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Short Communication

# Human endogenous retrovirus HERV-K113 is capable of producing intact viral particles

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Of all human endogenous retroviruses known today, HERV-K is the only one that has been shown to produce viral particles. While the first of the approximately 30 HERV-K sequences integrated into the human genome more than 40 million years ago, evidence is accumulating that HERV-K was active more recently, provirus HERV-K113 being the youngest sequence found. However, it is unclear which HERV-K sequences code for the viral particles that are produced by human germ-cell tumours or melanomas. Here, we show that the provirus HERV-K113, cloned into a baculovirus expression vector, is capable of producing intact particles of retroviral morphology, exhibiting the typical structure of those particles that were characterized in cell lines derived from human germ-cell tumours. Thus, the HERV-K113 sequence is a candidate for particle production *in vivo* and for an active human endogenous retrovirus of today.

Endogenous retroviruses entered the germ line of their host during evolution and are now passed from parents to offspring like Mendelian genes. While 8 % of the human genome consists of sequences of retroviral origin (Lander *et al.*, 2001), most of these sequences are highly defective due to mutations or deletions. Only very few human endogenous retroviruses have been shown to express functional proteins, some of which have been hypothesized to play a physiological role either during embryogenesis, in immunosuppression or in antiviral defence (for a review, see Bannert & Kurth, 2004). As yet, only one human endogenous retrovirus, HERV-K, could be shown to produce viral particles, which are expressed in germ-cell tumours and melanomas, although no infectivity could be demonstrated (Boller *et al.*, 1993; Muster *et al.*, 2003).

HERV-K is a multigene family that consists of more than 30 different sequences, of which the oldest entered the human genome more than 40 million years ago. It has been suggested that most HERV-K sequences were raised by germ line reinfection (Belshaw *et al.*, 2004), with HERV-K113 being the provirus that was acquired most recently. HERV-K113 is polymorphic and has a prevalence in the human population of about 30 % (Turner *et al.*, 2001). While in Britain 4 % of the population bears the HERV-K sequence in the genome, the prevalence in eastern Africa is 22 % (Moyes *et al.*, 2005). From these data, it has been calculated that the sequence of HERV-K might have been active less than 100 000 years ago (Turner *et al.*, 2001).

The existence of functional proteins for most viral components of HERV-K has been demonstrated, including the viral protease (Mueller-Lantzsch *et al.*, 1993) and the envelope surface protein (Dewannieux *et al.* 2005). It has also been shown by morphological and immunological criteria that several different types of HERV-K virus particles are expressed in cell lines established from different germ-cell tumour patients, perhaps derived from different proviruses (Bieda *et al.*, 2001). As yet, it is unclear which of the more than 30 different HERV-K sequences is responsible for production of the virus particles that are produced by germ-cell tumours (Boller *et al.*, 1983; Löwer *et al.*, 1984; Bieda *et al.*, 2001) and melanomas (Muster *et al.*, 2003). Thus, despite the failure to demonstrate infectivity of HERV-K particles, several lines of evidence originating from molecular biology, serology and morphology point to the possibility that a HERV-K variant was active until recently or is still active today. Recently, Heidmann and co-workers (Dewannieux *et al.*, 2006) and, independently, Lee & Bieniasz (2007) have reconstructed the putative progenitor of HERV-K sequences and have demonstrated its infectivity. They pointed out that an infectious HERV-K variant might be generated by recombination of different HERV-K sequences of today. Here, we report that the sequence of HERV-K113 is capable of producing intact particles of retroviral morphology resembling those that are observed in cell lines derived from human germ-cell tumours.

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To obtain high-level expression of recombinant HERV-K113, the full-length coding region, except the long terminal repeat (LTR), has been cloned as a proviral cassette downstream of the polyhedrin promoter in a baculovirus expression system. Baculoviruses infect insect cells that show a slightly different glycosylation pattern but mammalian-like splicing (Jeang *et al.*, 1987). For generation of this construct, PCR amplification was performed with a bacterial artificial chromosome (BAC RP11-398B1) (source: Children's Hospital Oakland Research Institute, Oakland, CA, USA) bearing HERV-K113 (GenBank accession no. AY037928) using oligonucleotide primers (MWG Biotech) encompassing a ligation-independent (LIC) site for cloning purposes. Oligonucleotides used for PCR were HERV-K113for (5′-GACGACGACAAGAT-TGGTGCCCAACGTGGAGGC-3′; binding site on the HERV-K113 full-length genome, nt 970–989) and HERV-K113rev (5′-GAGGAGAAGCCCGGTCTACACAGACAC-AGTAAC-3′; nt 8533–8550).

