Puuma malware (PUUV) is a hantavirus (family Bunyaviridae); it is carried by bank voles (Myodes glareolus) (Nichol et al., 2005) and causes a lifelong persistent and asymptomatic infection in these rodents (Plyusnin et al., 1996b). PUUV is pathogenic in humans, causing nephropathia epidemica (NE), a mild form of haemorrhagic fever with renal syndrome (Vapalahti et al., 2003). Hantavirus transmission occurs horizontally via direct contacts or through contaminated environment or aerosols in animals (Meyer & Schmaljohn, 2000). Humans acquire the infection by inhalation of contaminated rodent excreta.

For years, PUUV has been a model hantavirus in research on viral genetics and evolution. To our benefit, this virus can be studied both in cell culture and in the natural rodent host. The hantavirus genome consists of three segments of negative-polarity RNA (S, M and L) (Nichol et al., 2005) and, similarly to other RNA viruses, populations of PUUV in individual hosts are represented by quasispecies (Lundkvist et al., 1997; Plyusnin et al., 1995). Hantaviruses have adapted to their natural hosts during millions of years of co-evolution (Plyusnin & Morzunov, 2001) and thus the hantavirus quasispecies populations in the rodent hosts usually have a high fitness. Changes in the viral environment may, however, lead to rapid adaptive changes. For example, the adaptation of wild-type (wt) PUUV (passaged in colonized bank voles) to cultured primate Vero E6 cells is accompanied by increased genetic heterogeneity of the viral population and fixation of nucleotide changes in the S and L segments (Lundkvist et al., 1997; Nemirov et al., 2003). Even with minimal genetic differences between them, the two PUUV variants were clearly distinct phenotypically: the cell culture-adapted variant was non-infectious to bank voles. Interestingly, it is also non-pathogenic to cynomolgus macaques, which are used as a non-human primate model of NE (Groen et al., 1995). In contrast, the wt PUUV strain, passaged only in bank voles, caused in macaques a disease that mirrors NE of humans (Klingström et al., 2002).

Our aim was to investigate the quasispecies dynamics in bank voles when PUUV is transmitted from one animal to another in a controlled experiment. In vivo experiments on PUUV transmission have been described previously (Kallio et al., 2006) and are presented in Fig. 1. Briefly, colonized bank voles were inoculated subcutaneously with 100 bank vole ID₅₀ of PUUV, strain Kazan-wt (Gavrilovskaya et al., 1983). Ten days later, they were placed into individual experimental cages for 7 days. The donor voles (labelled D) were removed and recipient voles (labelled R) were placed into the cages, where they were exposed to the excrement-contaminated bedding for 3 days. All precautions were taken to ensure that no aerosol transmission between the cages was possible (Kallio et al., 2006). After exposure, the recipient voles were moved to individual management cages for 15 days to develop the infection, after which the animals were sacrificed and samples collected. Five voles were subsequently exposed to each donor’s bedding. PUUV infection was confirmed by using nested RT-PCR to detect PUUV S segment RNA in lung tissue. Bank voles positive for PUUV RNA in the RT-PCR test were used in this study.
The region selected for analysis (nt 631–1630) included both the coding region (CR) and the 3' non-coding region of the S segment RNA. The selected part of the CR encoded both a well-conserved C terminus and a variable part of the N protein (Kaukinen et al., 2005). All three regions seem to evolve under different selective pressures. Altogether, approximately 60 kb was sequenced for analysis of the S segment quasispecies from two donor and four recipient bank voles. RNA was extracted from the lung tissue and the entire S segment was amplified by RT-PCR as described previously (Plyusnin et al., 1994). PCR products were cloned by using the pGEM-T cloning system (Promega). Ten cDNA clones from each of the two donors and from four recipients were sequenced (nt 631–1630), using primers PuuSF572 (5'-TATATGTATCCATGCCTACTG-CC-3') and PuuSR1689 (5'-GATAGCTGTTTTACATTTTG-3'). Sequencing with these two primers resulted in twofold coverage of nt 850–1350. Thus, regions 631–850 and 1350–1630 were initially sequenced in one reading, but if any ambiguous nucleotides were detected, the sequencing reaction was repeated.

