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Haplotype Analysis of the Human Endogenous Retrovirus Locus HERV-K(HML-2.HOM) and Its Evolutionary Implications

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Abstract. We and others recently identified an almost-intact human endogenous retrovirus (HERV), termed HERV-K(HML-2.HOM), that is usually organized as a tandem provirus. Studies on HERV proviral loci commonly rely on the analysis of single alleles being taken as representative for a locus. We investigated the frequency of HERV-K(HML-2.HOM) single and tandem alleles in various human populations. Our analysis revealed that another HERV-K(HML-2) locus, the so-called HERV-K(II) provirus, is also present as a tandem provirus allele in the human population. Proviral tandem formations were identified in various nonhuman primate species. We furthermore examined single nucleotide polymorphisms (SNPs) within the HERV-K(HML-2.HOM) proviral *gag*, *prt*, and *pol* genes, which all result in nonsense mutations. We identified four proviral haplotypes displaying different combinations of *gag*, *prt*, and *pol* SNPs. Haplotypes harboring completely intact proviral genes were not found. For the left provirus of the tandem arrangement a haplotype displaying intact *gag* and *prt* genes and a mutated *pol* was found in about two-thirds of individuals from different ethnogeographic origins. The same haplotype was always found in the right provirus. The various haplotypes point toward multiple recombination events between HERV-K(HML-2.HOM) proviruses. Based on these findings we derive a model for the evolution of the proviral locus since germ line integration.

Key words: Human endogenous retrovirus — Provirus — Haplotype — Recombination — ARMS-PCR

Introduction

Human endogenous retroviruses (HERVs) were formed in an ancestral genome millions of years ago by integration of exogenous retroviruses in the germ line, subsequent inheritence of proviruses from parent to offspring, and fixation within the population. Approximately 8% of the human genome is of retroviral origin, and a number of distinct HERV families were defined (International Human Genome Sequencing Consortium 2001; Li et al. 2001). Following integration, proviruses retained replicative capabilities leading to copy number increases of up to several thousand for some HERV families. Over time, proviral open reading frames (ORFs) and long terminal repeats (LTRs) accumulated mutations, resulting in coding-deficient proviruses. However, a few HERV families still include coding-competent proviruses, e.g., the HERV-K(HML-2) family displaying ORFs for *gag*, *protease*, *polymerase*, and/or *envelope* (Lower et al. 1996; Tonjes et al. 1996).

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Previously, we described a locus on human chromosome 7 containing an almost-intact HERV-K(HML-2) provirus, named HERV-K(HML-2.HOM), that was subsequently also identified by others (Barbulescu et al. 1999; Mayer et al. 1999; Tonjes et al. 1999). Only a mutation within the conserved YXDD motif in the proviral *polymerase* (*pol*) renders the reverse transcriptase domain presumably inactive. We subsequently reported that, in most cases, HERV-K(HML-2.HOM) displays a tandem organization, with a central LTR shared by both proviruses. Furthermore, proviruses displaying intact YXDD motifs were identified (Reus et al. 2001). A recent study reported two full-length, one of them apparently completely intact HERV-K(HML-2), proviruses in a fraction of the human population (Turner et al. 2001). Taken together, these data provide evidence for potentially important single nucleotide polymorphisms (SNPs), as well as the presence/absence of HERV proviruses in the human population.

It was recently pointed out by J.P. Stoye that the majority of HERV-related investigations considers a single sequenced HERV locus as representative of that HERV locus (Stoye 2001). Potentially consequential sequence variations for a particular HERV locus were, as of yet, not investigated in detail. However, as evidenced by the above examples, such variations may change an individual's HERV content significantly regarding, for instance, HERV protein coding capacity. In the present study we examined occurence of distinct HERV-K(HML-2.HOM) SNPs, as well as the frequency of proviral tandem and single alleles in the human population. Results for the latter part of our study may have direct implications for two recently published studies of the HERV-K(HML-2.HOM) tandem provirus (Hughes and Coffin 2004; Macfarlane and Simmonds 2004). We identified a limited number of SNP haplotypes that furthermore varied between different ethnogeographic groups. Based on the available data we derived a model for the evolution of the HERV-K(HML-2.HOM) locus that indicates multiple recombination events since initial provirus formation.

Methods

Collecting HERV-K(HML-2.HOM) Variants

We compiled different HERV-K(HML-2.HOM) provirus isolates from the literature (see Introduction). In addition, we retrieved from GenBank corresponding proviral sequences produced in the course of the human genome project. HERV proviral sequences were multiple aligned to identify sequence variations.

DNA Samples

DNA samples from African, Asian Indian, Southeast Asian, and Papua New Guinean individuals were kindly provided by Norbert Kienzle and Catherine Stolle. Total genomic DNA from additional Caucasian, African, and Southeast Asian donors was prepared according to standard procedures (Sambrook 1989). Genomic DNA from Aborigine individuals was obtained from the European Collection of Cell Cultures (ECACC).