Gel-purified PCR products were cloned into baculovirus transfer plasmid pBac-2cp EK/LIC (Novagen Merck) and amplified DNA was sequenced (Seqlab). The clone used showed one single amino acid exchange in the *env* gene region (nt 7146 C to A; proline to histidine) compared with the published sequence of HERV-K113.

The recombinant baculovirus transfer plasmid was co-transfected with BaculoGold DNA (BD Bioscience) using GeneJuice (Novagen Merck) into *Sf9* insect cells (Invitrogen) for homologous recombination. Recombinant baculoviruses were harvested and amplified in the course of two rounds of infection using *Sf9* cells. The resulting baculovirus stocks were analysed by PCR and subsequently used for protein expression.

*Sf9* cells were infected with virus stocks and lysed with Qiagen Shredder for Western blot analyses or fixed with glutaraldehyde for EM analyses 3–5 days post-infection.

In order to obtain a tool for sensitive detection of HERV-K proteins, we developed a panel of monoclonal antibodies (mAbs) to HERV-K viral particles, suitable for immunofluorescence and Western blotting. Initially, BALB/c mice were immunized with either whole formaldehyde-fixed cells or crude preparations of membranes of HERV-K-producing GH teratocarcinoma culture cells (Löwer *et al.*, 1984). After fusion of spleen cells with AG9 myeloma cells, hybridomas were grown in HAT selection medium according to standard methods. Clones were screened by using hybridoma supernatant for immunofluorescence labelling on HERV-K-producing culture cells, leading to a typical fluorescence pattern in the case of a positive clone. Confirmation was done by immunofluorescence double labelling with goat anti-HERV-K antiserum (Boller *et al.*, 1997; Tönjes *et al.*, 1997), by Western blotting and by immunoelectron microscopy. Five different positive clones were obtained, designated HERMA-1, -4, -6, -7 and -8, which turned out to be specific for the Gag protein in HERV-K-expressing human teratocarcinoma cell lines GH and NCCIT (Fig. 1), but not in Jurkat cells, which have been shown not to produce HERV-K proteins (Boller *et al.*, 1997). For subsequent characterization of HERV-K113 produced by insect cells, supernatant of hybridomas was used as such or as an equal mixture of the five clones.

For Western blot analyses, cell lysates were separated by PAGE in a 10 % SDS gel and HERV-K proteins were visualized after blotting onto nylon membranes by incubation with mAb HERMA-6, followed by a secondary HRP-coupled anti-mouse antibody and ECL detection. HERMA-6 was chosen because it reacts most strongly with

