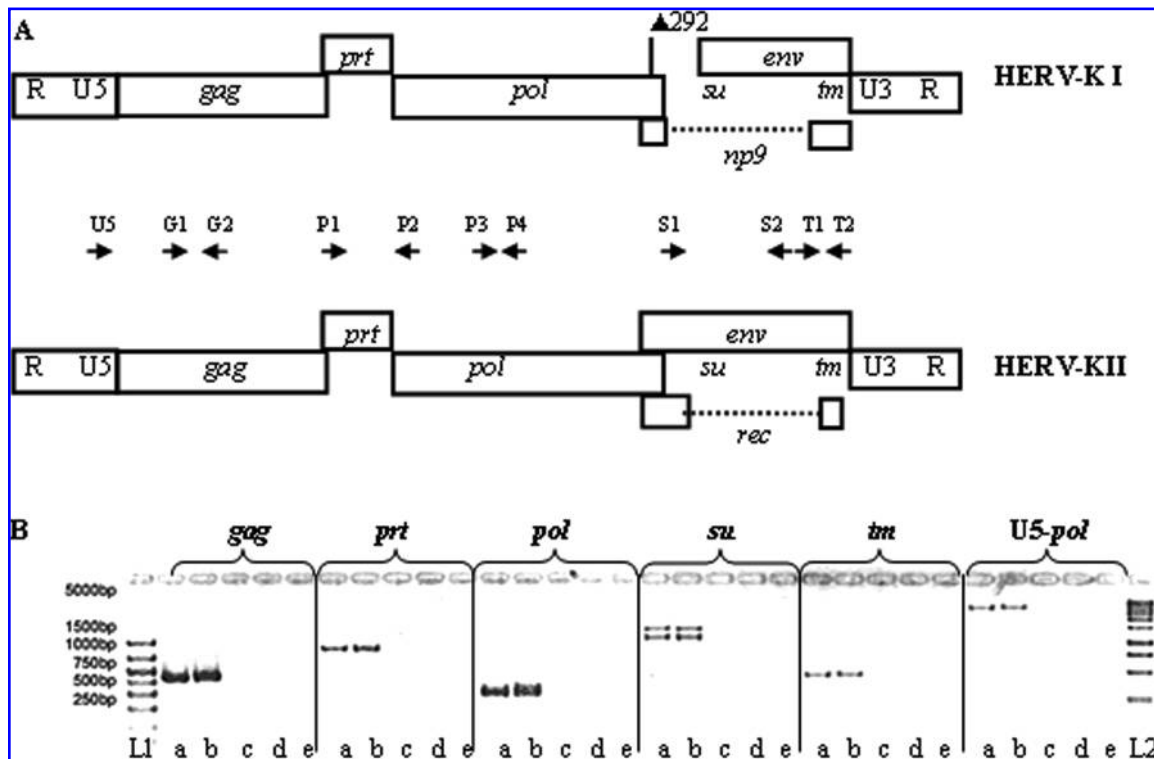


FIG. 1. Phylogenetic dendrogram of 244-bp HERV-K *pol* sequences amplified from HIV-1 patients (black circles), together with reported HERV-K subfamilies (HML1 to HML10) and type A, B, C, and D retroviruses. Queries sequences are available upon request.

To rule out the possibility of that only short *pol* RNA transcripts were present in plasma, we amplified different gene segments of the HERV-K(HML-2) viral RNA genome using the set of primers described in Table 1. We used six plasma samples taken from HIV-1<sup>+</sup>, HIV-1<sup>+</sup>/HCV-1<sup>+</sup>, HCV-1<sup>+</sup>, and seronegative patients. All HERV-K genes were amplified from HIV-1-seropositive patients but not from HCV-1<sup>+</sup> patients or control subjects (Fig. 2). We also performed an amplification reaction without the reverse transcription step to eliminate the possibility of DNA contaminants in plasma samples.  $\beta$ -Actin primers that span spliced mRNA regions did not amplify in six HIV-1 RNA extractions, suggesting that the HERV-K amplified is not a product of cellular RNA contamination. In addition, primers specific for HERV-H *pol* sequences,<sup>18</sup> previously found in plasma from rheumatoid arthritis patients,<sup>19</sup> did not amplify in HIV-1 RNA extracts. The authenticity of the RT-PCR products was confirmed by sequencing. The size of the amplification product obtained with the *env* (*su*) primers was used to determine the type of HERV-K(HML-2) present in the amplification reactions. A 292-bp deletion in type 1 viruses gives rise to an ~1105-bp amplification product. On the other hand, HERV-K type 2 genomes are characterized by an ~1397-

bp amplicon. The amplification of *env* (*su*) showed both type 1 and type 2 HERV-K(HML-2) genomes to be present in plasma samples from HIV-1 patients (Fig. 1).<sup>20-21</sup>