Long-PCR Primers and Conditions

The left provirus of the HERV-K(HML-2.HOM) tandem repeat was amplified from total genomic DNA by long-PCR using primers 5′flank2FOR (5′TACCCACAGCACCCAAGAGATG3′) and envendREV (5′GTCATCATGGCCCGTTCTCG3′). PCR cycling conditions were as follows: 93°C for 5 min; 9 cycles of 93°C for 15 s, 54°C for 30 s, and 68°C for 9 min; followed by 19 cycles of 93°C for 15 s, 54°C for 30 s, and 68°C for 11 min; and final incubation for 10 min at 68°C. The right provirus was amplified by long-PCR using primers 5′gagFOR (5′CGCTCGGAAGAAGCT AGG3′) and 3′flankREV (5′GCTTTCGGGACTTGAACATTG G3′). PCR cycling conditions were as above. Single proviruses were amplified from total genomic DNA by long-PCR using primers 5′flank2FOR and 3′flankREV with cycling conditions as above. Tandem proviruses were amplified by an extended long-PCR using primers 5′flank2FOR and 3′flankREV. PCR cycling conditions were as follows: 93°C for 5 min; 9 cycles of 93°C for 15 s, 54°C for 30 s, and 68°C for 12 min; followed by 19 cycles of 93°C for 15 s, 54°C for 30 s, and 68°C for 12 min; and final incubation for 12 min at 68°C. HERV-K(HML-2.HOM) and HERV-K(II) tandem provirus portions, spanning a 292-bp region within pol–env and the central LTR, were amplified with primers HK290FOR (5′GGGGAGAGGTTTTGCTTGT3′) and LTRgagREV2. Cycling conditions were as follows: 94°C for 5 min; 10 cycles of 94°C for 15 s, 58°C for 30 s, and 68°C for 4 min; 20 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 4 min (+5 s); and 72°C for 7 min. The TripleMaster PCR-System (Eppendorf), was used for long-PCR reactions as recommended by the manufacturer. Long-PCR products were TA-cloned employing the pGEM T Easy Vector System (Promega), and inserts were sequenced using vector-specific primers.

PCR Primers and Conditions

For verification of target site duplications a sequence portion flanking the left provirus was amplified from total genomic DNA by PCR using primers 5′flank2FOR and LTRgagREV2 (5′TTTGCCCCATTATCACCCTA3′). PCR cycling conditions were as follows: 94°C for 5 min; 30 cycles 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min; and final incubation for 1 min at 72°C. A sequence portion flanking the right provirus was PCR-amplified from total genomic DNA using primers 3′flankREV and ENV8406 (5′GTCTGCAGGTGTACCCAACAG3′). PCR cycling conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min; and final incubation for 1 min at 72°C. The central LTR in the tandem provirus was amplified from total genomic DNA using primers ENV8406F and LTRgagREV2. PCR cycling conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min; and final incubation for 1 min at 72°C. Taq DNA polymerase was used for all those PCRs as recommended by the manufacturer (Invitrogen).

ARMS-PCR

ARMS-PCR (Amplification Refractory Mutation System) (Newton et al. 1989) was applied to genotype SNPs in the HERV-K(HML-2.HOM) *gag*, *prt*, and *pol* genes. ARMS-PCR is based on the fact that oligonucleotides with a mismatched 3′ residue will not function as primers in the PCR under stringent conditions. Each SNP is genotyped by two separate PCR reactions amplifying the two different SNP alleles. Therefore, each sample was tested with an appropriate "normal" or "mutant" forward primer for the respective proviral gene, paired with a universal reverse primer. Specificity of ARMS-PCR was optimized by varying Mg^{2+} concentrations and annealing temperature, until the "normal" forward primer was refractory to PCR on "mutant" template DNA, and vice versa. Purified and diluted PCR product from the long-PCRs was used as template for ARMS-PCR. Sequence analysis of a number of long-PCR products confirmed the ARMS-PCR results. As controls, we included in each experiment two ARMS-PCRs containing template with known SNP sequences. Primers gagARMS1for (C-allele) (5′GTCTCTCTCAC CCTCTCAATTTTTAC3′), gagARMS2for (T-allele) (5′GTCTCT CTCACCCTCTCAATTTTTAT3′), and gagARMSrev (5′GCTTT ATGCATAGCTCCTCCG3′) were used to characterize the SNP in the proviral *gag* gene. PCR parameters were as follows: 94°C for 5 min; 25 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 25 s; and final incubation for 10 min at 72°C. Reaction mixtures contained 0.75 mM $MgCl_2$. Primers polARMS1for (G allele) (5′AAG TTTTCAGACTGTTATATTATTCATTG3′), polARMS2for (A allele) (5′AAGTTTTCAGACTGTTATATTATTCATTA3′), and polARMSrev (5′ACTGTGAGGAAGGAATGACCA3′) were selected to characterize the *pol* gene SNP. PCR parameters were as for *gag*. Reactions included 1.2 mM $MgCl_2$. Since the mutation in the *prt* gene is a deletion, but no substitution, we restricted the PCR to detection of the nonmutated allele. Presence of the mutated allele in those samples failing to amplify a PCR product under stringent conditions was confirmed by PCR under less stringent conditions. We designed forward primer prtARMS for (5′CTTCCAGGGGA GCCCCC3′), corresponding to the nonmutated allele, and reverse primer prtARMSrev (5′GCTATATGATG GAGCGGGC3′). PCR parameters were as follows: 94°C for 5 min; 25 cycles of 94°C for 30 s, 67°C for 30 s, and 72°C for 25 s; and a final incubation of 10 min at 72°C. Reactions included 0.5 mM $MgCl_2$. All ARMS-PCRs furthermore contained a 100 μM concentration of each dNTP, and 2.5 U of Taq polymerase in standard PCR buffer (Gibco, BRL, Life Technologies).

Characterization of Homo- and Heterozygotes

Homozygotes are characterized by a PCR product in only one of two reactions required to type each SNP (Fig. 4). Presence of PCR products in both reactions potentially results from heterozygosity. We then cloned the long-PCR product and performed ARMS-PCR onto plasmid DNA from several independent clones as template, confirming the presence of both alleles.

DNA Sequencing and Analysis

Cloned PCR products were sequenced using the SequiTherm Excel II DNA Sequencing Kit-LC (Biozym) on an automated DNA sequencer (Licor 4000-L, MWG). Sequence data were analyzed using the Sequencher software (Gene Codes Corp. Inc.).