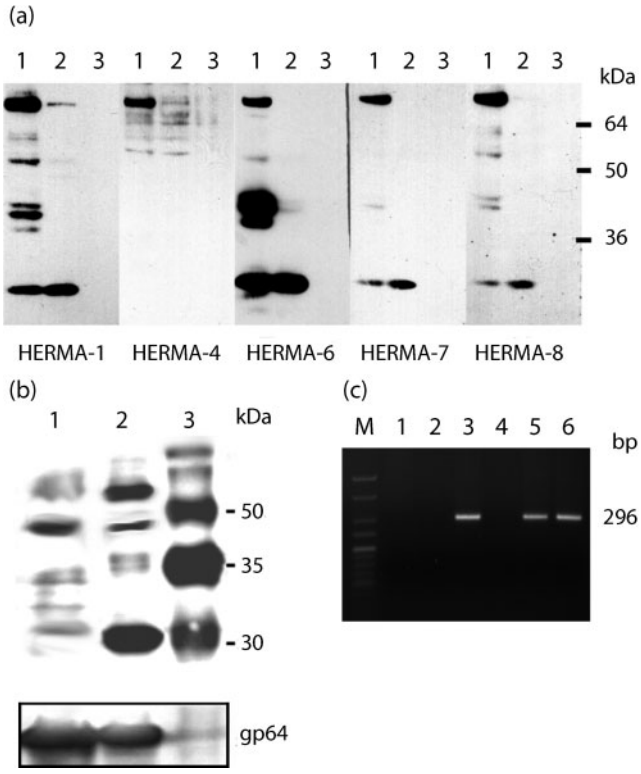


**Fig. 1.** (a) Western blots using the different HERMA mAbs produced against HERV-K/Gag, reacting with lysate from HERV-K-producing human teratocarcinoma cell lines GH (lanes 1) and NCCIT (lanes 2). Lanes 3 show Jurkat cell lysate as a negative control. Immunoblots show major bands at 80 kDa (Gag precursor protein) and at 30 kDa (cleaved Gag protein, except HERMA-4) as well as minor bands that presumably represent intermediate cleavage products. (b) Western blot using mAb HERMA-6 reacting with lysate of *Sf9* cells infected with wild-type baculovirus as a control (*Sf9* Baculo, lane 1) and of *Sf9* cells infected with HERV-K113-encoding baculovirus (*Sf9* Baculo/HERV-K113, lane 2). Lane 3 contains a 'rainbow' molecular mass marker. The lower panel shows anti-baculovirus gp64 antibody as a loading control. (c) RT-PCR using cell lysates and supernatants. Lanes: 1, uninfected *Sf9* insect cells; 2, insect cells infected with recombinant baculovirus encoding GFP (negative control); 3, purified virus isolate Baculo/HERV-K113; 4, water (negative control); 5 and 6, human teratocarcinoma cell lines GH and NCCIT (positive controls).

the 30 kDa band of cleaved HERV-K/Gag protein (Fig. 1a; HERMA-6, lanes 1 and 2). When *Sf9* cells infected with the Baculo/HERV-K113 construct were analysed, a strong band of 30 kDa was observed that represents the viral Gag capsid protein (Fig. 1b, lane 2). This protein band was not present in cell lysates of *Sf9* cells infected with wild-type baculovirus (Fig. 1b, lane 1). Thus, HERV-K113 sequences are specifically expressed by recombinant *Sf9* Baculo/HERV-K113. Baculovirus glycoprotein gp64 served as a loading control.

To visualize HERV-K113 expression in *Sf9* cells infected with the Baculo/HERV-K113 construct by immunofluorescence, we stained formaldehyde-fixed and Triton X-100-permeabilized cells with the mixture of mAbs to HERV-K Gag protein or with serum of a germ-cell tumour patient followed by FITC-labelled anti-mouse antibody or rhodamine-labelled anti-human antibody, respectively. Analysis by confocal microscopy revealed a speckled pattern around the cells (Fig. 2a), reminiscent of the characteristic labelling pattern in human germ-cell tumour lines (Boller *et al.*, 1997). This indicated expression in the form of viral particles. When a commercially available mAb to HERV-K/Env (Austral Biologicals) was used, no labelling was detected (Fig. 2c), indicating that no glycoproteins are incorporated into viral particles.

To confirm the assumption that particles are produced, we processed the cells for electron microscopy according to standard procedures (Boller *et al.*, 1993). By examination of ultrathin sections, retroviral particles were easily detected (Fig. 2e–i). They revealed a structure nearly identical to that observed in the human teratocarcinoma cell line GH. All particles showed the dark electron-dense area between viral core and envelope, characteristic of HERV-K particles (Boller *et al.*, 1983; Bieda *et al.*, 2001), and most particles adhered to the producing cell. No mature particles with condensed core could be observed.