Total mutation frequency in the donor sequences was \(1.6 \times 10^{-3}\) [31 mutations in 20 kb sequenced, mean \(\pm SD = (1.6 \pm 1.273) \times 10^{-3}\)]; in the recipient sequences, it was almost twice as high \([2.6 \times 10^{-3}, \text{mean } \pm \text{SD} = (2.63 \pm 1.234) \times 10^{-3}]\) (Table 1), and the difference is statistically significant (Student’s t-test, \(P = 0.004\)). Nucleotide substitutions were distributed evenly across this region (data not shown) and also evenly across the different codon positions (Table 1), suggesting no stabilizing selection at the protein level for individual de novo-generated mutations. Mutations were predominantly transitions (92%), and most mutations (78%) were either A→G or U→C substitutions.

One synonymous mutation (A759G) appeared to become fixed in the viral quasispecies population during a single virus-transmission event. First, donor D1 and its two recipients, D1-R3 and D1-R4, were analysed and the mutation was seen in all 20 cDNA clones from the recipient voles, but in none of the D1-originating cDNA clones. Next, donor D2 and its two recipients, D2-R3 and D2-R4,
were analysed. Again, the A759G mutation was observed in all of the recipient cDNA clones. Surprisingly, it was also seen in 60% of donor D2 clones. An additional 30 clones of donor D1 were sequenced to see whether the mutation was pre-existing in its quasispecies population, but the mutation was not observed. Five additional donors were examined for the presence of this mutation by using direct sequencing of the partial S PCR product that was amplified by nested RT-PCR essentially as described previously (Plyusnin et al., 1997), but using the primers PUUSF688 [5'-ATGAGTCC(A/T)GTIAATGGG(A/G)GTIAATT-3'] and PUUSR1106 [5'-GCTGTICCTACAGT(C/T)TTIGATGCC-AT-3'] in the second round. The mutation was found in only one of the donors. All PUUV-positive recipients of the five donors were also examined for the presence of this mutation (Fig. 2). In some recipient voles, the sequence with the A residue remaining at position 759 could also be seen in the sequencing file as a minor peak (data not shown). This suggested that, at least for one transmission event, the original nucleotide was preserved in the quasispecies swarm. Four additional donors and their five PUUV-positive recipients from the second trial of PUUV transmission (Kallio et al., 2006) were also studied, but the mutation A759G was not found in any of them.

The wt S segment sequence (without the A759G mutation) was found in the quasispecies population, which has an overall mutation frequency of $1.4 \times 10^{-3}$ at the nucleotide level. Notably, the mutation was found on a background of substantially higher genetic variability: the mutation frequency was $2.6 \times 10^{-3}$ at the nucleotide level ($t$-test, $P=0.002$). The mutation is located at the third codon

**Table 1. Summary of mutations detected in the quasispecies swarm**

The level of PUUV diversity seen in this study is seven to 15 times higher than the level of misincorporations produced by AmpliTaq polymerase (Lundkvist et al., 1997; Plyusnin et al., 1996a).

<table>
<thead>
<tr>
<th></th>
<th>Donor</th>
<th>Recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D1</td>
<td>D2</td>
</tr>
<tr>
<td>Min. mutation frequency (no. different mutations $\times 10^{-3}$)</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Max. mutation frequency (total no. mutations $\times 10^{-3}$)</td>
<td>1.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Transitions/transversions</td>
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<td>12/2</td>
</tr>
<tr>
<td>Synonymous/non-synonymous amino acid changes</td>
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<td>2/5</td>
</tr>
<tr>
<td>Codon positions 1/2/3</td>
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<td>2/3/2</td>
</tr>
<tr>
<td>Deletions/insertions</td>
<td>0/1</td>
<td>–</td>
</tr>
<tr>
<td>Frameshift mutations</td>
<td>1</td>
<td>–</td>
</tr>
</tbody>
</table>

*There was a deletion of 80 nt in two cDNA clones.

**Fig. 2.** Occurrence of the mutation A759G. White voles carry the wild-type S sequence; black voles carry the mutation. In donor D2 (grey vole), 60% of the cDNA clones are mutated. A dash represents either a PUUV-negative vole or a sample that was no longer available. Donors D1 and D2 and their recipient voles in groups R3 and R4 were used for quasispecies analysis via generation of cDNA clones. Partial S sequences from other voles were recovered by direct sequencing.
position of phenylalanine 239 and it changes the triplet in mRNA from UUU to UUC. Codon-usage analysis (Nakamura et al., 2000) showed that the UUC codon is more frequent in bank voles and humans, but in PUUV it is the rarer codon.