Results

Frequency of Tandem and Single Proviral Alleles

As opposed to the common HERV-K(HML-2.HOM) tandem arrangement where a central LTR is shared by both proviruses, we recently identified two individuals from Papua New Guinea (PNG) that harbor single HERV-K(HML-2.HOM) proviruses in a homozygous state (Reus et al. 2001). Here we examined 67 human DNA samples, representing 134 chromosomes, from various ethnogeographic origins for the presence of tandem and single proviruses. Tandem proviruses were identified by PCR using a forward primer located in the retroviral *env* gene and a reverse primer in the *gag* gene, encompassing the central LTR. A PCR product of 1216 bp indicated the presence of the tandem allele. Single proviruses were amplified by long PCR using primers located in the 5′ and 3′ flanking regions, resulting in a 9891-bp PCR product. Among the 69 DNA samples included in this study (67 + 2 PNG) we identified five more samples to harbor single proviruses in a homozygous state, totaling 10% of individuals. Sixteen samples (23%) harbored tandem proviruses in a homozygous state, and 46 individuals (67%) were heterozygous for single and tandem alleles. Single proviruses were found in all ethnogeographic groups. Homozygous states for single proviruses were found in two individuals from Papua New Guinea (Reus et al. 2001), one African, and four Asian individuals.

Another HERV-K(HML-2) Tandem Provirus in the Human Population

The observed frequencies of single and tandem alleles of the HERV-K(HML-2.HOM) locus appeared different from those predicted by the Hardy–Weinberg equilibrium. Heterozygous individuals were significantly more frequent, and individuals carrying single proviruses in a homozygous state were observed less often than expected. We evaluated statistical significance of observed versus expected frequencies, employing a *G*-test. A *G*-value of 15.694 indicated that observed genotype frequencies differ significantly ($p < 0.001$) from the Hardy–Weinberg equilibrium. The observed disequilibrium is not due to PCR artifacts. It is imaginable that false-positive PCR products indicative of single proviruses may be due to annealing and extension of partly overlapping DNA strands that actually stem from a tandem provirus. Such PCR products could feign a single provirus. We verified the presence of single and tandem proviruses by an extended long-PCR approach able to amplify both tandem and single alleles. Long-PCR results confirmed previous findings for 10 representative samples supposedly homozygous or heterozygous for single and/or tandem proviruses. PCR products with lengths characteristic for single and tandem alleles were amplified. Importantly, homozygous tandem alleles never resulted in artifactual PCR products characteristic of single proviruses (Fig. 1).

The observed disequilibrium—more tandem provirus alleles than expected—could also be explained by the presence of a tandem provirus other than the HERV-K(HML-2.HOM) locus in the human popu-

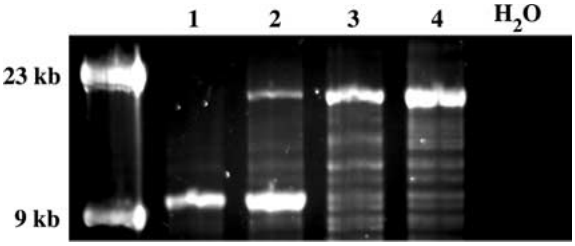

Fig. 1. Representative results for amplification of single and tandem HERV-K(HML-2.HOM) proviruses, employing an extended long-PCR approach. Single proviruses in homozygous and heterozygous states are shown in lanes 1 and 2, while lanes 3 and 4 show results for tandem proviruses in a homozyous state. Note that the latter PCRs did not yield products characteristic of single proviruses, excluding possible PCR artifacts (see text). PCR products were separated by electrophoresis in a 0.8% TAE agarose gel.

lation. Another tandem provirus could equally produce a 1.2-kb PCR product when testing for the presence of a central LTR (see above). We therefore cloned the 1.2-kb PCR product obtained from various individuals, indicative of a tandem provirus allele, and sequenced a number of clones each. Indeed, several cloned PCR products were clearly different from the HERV-K(HML-2.HOM) provirus sequence. Further sequence comparison showed that those sequences matched a HERV-K(HML-2) provirus located on chromosome 3q12.3, which was previously reported by others as HERV-K(II) (Sugimoto et al. 2001). Further sequence comparisons show that the central LTR of the HERV-K(II) tandem provirus arrangement comprises the proviral 5′LTR that displays a number of differences compared to the 3′LTR (Fig. 2). We further confirmed the presence of a HERV-K(II) tandem provirus in human individuals by PCR. We made use of the fact that HERV-K(II) is a type 1 provirus, lacking a 292-bp sequence within the *pol–env* boundary that is present within the type 2 HERV-K(HML-2.HOM) provirus (Lower et al. 1995; Ono et al. 1986). Thus, a PCR product spanning that region and the central LTR distinguishes HERV-K(II) from HERV-K(HML-2.HOM) tandem provirus alleles by a 292-bp size difference. Indeed, PCR primers HK290FOR and LTRgagREV2 yielded in some individuals PCR products of sizes characteristic for either HERV-K(II) or HERV-K(HML-2.HOM) tandem alleles (Fig. 2). We therefore conclude that the human population harbors not only a tandem allele of the HERV-K(HML-2.HOM) provirus, but also a tandem allele of the HERV-K(II) provirus. The observed higher number of tandem proviruses can be fully explained by this finding (Fig. 2 and Discussion).

We furthermore note that formation of HERV-K(HML-2) tandem proviruses is obviously not restricted to humans. We also examined various primate species using the above-mentioned *env* forward and *gag* reverse primers. Besides a PCR product characteristic of tandem alleles in *Pan troglodytes* (Reus et al. 2001), we obtained PCR products from genomic DNA from *Gorilla gorilla*, *Macaca mulatta*, and *Macaca fascicularis*. Sequences of PCR products obtained from *Gorilla gorilla* and *Macaca fascicularis* (GenBank accession nos. AY425962 and AY425963) confirmed *env*–LTR and LTR–*gag* boundaries. No PCR products were obtained from *Pan paniscus*, *Pongo pygmaeus*, *Hylobates lar*, *Mandrillus sphinx*, and *Colobus guereza* (data not shown).