We further amplified a longer region of the HERV-K viral genome. By using a forward primer that spans the U5 RNA segment and a reverse primer that anneals to *pol*, we detected an ~5135 full length HERV-K genome in four of six HIV-1-positive plasma samples (Fig. 1). The results strongly suggest that full-length HERV-K RNA genomes are present in HIV-1<sup>+</sup> individuals. To protect these RNA genomes from abundant serum RNases, retroviruses have preserved the *gag* gene to encode the matrix, capsid, and nucleocapsid structures, which is a prerequisite for particle formation.<sup>22</sup> Our results suggest the presence of HERV-K viral particles in the circulating blood of HIV-1-infected individuals and provide a rationale for the detection in plasma of antibodies reactive to HERV-K.<sup>13,14</sup> Even though the difference in sensitivity between an immunoassay and an RT-PCR may be responsible for the variation in the percentage of HERV-K/HIV-1-positive patients, it is also conceivable that the difference between patients with detectable HERV-K plasma RNA and patients exhibiting an anti-HERV-K response de-



**FIG. 2.** Amplification of HERV-K viral RNA from HIV-1<sup>+</sup> plasma samples. (A) Genomic organization of HERV-K viral RNA of type 1 and type 2 viruses. HERV-K type 1 lacks a 292-bp nucleotide boundary (▲) that fuses the viral genes *pol* and *env*. The 292-bp segment in type 2 viruses has nucleotide sequences that code for the first exon of *rec*. On the other hand, type 1 HERV-K viruses code for the accessory protein, *np9*, whose viral function is unknown. In the illustrations between the HERV-K genomes are the primers that were used in this study; they are located in perspective to the regions they anneal. (B) Amplification of HERV-K genes in HIV-1 patients. RT-PCR was performed on 5  $\mu$ l of viral RNA extractions (from plasma samples) as described in Materials and Methods. Shown are the amplifications of *gag*, *prt*, *pol*, *env*, and the U5-*pol* segment representing (a) the six HIV-1<sup>+</sup> patients, (b) the six HIV-1<sup>+</sup>/HCV<sup>+</sup> patients, (c) the six HCV<sup>+</sup> patients, (d) the six healthy volunteers, and (e) the negative controls: dH<sub>2</sub>O. L1: Biomarker low (Bioventures, Inc.), L2: 1 kb Ladder (Promega). As observed in the picture of the *env* SU amplification, the lower band represents type 1 viruses (~1100 bp) and the upper band represents type 2 viruses (~1397 bp).

ected is due to HERV-K antigen titers that are sufficient to trigger an antibody response.

To date, the only HERV-K subfamily that can produce viral particles is HERV-K(HML-2).<sup>6-9</sup> We have provided evidence that HERV-K(HML-2) RNA genomes are present in HIV-1-infected plasma samples. Sequencing analyses of the proviruses that are expressed in HIV-1-positive patients indicated the activation of 32 of 128 HERV-K(HML-2) members with sequence similarities between 98.5% and 100%. Notably, these proviruses all have flanking LTRs and none of them is merely an HERV-K(HML-2 fragment). Compared to the 18 type 2 elements expressed in HIV-1 patients, many sequences were highly similar to the most recent K108, K109, K115, and K113 viruses.<sup>4,10</sup> However, some sequences were more than 2% divergent from these proviruses, which might indicate unfixed HERV-K elements may have formed in recent times. With the discovery of K113, and perhaps other replication-competent unfixed viruses,<sup>12</sup> the pathogenic roles of HERV-K in human disease must be explored further. Recent evidence suggests that humans may retain a pool of replication-competent viruses.<sup>12</sup> In addition, almost intact HERV-K polymorphisms might be functional in a fraction of individuals. Pseudotyping of HERV-K particles with functional envelopes may also yield infectious viruses, which could have a pathogenic potential.

Besides the pathogenic impact that a replication-competent virus may produce, abnormal expression of HERV-K particles and associated proteins may provoke an autoimmune response. Overexpression of HERV-K has been linked to autoimmune diseases, cancer, and neuropathological conditions, such as HIV-1-associated dementia.<sup>23-27</sup> Further, the expression of the Np9 HERV-K(HML-2) protein by type 1 viruses has been implicated in tumorigenesis.<sup>28</sup> The pathogenic impact of HERV-K in HIV-1 infection is still an open question and deserves further study.

### SEQUENCE DATA

The sequences obtained for this publication have been deposited at GenBank, accession numbers DQ166905–DQ166934, DQ157705–DQ157734, DQ159100–DQ159119, and DQ360503–DQ360809.

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