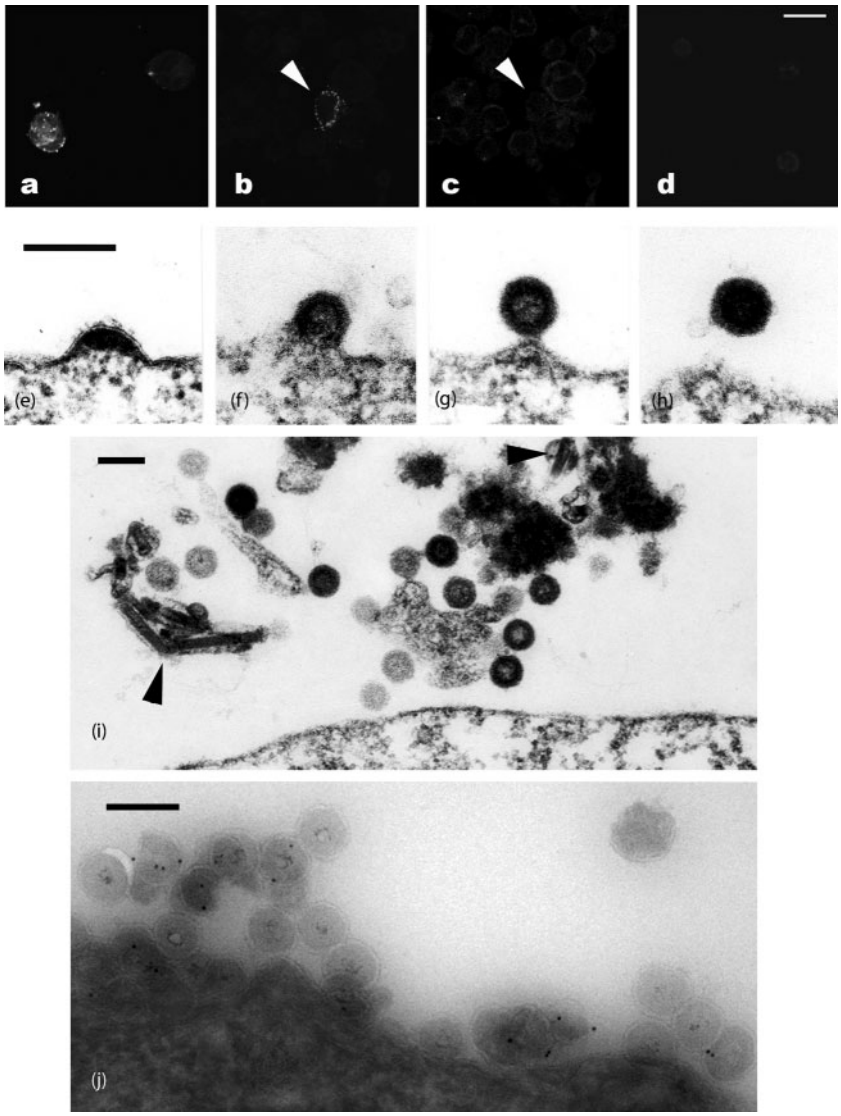


**Fig. 2.** HERV-K113 sequence is competent to encode intact retroviral particles. (a–d) Confocal immunofluorescence of *Sf9* cells, infected with construct Baculo/HERV-K113. (a) Immunolabelling with a mixture of the mAbs HERMA-1, -4, -6, -7 and -8. (b, c) Double labelling with human serum no. 2194, originating from a seminoma patient, and mAb HERM 1811, specific for the Env protein of HERV-K. While the cell expressing HERV-K/Gag (arrowhead) reveals strong binding to the patient serum, only background staining is seen with the anti-Env antibody. (d) Negative control omitting primary antibody. (e–h) Budding states of HERV-K113; the particles clearly show retroviral morphology, but free mature particles with condensed cores are not observed. (i) A group of HERV-K virus particles (note the baculovirus particles indicated by arrowheads). (j) Immunoelectron microscopic labelling of HERV-K113 particles with a mixture of the monoclonal anti-HERV-K/Gag antibodies HERMA-1, -4, -6, -7 and -8. Bars, 20 μm (a–d) and 150 nm (e–j).

**Table 1.** Comparison of the deduced protease and polymerase amino acid sequences of HERV-K113 and other HERV-K sequences

In the upper part of the table, the amino acid sequence of the HERV-K113 protease is compared with that of HERV-K10 clone pKG-P, reported by Schommer *et al.* (1996). Three differing amino acids within the active site region of the protease (aa 179–284) are highlighted in bold. In the lower part, the polymerase amino acid sequences of HERV-K113 and HERV-K cDNA in pcGPK31ΔLTR (Tönjes *et al.*, 1997) are compared. Differences surrounding the intact YIDD motifs in the RT domain are shown. One differing amino acid within the active site region (aa 185) is highlighted.

| Position | HERV-K113 | Compared sequence |
|---|---|---|
| **Protease** | | |
| 9 | R | – |
| 25 | N | S |
| 58 | P | L |
| 59 | A | T |
| 95 | E | K |
| 140 | S | C |
| 161 | I | L |
| 163 | R | K |
| 164 | T | I |
| **234** | **S** | **G** |
| **242** | **T** | **I** |
| **280** | **V** | **A** |
| 300 | M | R |
| 306 | K | R |
| 307 | G | D |
| 308 | L | Stop |
| 311 | N | – |
| 312 | E | – |
| 313 | D | – |
| 314 | G | – |
| 315 | I | – |
| 317 | V | – |
| 318 | P | – |
| 319 | V | – |
| 320 | E | – |
| 321 | A | – |
| 324 | N | – |
| 325 | Q | – |
| 327 | R | – |
| 331 | G | – |
| 332 | Y | – |
| 333 | P | – |
| 334 | F | – |
| **Polymerase** | | |
| 16 | A | V |
| 84 | R | H |
| 85 | M | T |
| **185** | **D** | **E** |
| 195–198 | YIDD | YIDD |
| 288 | V | A |
| 305 | K | Q |
| 307 | M | I |
| 312 | T | A |
| 338 | R | Q |
| 349 | I | T |
| 391 | I | T |
| 430 | I | L |
| 481 | L | P |
| 563 | H | Y |

To prove that these particles are encoded specifically by the cloned HERV-K113 sequence, ultrathin frozen sections were prepared and incubated with HERMA mAbs followed by anti-mouse IgG coupled to 10 nm gold particles (Tokuyasu *et al.*, 1981). Examination in the electron microscope showed that recombinant retroviral HERV-K particles were labelled specifically (Fig. 2j).