HERV-K(HML-2.HOM) SNPs

The HERV-K(HML-2.HOM) proviral locus has been repeatedly isolated by us and others in specific studies and in the course of the human genome sequencing project (Barbulescu et al. 1999; International Human Genome Sequencing Consortium 2001; Mayer et al. 1999; Tonjes et al. 1999). We identified sequence variants among those different proviral isolates. We found that the different reported proviral sequences displayed single nucleotide polymorphisms (SNPs) within the *gag*, *prt*, and *pol* genes, resulting in a stop codon, a frameshift, and a mutation within the polymerase YXDD motif, respectively. With respect to HERV-K(HML-2.HOM), a C/T exchange at nt 2204 resulted in a stop codon in *gag*. An extra C at nt 3158 resulted in a frameshift and a premature stop codon in *prt*. An A/G exchange at nt 4462 altered a highly conserved reverse transcriptase motif from YIDD to CIDD. Differences between various HERV-K(HML-2.HOM) isolates are summarized in Fig. 3. SNPs in *gag*, *prt*, and *pol* occurred in different combinations. The recently reported HERV-K(HML-2.HOM) proviral sequence, isolated from an Asian sample, displayed mutated *gag* and *prt* genes and an intact YXDD motif (GenBank accession no. AF490464) (Reus et al. 2001). Barbulescu et al. (1999) reported a similarly mutated proviral sequence (AF261945). The provirus isolated by Tonjes et al. (1999) (Y17832) harbored an intact *gag* but mutated *prt* and *pol* genes. The corresponding chromosome 7 proviral entry from the human genome sequencing project (AC072054) displays intact *gag* and *prt* genes but a mutated *pol*. Additional nonsense mutations were not detected in the respective genes.

We next examined the occurrence of the above-mentioned *gag*, *prt*, and *pol* gene SNPs in the human population. Using ARMS-PCR as an allele-specific PCR approach (Newton et al. 1989), we analyzed 67 human DNA samples, representing 134 chromosomes, from various ethnogeographic origins. Since the HERV-K(HML-2.HOM) provirus is commonly organized in tandem (Reus et al. 2001), we separately amplified and analyzed the left and the right portion of the tandem provirus.

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         nt 543/9047
HOM Tandem  TTG----TATGCTCCATCTACTGAGATAGGGAAAAACCGCCTTAGGGCTGGAG--GTGGGACCTGCGG
HERV-K(II)  ...----..C.T..................A.G...A................--.......A...A.
K(II) 5'LTR ...ATTG..C.T..................A.G...A................GT.......AA..C.
K(II) 3'LTR ...----..C.T..................A.G...A................--.......A...A.
clone4      ...----..............................................--.............
clone5      ...----..............................................--.............
clone6      ...ATTG..C.T..................A.G...A................GT.......AA..C.
clone7      ...----..............................................--.............
clone8      ...ATTG..C.T..................A.G...A................GT.......AA..C.
clone9      ...----..............................................--.............
clone11     ...ATTG..C.T..................A.G...A................GT.....-.AA..C.
clone12     ...ATTG..C.T..................A.G...A....-...........GT.......AA..C.
clone13     ...----..............................................--.............
clone14     ...ATTG..C.T..................A.G...A................GT.......AA..C.
clone15     ...ATTG..C.T..................A.G...A................GT.......AA..C.
clone16     ...ATTG..C.T..................A.G...A................GT.......AA..C.
clone19     ...----..C.T..................A.G...A................--.......A...A.
clone20     ...----.-............................................--.............
clone21     ...ATTG..C.T................A.A.G...A................GT.......AA..C.
clone22     ...----..C.T..................A.G...A................--.......A...A.
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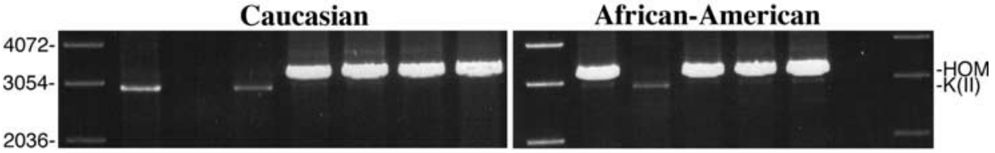

	$S^{H}S^{II}$	$S^{H}T^{II}$	$T^{H}S^{II}$	$T^{H}T^{II}$
$S^{H}S^{II}$	$S^{H}S^{II}$ $S^{H}S^{II}$	$S^{H}S^{II}$ $S^{H}T^{II}$	$S^{H}S^{II}$ $T^{H}S^{II}$	$S^{H}S^{II}$ $T^{H}T^{II}$
$S^{H}T^{II}$	$S^{H}T^{II}$ $S^{H}S^{II}$	$S^{H}T^{II}$ $S^{H}T^{II}$	$S^{H}T^{II}$ $T^{H}S^{II}$	$S^{H}T^{II}$ $T^{H}T^{II}$
$T^{H}S^{II}$	$T^{H}S^{II}$ $S^{H}S^{II}$	$T^{H}S^{II}$ $S^{H}T^{II}$	$T^{H}S^{II}$ $T^{H}S^{II}$	$T^{H}S^{II}$ $T^{H}T^{II}$
$T^{H}T^{II}$	$T^{H}T^{II}$ $S^{H}S^{II}$	$T^{H}T^{II}$ $S^{H}T^{II}$	$T^{H}T^{II}$ $T^{H}S^{II}$	$T^{H}T^{II}$ $T^{H}T^{II}$

genotype	frequency	expected	observed
S/S	1/16	6.25%	10%
S/T	11/16	68.75%	67%
T/T	4/16	25%	23%