Quasineutral mutations have been detected in hantavirus genomes (Plyusnin et al., 1995, 1996a; Feuer et al., 1999), but in only one study (Lundkvist et al., 1997) has it been possible to follow whether they become dominant. In our study, we observed two alleles of the PUUV S gene in the population used to infect the donor voles. In the first trial (donors D1–D10) of the transmission experiment (Kallio et al., 2006), the mutation A759G was detected in all PCR-positive recipients (Fig. 2). In the second trial (donors D16–D21), however, it was not detected. The reason for this might be the somewhat lower transmission efficiency in the second trial (Kallio et al., 2006), which could result from as-yet-unknown factors, such as the humidity. Alternatively, it could have occurred because of sampling from the genetically heterogeneous virus stock used for inoculation of donor voles. When the variant with the A759G mutation nevertheless appeared, it seemed to have an advantage in natural transmission, as it was found in all cDNA clones from the recipients. This is, to our knowledge, the first time that it has been possible to follow the mutations appearing in a hantavirus quasispecies swarm in vivo, and to detect fixation of a synonymous mutation in transmission from one animal to another.

Viral RNA-dependent RNA polymerases are notorious for their high mutation rates (Domingo & Holland, 1997). New RNA virus mutants are continuously generated and assessed by positive (diversifying) and negative (stabilizing) selective forces (Domingo et al., 2001), which act at the population level rather than on individual variants (Vignuzzi et al., 2006). The evolution of PUUV is driven mainly by genetic drift through accumulation of point mutations or deletions/insertions (Plyusnin et al., 1995; Sironen et al., 2001). In theory, population bottlenecks – common events in virus transmission – should also affect the evolution of PUUV externally. A bottleneck reduces both the effective size and the genetic diversity of a viral population, as only a few particles are sampled randomly to found the novel population of the virus (Manrubia et al., 2005).

As mutation A759G is silent, the positive advantage brought by the mutation cannot arise on the level of protein structure. Changes in the predicted secondary structure of either the viral RNA or the mRNA appeared minor (not shown) and thus are unlikely to be the sole, if any, reason for the advantageous phenotypic effect of the mutation. Another possibility might be a general enhancement of viral replication, a phenomenon described recently for vesicular stomatitis virus and human immunodeficiency virus type 1 (Hamano et al., 2007; Novella et al., 2004).

In our experiments, the mutation A759G appears to be advantageous and selected for, as it becomes fixed in the PUUV master sequence in the recipient voles. As it was not feasible to sequence whole genomes in the frame of this project, the possibility remains that this substitution is a marker for an accompanying amino acid substitution located in a region not yet analysed. Further experiments with additional passages in animals would also reveal whether this mutation becomes fixed permanently in the virus genome. An important question is whether the mutation A759G is generated de novo in the process of viral adaptation or whether it is preserved, as a minority, in the quasispecies swarms within the donor voles. Notably, this particular mutation was seen earlier in two of 25 cDNA clones originating from the wt PUUV adapting to cell culture (Lundkvist et al., 1997). This suggested that the mutation can be maintained permanently, even as a minor variant, in the quasispecies swarm. This view is also supported by the finding of this mutation in two of the donors (D2 and D5) (Fig. 2). The adaptation to a completely different host (from voles to cell culture) was accomplished via only a few changes in the PUUV genome (Lundkvist et al., 1997; Nemirov et al., 2003). These small alterations, mainly on the RNA level, nevertheless changed the virus phenotype profoundly, and the virus was no longer able to infect the original host, the bank vole. Similarly, the silent mutation A759G may provide the virus with a strong advantage in transmission. Interestingly, both A and G nucleotides occur in the consensus S segment sequences of PUUV strains (all sequences from the Russian lineage, Kazan strain included, have A), suggesting that neither purine at position 759 is deleterious to the virus.

Genetic drift is the main mechanism of PUUV evolution (Plyusnin et al., 1995; Sironen et al., 2001). This study, for the first time, confirms the result in vivo. Furthermore, these data add to the increasing evidence (Hamano et al., 2007; Novella et al., 2004) that silent mutations in RNA viruses may be far from neutral and, on the contrary, can contribute significantly to adaptation of these agents to the ever-changing environment.

Acknowledgements
Sonja Ahlfors is thanked for her assistance in sequencing. This work was supported by grants from the Academy of Finland, Helsinki Biomedical Graduate School, Sigrid Juselius Foundation, the Swedish Research Council (project no. 12177), Oscar Offlund Foundation, Finnish Cultural Foundation and Nordforsk.

References
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