The ultrastructural appearance of virions, without mature forms with condensed cores, suggests the lack of complete processing of Gag precursors and thus lack of an intact and active protease. Comparison of the protease amino acid sequences of HERV-K113 and HERV-K10 clone pKG-P (Mueller-Lantzsch *et al.*, 1993), which had been used for recombinant protease expression (Schommer *et al.*, 1996), shows three differences within the active HERV-K protease region (aa 179–284) that may account for incomplete activity of the enzyme (Table 1).

Presence of a viral RNA packaged into HERV-K particles was demonstrated by RT-PCR using HERV-K polymerase oligonucleotides ABDPOL/ABDPOR as described previously (Medstrand *et al.*, 1992; Tönjes *et al.*, 1997) and viral RNA derived from purified virus isolates of supernatants of Baculo/HERV-K113-infected *Sf9* insect cells (Fig. 1c).

Reverse transcriptase (RT) activity associated with virus-like particles (VLPs) was investigated by employing a PCR-based protocol as established by Silver *et al.* (1993) and further improved by Lugert *et al.* (1996). Samples were harvested from tissue-culture supernatants and particulate material was tested. In addition, cell-free membrane-filtered supernatants were tested for RT activity using $Mn^{2+}$ or $Mg^{2+}$ in a classical RT activity assay (Cavidi Tech Ab) according to the instructions of the manufacturer. These assays demonstrated no RT activities for purified VLPs produced by the recombinant virus Baculo/HERV-K113 (data not shown).

The polymerase amino acid sequence of HERV-K113 was compared with the polymerase sequence of baculovirus pBac-HERV-K, which demonstrated weak but specific RT activity (Tönjes *et al.*, 1997). The polymerase sequences show a number of differences (Table 1). Both sequences include intact YIDD motifs, but HERV-K113 shows some differences around this motif which may explain the absence of polymerase activity. Particularly, amino acid position 185 is an aspartate (D) in HERV-K113 whereas, in active polymerases of pcGPK31ΔLTR (Tönjes *et al.*, 1997),

HERV-K-*Phoenix* (Dewannieux *et al.*, 2006) and HERV-K-CON (Lee & Bieniasz, 2007), it is a glutamate (E).

The existence of HERV-K-encoded particles *in vivo* is well documented (Löwer *et al.*, 1993; Boller *et al.*, 1993). Virions were first characterized in cell lines derived from human germ-cell tumours and characterized by morphological features. Viral proteins have been demonstrated in seminoma and teratocarcinoma tissue, and it is reasonable to assume that particles are produced in these tumours, as a strong and specific immune reaction against HERV-K core and surface proteins can be observed in 67 % of the corresponding patients (Sauter *et al.*, 1995; Boller *et al.*, 1997; Kleiman *et al.*, 2004). The observation of HERV-K particles was also published for melanomas and melanoma cell lines (Muster *et al.*, 2003; Büscher *et al.*, 2006). *In vitro*, particles with HERV-K-like morphology could repeatedly be observed after expression of HERV-K proviral sequences in baculovirus or cytomegalovirus expression vectors (Tönjes *et al.*, 1997; Dewannieux *et al.*, 2006). However, these proviruses were constructed by prediction of coding-competent sequences and gene engineering. Here, we present for the first time a naturally occurring HERV-K sequence that is capable of producing structurally intact viral particles. It is noteworthy that the HERV-K113 sequence is, in contrast to most other HERV-K sequences, highly polymorphic in the human population and is therefore probably the latest acquired HERV-K sequence during human evolution. It could therefore be speculated that HERV-K113 is responsible for the expression of particles in germ-cell tumour and melanoma patients.

For the putative progenitor of the present HERV-K sequences, infectivity has been shown at a very low level (Dewannieux *et al.*, 2006). The question arises whether HERV-K113 is also infectious. This seems unlikely, because our experiments show the absence of an active RT. In addition, free mature HERV-K113 virions with condensed cores, the morphological equivalent of infectious retroviruses, could not be observed, perhaps because of the lack of functional protease. Moreover, envelope proteins do not seem to be incorporated into viral particles as shown by immunofluorescence, an observation that is congruent with data from Heidmann and co-workers (Dewannieux *et al.*, 2005), who were unable to demonstrate a functional Env of HERV-K113. Therefore, we conclude that HERV-K113, similar to other human endogenous retroviruses, is probably defective.

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