Fig. 2. Detection of an additional HERV-K(HML-2) tandem provirus allele in the human population. **Top** A multiple alignment of a diagnostic LTR sequence portion from various PCR products, indicative of the presence of a tandem provirus (see text), with the corresponding portion of the HERV-K(HML-2.HOM) and HERV-K(II) proviruses. The latter sequence was obtained from the most recent human genome sequence. HERV-K(II) 5′ and 3′ LTRs are also included in the alignment to demonstrate the higher sequence similarity of PCR products with the HERV-K(II) 5′ LTR rather than the 3′ LTR. Numbers above the alignment indicate the location of the regarded sequence region with respect to the HERV-K(HML-2.HOM) sequence (GenBank accession no. AF074086). **Middle** Representative results of a PCR from Caucasian and from African-American individuals that specifically distinguishes HERV-K(HML-2.HOM) from HERV-K(II) tandem proviruses, owing to the lack of a 292-bp sequence within the amplicon of HERV-K(II) (see text). PCR products from the HERV-K(HML-2.HOM) and the HERV-K(II) tandem provirus are expected to be 3257 and 2965 bp, respectively, in size. DNA marker bands (bp) are indicated on the left. **Bottom** Summary of expected genotype frequencies when a second tandem provirus allele is considered. Pale gray fields indicate that corresponding genotypes (human DNA samples) will appear in corresponding PCR tests as heterozygous for the HERV-K(HML-2.HOM) tandem allele, although they may be heterozygous for HERV-K(HML-2.HOM) single proviruses, because PCR products actually stem from the HERV-K(II) tandem provirus. Dark gray fields indicate homozygosity for tandem proviruses. Genotypes and expected and observed frequencies are summarized at the bottom.

We amplified the left provirus from the HERV-K(HML-2.HOM) tandem by long-PCR using a forward primer in the 5′ flanking chromosomal sequence and a reverse primer in *env*.

Gel-purified long-PCR products served as templates for ARMS-PCR that consisted of two reactions, each containing an allele-specific forward primer and a conserved reverse primer. A PCR product in only one reaction indicated homozygosity of the analyzed SNP while PCR products in both reactions indicated heterozygosity (Fig. 4). The latter was further confirmed by ARMS-PCR analysis of several independent plasmids cloned from long-PCR products. Furthermore, sequencing of the SNP-con-

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 Position                nt 2204
 Accession no.             |
gag  AF074086  CCCTCTCAATTTTTACAATTTAAGACTTGGT
     AF490464  ...............T...............
     AF261945  ...............T...............
     AC072054  ...............C...............
     Y17832    ...............C...............
  correct ORF    S  Q  F  L  Q  F  K  T  W
  mutated ORF    S  Q  F  L  *

                         nt 3158
                           |
prt  AF074086  TTCCAGGGGAGCCCCCACAAAAAACCCCCAC
     AF490464  ...............-...............
     AF261945  ...............-...............
     AC072054  ...............................
     Y17832    ...............................
  correct ORF    P  G  E  P  P  Q  K  T  P
  mutated ORF    P  G  E  P  H  downstream stop

                         nt 4462
                           |
pol  AF074086  GTTATATTATTCATTGTATTGATGATATTTT
     AF490464  ...............A...............
     AF261945  ...............A...............
     AC072054  ...............G...............
     Y17832    ...............G...............

  correct ORF    Y  I  I  H  C  I  D  D  I
  mutated ORF    Y  I  I  H  Y  I  D  D  I
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Fig. 3. Sequence comparison of different HERV-K(HML-2.HOM) provirus isolates, as previously reported by us and others (for references, see text). Relevant *gag*, *prt*, and *pol* regions and corresponding reading frame changes are shown. Polymorphisms in *gag* and *pol* result in a stop codon and an amino acid exchange, respectively. An extra C in *prt* causes a premature stop. SNP positions are numbered with respect to the HERV-K(HML-2.HOM) sequence. Correct and mutated reading frames are indicated. Asterisk indicates a stop codon.

taining gene regions of three retroviral inserts confirmed the ARMS-PCR results, and excluded additional mutations altering *gag*, *prt*, and *pol* for those clones.

As for the left provirus, ARMS-PCR identified 27 chromosomes (20%) displaying the stop mutation within *gag*, 28 (21%) having the frameshift within *prt*, and 92 (69%) showing a defective YXDD motif. The identified single nucleotide changes could be summarized in four different haplotypes, in the following referred to as haplotypes A, B, C, and D. Haplotype A shows mutant *gag* and *prt* genes and a *pol* with an intact YXDD motif. Haplotype B shows an intact *gag* but mutated *prt* and *pol*. In haplotpye C only *pol* is mutated. In haplotype D all three genes carry mutations. Haplotype C was most frequent (92 chromosomes; 69%), followed by haplotype A (24; 18%), haplotype D (5; 3.7%) and haplotype B (1; 0.7%) (Table 1). Notably, we did not identify any of the other four possible haplotypes. Of further importance, a haplotype displaying ORFs for all retroviral genes was not detected either.

The right provirus was amplified from the tandem arrangement by long PCR using a forward primer located at the start of *gag* and a reverse primer located in the 3′ flanking region. SNP analysis was as described above. We chose eight DNA samples that displayed different haplotypes in the left provirus and that were of different ethnogeographic origin. The 16 analyzed chromosomes uniformly displayed haplotype C, that is, intact *gag* and *prt*, but a mutated YXDD-motif in *pol*. ARMS-PCR results are summarized in Table 1. All HERV-K(HML-2.HOM) variants identified in this study are summarized in Fig. 5.

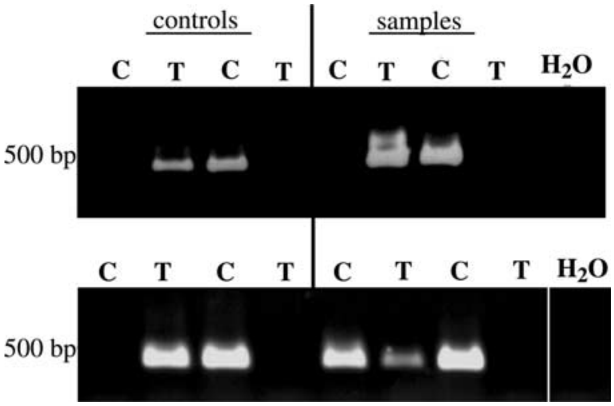

Fig. 4. Representative ARMS-PCR results for detection of HERV-K(HML-2.HOM) *gag* SNPs. The left portion of the top and bottom panels shows control reactions employing sequences with either thymine (T) (lanes 1 and 2) or cytosine (C) (lanes 3 and 4) in the relevant position. The right portions of both panels each show analyzed genomic DNA samples. Samples 1, 2, and 4 display a homozygous state, while sample 3 is heterozygous for the regarded SNP.

We furthermore analyzed haplotype distribution depending on ethnogeographic groups included in this study (Table 2). Haplotype C was most frequent in all groups, followed by haplotype A. Compared to Caucasians, Southeast Asian and African individuals displayed greater numbers of haplotype A. Notably, Aborigine and Papua New Guinea individuals exclusively displayed haplotype C. Haplotypes B and D were found in African individuals in one case each, and haplotype D was also present in Southeast Asians. As revealed by Fisher's exact test for 3×2 tables, haplotype distributions were significantly different ($p = 0.0051$) between Caucasians and Southeast Asians and between Aborigine and Southeast Asian individuals ($p = 0.005$). Sample numbers for other groups were too small to determine statistical significance.

Discussion

There is limited information on HERV polymorphisms. A recent study reported full-length retroviruses, termed HERV-K113 and HERV-K115, in 15% and 29% of the human population, respectively (Turner et al. 2001). A relatively recent formation of

Table 1. HERV-K(HML-2.HOM) haplotypes and frequencies as revealed in this study[a]

				Frequency	
Haplotype	*gag*	*prt*	*pol*	Left PV	Right PV
A	Stop	Frameshift	YIDD	28	0
B	No stop	Frameshift	CIDD	1	0
C	No stop	No frameshift	CIDD	100	16
D	Stop	Frameshift	CIDD	5	0

[a] Four different haplotypes were identified in this study displaying intact or defective *gag* and *prt* reading frames and an intact (YIDD) or defective (CIDD) reverse transcriptase motif in *pol*. As HERV-K(HML-2.HOM) is usually organized as a tandem provirus, haplotype frequencies for the left (left PV) and the right provirus (right PV) are given.

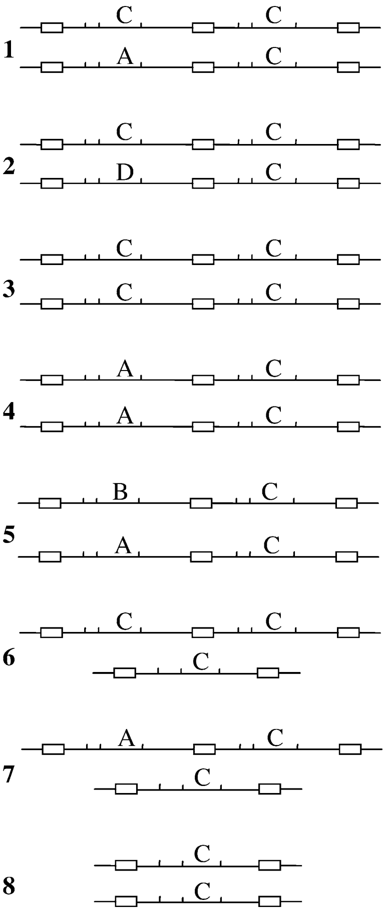

Fig. 5. Summary of HERV-K(HML-2.HOM) haplotypes identified in different individuals. Tandem and single proviruses are illustrated. Haplotypes are indicated above each provirus. Rectangles represent LTRs. Boundaries between proviral *gag*, *prt*, *pol*, and *env* genes are depicted by short vertical lines. Haplotypes 6–8 are heterozygous or homozygous for tandem and single proviruses, respectively. See text and Table 1 for haplotype details.

Table 2. HERV-K(HML-2.HOM) haplotypes identified in human individuals[a]

	Haplotype			
Ethnogeographic group	A	B	C	D
Caucasian	5		31	
Southeast Asian	11		17	4
African	11	1	27	1
Indian	1		7	
Aborigine			14	
Papua New Guinea			4	
Total	28	1	100	5

[a] The various haplotypes are defined by SNPs in the proviral *gag*, *prt,* and *pol* genes (Table 1). Overall haplotype frequencies are summarized in the last two columns.

these proviruses in evolution may explain why HERV-K113 and HERV-K115 are not fixed in humans. Hughes and Coffin (2004) revealed several polymorphic HERV-K(HML-2) loci in the human population. Polymorphic solitary LTRs in the HLA-DQ locus were previously reported (Kambhu et al. 1990). As for single nucleotide polymorphisms (SNPs) within particular proviral sequences, it was shown that a SNP within the ERV-3 *env* gene results in a stop codon. About 1% of Caucasian individuals are homozygous for the defective allele, thus coding deficient for the *Env* protein for which a role in placental development had been postulated (de Parseval and Heidmann 1998). Furthermore, SNPs in the so-called HERV-K18 provirus were analyzed in diabetes mellitus type 1 patients but were found not to be correlated with the disease (Kinjo et al. 2001). We recently reported HERV-K(HML-2.HOM) variants with and without intact YXDD motifs within the reverse transcriptase domain, a tandem organization of HERV-K(HML-2.HOM) in a majority of human genomic DNA samples, and a single provirus in two samples from Papua New Guinea (Reus et al. 2001). From several independent investigations it became apparent that the HERV-K(HML-2.HOM) locus displays defined sequence variations, SNPs, within the *gag*, *prt*, and *pol* genes that effect the coding capacity of those genes by introducing stop mutations or altering a conserved catalytic motif. We present here a systematic investigation of those HERV-K(HML-2.HOM) polymorphisms in various ethnogeographic groups.

When we investigated the presence of HERV-K(HML-2.HOM) single versus tandem provirus alleles, observed frequencies significantly differed from

Hardy–Weinberg equilibrium ($p < 0.001$). Further analysis revealed that the so-called HERV-K(II) provirus on chromosome 3q12.3 is likewise present in the human population as a tandem provirus allelic variant. The HERV-K(II) tandem allele produced similar-sized PCR products when human DNA samples were tested for the *env*–central LTR–*gag* arrangement, and thus feigned HERV-K(HML-2.HOM) tandem provirus alleles. While the overall structure of the HERV-K(II) tandem provirus is currently not known, the presence of a second tandem provirus allele in the human population explains very well the genotype frequencies observed in our analysis. In our PCR-based analysis, we tested for single HERV-K(HML-2.HOM) proviruses and for tandem alleles (see Methods). Thus, the presence of a second tandem provirus will increase the overall number of genotypes heterozygous for single and tandem proviruses and will reduce the overall number of genotypes homozygous for single proviruses. Indeed, our observed genotype frequencies match very well the expected frequencies (Fig. 2). In detail, 25% of genotypes are expected to represent single HERV-K(HML-2.HOM) proviruses in a homozygous state. However, of those genotypes with two HERV-K(HML-2.HOM) single proviruses, three will contain at least one HERV-K(II) tandem provirus (Fig. 2). In PCR analysis, those genotypes will thus appear as heterozygous for a HERV-K(HML-2.HOM) single and tandem allele. Hence, the overall number of homozygous single HERV-K(HML-2.HOM) provirus alleles is reduced from 4 of 16 (=25%) to 1 of 16 (=6.25%) since the other 3 of 16 (=18.75%) will appear as heterozygous for a single and a tandem allele of HERV-K(HML-2.HOM). Indeed, we observe 10% of individuals to be homozygous for HERV-K(HML-2.HOM) single proviruses. Accordingly, the 3 of 16 genotypes with homozygous HERV-K(HML-2.HOM) single proviruses, but at least one HERV-K(II) tandem provirus, will add to the number of expected heterozygous single/tandem alleles, increasing their frequency from 50% to 68.75%. Indeed, we observed 67% of individuals to contain single and tandem proviruses in a heterozygous state.

We note in this context that two previous studies on the HERV-K(HML-2.HOM) provirus allele did not reveal a second HERV-K(HML-2) tandem provirus allele in the human population (Hughes and Coffin 2004; Macfarlane and Simmonds 2004). Since the detection of a tandem provirus by means of PCR amplification of an *env*–central LTR–*gag* portion was very similar, or identical, to our methodology, it is possible that some of the observed HERV-K(HML-2.HOM) tandem allele frequencies, that likewise deviate from Hardy–Weinberg equilibrium, actually stem from HERV-K(II) tandem proviruses. It is currently not known whether the human population harbors HERV-K(HML-2) tandem proviruses in addition to the two examined/revealed in our study. As the recent study by Hughes and Coffin (2004) shows considerable allelic variation for several HERV-K(HML-2) proviruses in different chromosomal locations, it seems possible that other HERV-K(HML-2) tandem provirus alleles indeed exist.

Besides the frequencies of tandem and single alleles, we also characterized particular SNPs within the *gag*, *prt*, and *pol* genes of the HERV-K(HML-2.HOM) provirus. We specifically amplified the leftmost or rightmost proviruses from human genomic DNA samples and determined their specific SNP status. For the left provirus of the HERV-K(HML-2.HOM) tandem arrangement we identified four haplotypes, of eight possible haplotypes, regarding the presence of intact versus defective *gag*, *prt*, and *pol* genes. Obviously, not all possible haplotypes were created during evolution or fixed in the human population. The majority of proviruses/individuals displayed intact *gag* and *prt* genes and a defective YXDD motif, that is, haplotype C. However, a significant number of individuals harbored different haplotypes in that, for instance, *gag* and *prt* genes were defective but the YXDD motif was intact. We note in this context that we did not identify HERV-K(HML-2.HOM) proviruses with intact *gag*, *prt*, and *pol* genes, although there was opportunity for creating such a completely intact provirus by recombination events (see below). According to our results such an allele was not fixed in the human population, at least not at higher frequencies. One may hypothesize a selection pressure against a completely intact provirus. However, the effect of such a completely intact provirus seems vague. As a recent report shows, the so-called HERV-K113 provirus—having an allele frequency of 0.19 and displaying ORFs for all retroviral genes and an intact YXDD motif—probably is not deleterious (Turner et al. 2001). On the other hand, more elaborate studies investigating more samples will be required to exclude with higher certainty completely intact HERV-K(HML-2.HOM) alleles in the human population.

Our study further demonstrates that particular HERV loci are not necessarily identical in sequence and coding capacity. Generally, the HERV-K(HML-2) family is characterized by relatively intact retroviral genes. Besides several loci with intact genes (Barbulescu et al. 1999; Mayer et al. 1997a, b, 1999; Tonjes et al. 1999), defective genes are due to one or a few nonsense mutations. As exemplified by the observed polymorphisms within the HERV-K(HML-2.HOM) locus, other HERV-K(HML-2) loci may as well display sequence polymorphisms, and genes with or without ORFs could be rendered defective or intact, respectively, in various individuals. Therefore, each individual could display a particular set of intact and defective

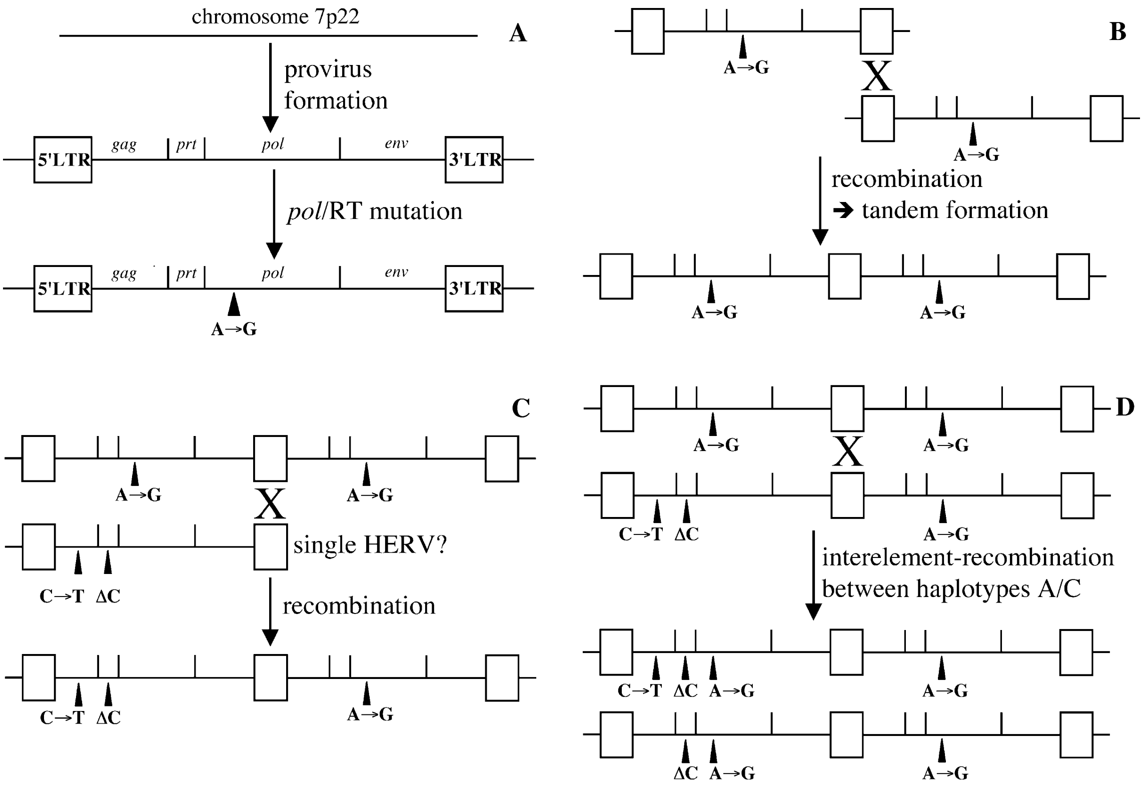

Fig. 6. Proposed evolutionary history of the HERV-K(HML-2.HOM) locus indicating multiple recombination events between provirus loci. **A** Initial provirus formation in chromosome 7p22 followed by a mutation within the *pol* gene. **B** Tandem formation by recombination between two haplotype C proviruses. **C** Generation of haplotype A by interelement recombination with a provirus having mutated *gag* and *prt*. The latter may have been present as single or tandem provirus. **D** Generation of haplotypes B and D from recombination between haplotypes C and A. See Discussion for details.

HERV-K(HML-2) loci. Hence, some individuals may harbor a greater number of intact HERV-K(HML-2) genes than others. Whether there could be a biological consequence from smaller or greater numbers of intact retroviral genes is currently not clear, though.

The combined results of our haplotype analysis allows us to envisage the evolutionary history of this specific proviral locus (Fig. 6). First, a HERV-K(HML-2) provirus was formed in chromosome 7p22. It is not clear whether that provirus was completely intact or already harbored the transition in *pol*. However, the *pol* mutation probably occurred prior to other mutations. A tandem provirus, sharing the central LTR, was probably generated by recombination between 5′ and 3′ LTR of the same locus. This event could also explain the frequent occurrence of haplotype C (mutation in *pol*) in both the left and the right provirus. Tandem provirus formation implies simultaneous formation of a solitary LTR on the other chromosome. We did not find evidence for loci harboring only a solitary LTR. It is possible that such an allele was not fixed in the human population, at least not at high frequencies. The tandem arrangement seems to be the prevalent of HERV-K(HML-2.HOM). The nucleotide changes in the *gag* and *prt* genes were found only in the left provirus, making it unlikely that these mutations occurred before tandem formation. It seems very unlikely that observed haplotypes are due to recurrent mutations. We propose that observed haplotypes other than C were generated by recombination between a HERV-K(HML-2) provirus carrying mutations in *gag* and *prt* in the left part of the tandem, with the recombination site excluding the mutation in *pol*. The resulting tandem provirus then carried haplotype A (mutated *gag*, *prt*, and *pol*) in the left provirus and haplotype C (intact *gag* and *prt*, mutated *pol*) in the right provirus. Likewise, haplotypes B and D can be explained by recombination events. For example, haplotye D in the left provirus could be the result from recombination between haplotype C and haplotype A (Fig. 6). As others recently reported frequent recombination events between HERV-K(HML-2) proviruses resulting in rearrangements of flanking cellular sequences (Hughes and Coffin 2001), we sequenced target site duplication (TSD) regions for several tandem and single allele proviruses. All proviruses displayed identical TSDs (not shown). Thus, our results indicate recombination events within the HERV-K(HML-2.HOM) locus rather than between loci in different genomic regions.

The identification of several HERV-K(HML-2.HOM) haplotypes provides insight into the evolution of a single HERV locus. Recently, Hughes and Coffin (2001) showed that recombination between proviral HERV-K(HML-2) loci in different genomic regions generated rearrangements in flanking cellular sequences. Our analysis provides further evidence that recombination between HERV-K(HML-2.HOM) proviruses occurred several times since provirus formation. Therefore, exchange of HERV sequence portions by recombination seems to be a frequent event during evolution.

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