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Cell Culture Adaptation of Puumala Hantavirus Changes the Infectivity for Its Natural Reservoir, *Clethrionomys glareolus*, and Leads to Accumulation of Mutants with Altered Genomic RNA S Segment

ÅKE LUNDKVIST,[1,2]* YING CHENG,[3] KATARINA BRUS SJÖLANDER,[1] BO NIKLASSON,[1] ANTTI VAHERI,[3] AND ALEXANDER PLYUSNIN[3]

Swedish Institute for Infectious Disease Control, S-10521 Stockholm,[1] and Microbiology and Tumour Biology Centre, Karolinska Institute, S-17177 Stockholm,[2] Sweden, and Department of Virology, Haartman Institute, University of Helsinki, FIN-00014 Helsinki, Finland[3]

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This paper reports the establishment of a model for hantavirus host adaptation. Wild-type (wt) (bank vole-passaged) and Vero E6 cell-cultured variants of Puumala virus strain Kazan were analyzed for their virologic and genetic properties. The wt variant was well adapted for reproduction in bank voles but not in cell culture, while the Vero E6 strains replicated to much higher efficiency in cell culture but did not reproducibly infect bank voles. Comparison of the consensus sequences of the respective viral genomes revealed no differences in the coding region of the S gene. However, the noncoding regions of the S gene were found to be different at positions 26 and 1577. In one additional and independent adaptation experiment, all analyzed cDNA clones from the Vero E6-adapted variant were found to carry substitutions at position 1580 of the S segment, just 3 nucleotides downstream of the mutation observed in the first adaptation. No differences were found in the consensus sequences of the entire M segments from the wt and the Vero E6-adapted variants. The results indicated different impacts of the S and the M genomic segments for the adaptation process and selective advantages for the variants that carried altered noncoding sequences of the S segment. We conclude that the isolation in cell culture resulted in a phenotypically and genotypically altered hantavirus.

In common with other members of the *Bunyaviridae*, hantaviruses have a single-stranded, tripartite RNA genome, packed in enveloped helical nucleocapsids. The negative-strand genome segments of approximately 6, 4, and 2 kb encode at least four structural proteins: the RNA polymerase, a glycoprotein precursor (GPC) cotranslationally processed into two envelope proteins (G1 and G2), and a nucleocapsid protein (N) (for reviews, see references 37 and 43). The 3′ and 5′ termini of the hantavirus RNA genome are complementary and thus capable of forming panhandle structures, a hallmark of the family *Bunyaviridae* (2). The panhandles of hantaviruses are thought to serve as regulators of viral transcription and replication, similarly to what has been demonstrated for Bunyamwera virus, the only member of the *Bunyaviridae* studied in this respect (9), as well as other negative-strand RNA viruses such as vesicular stomatitis virus (50) and influenza viruses (12, 22, 47).

The mRNAs of hantaviruses, like those of all members of the family *Bunyaviridae* studied, have 5′-terminal capped extensions. They are of cellular origin and heterogeneous length (less than 20 nucleotides [nt]) and are cleaved preferably after a G residue (6, 13, 20). Different kinetics for the hantaviral N, GPC, and L protein mRNA accumulation in Vero E6 cells have been demonstrated, and different mechanisms for transcription termination for each of the three viral RNAs have been proposed (19). These data suggest complicated and most probably gene-specific mechanisms of regulation for hantavirus transcription and replication. Progress in this important field, however, is hampered by the poor growth of hantaviruses in cell culture (29).

* Corresponding author. Phone: 46-8-7351273. Fax: 46-8-7351424. E-mail: Ake.Lundkvist@smi.ki.se.

Small mammals, mainly rodents, are the natural reservoirs of hantaviruses. Transmission to humans occurs via aerosolized animal excreta. Hantaviruses are known to cause two serious and often fatal human diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome. Hantaan (HTN), Seoul (SEO), Dobrava (DOB), and Puumala (PUU) viruses cause HFRS, characterized by fever, renal failure, and, in severe cases, hemorrhagic manifestations (21, 25). Sin Nombre (SN) virus and related viruses cause hantavirus pulmonary syndrome, characterized by acute respiratory dysfunction (31). Prospect Hill (PH) virus, Tula (TUL) virus, Khabarovsk virus, and other hantaviruses are thought to be apathogenic for humans. Although little is known about the basis of hantavirus pathogenicity, there is a connection to the nature of the rodent host. Thus, HTN, SEO, and DOB viruses, carried by mice and rats, cause more severe illnesses in humans than PUU virus, carried by *Clethrionomys* voles, and no human diseases have been associated with hantaviruses carried by *Microtus* voles. However, the lack of an adequate animal model has prevented intensive studies in this field, and genetic dissection of the infectivity and pathogenicity, as well as host specificity, of hantaviruses is still at an early stage.

Previous studies have shown that experimental PUU virus inoculation of the natural reservoir, the bank vole (*Clethrionomys glareolus*), efficiently results in prolonged or persistent infection (52). Other studies have demonstrated that there are major differences in PUU virus susceptibility in other rodent species. Thus, although passaged three times in suckling hamster brains for its adaptation, PUU virus strain K-27 did not efficiently infect hamsters (5). In a recent study (27), a virus challenge model for PUU virus was developed, and it was found that colonized bank voles were highly susceptible to

infection with wild-type (wt) PUU virus (designated strain Kazan-wt, passaged only in bank voles) but not with cell culture-grown PUU virus (strain Sotkamo). These results indicated that there are differences in infectivity between wt and cell culture-isolated variants of PUU virus or, alternatively, that there are differences in the infectivity of various PUU virus strains. Moreover, hantaviruses are usually most difficult to isolate in cell culture, and many questions remain concerning factors that influence their ability to replicate in different hosts. To elucidate some of these questions, a model based on PUU virus strain Kazan was established. wt (bank vole-passaged) and cell culture-grown variants were analyzed for their virological properties in vitro and in vivo, and adaptation to cell culture was monitored by sequence analysis of the S and M genome segments.

MATERIALS AND METHODS

Virus strains. Strain Kazan-wt was passaged in colonized bank voles as described previously (14, 26). The Kazan-wt stock used in this study originated from pooled lung material of 10 infected bank voles. Strain Kazan-E6-I, originating from the same Kazan-wt stock described above and previously adapted to growth on Vero E6 cells (CRL 1586; American Type Culture Collection) (26), was further cultured up to 11 passages in cell culture. One additional cell culture isolation of Kazan-wt, yielding strain Kazan-E6-II, was performed as described elsewhere (26). PUU virus Kazan-E6 strains, PUU virus strain Sotkamo (48), and PUU virus strain Vindeln/83-L20 (16) were passaged on monolayers of Vero E6 cells in Eagle's minimal essential medium supplemented with 2% fetal calf serum, 2 mM L-glutamine, and antibiotics. Virus stocks were kept at −70°C until used.

Antibodies. Generation and characterization of PUU virus N-, G1-, and G2-specific monoclonal antibodies (MAbs) have been described elsewhere (23, 24). Large-scale MAb production was performed by culture of hybridomas in roller bottles followed by purification on protein G-Sepharose as previously described (23).

Animal models. To assess virus infectivity, 4- to 10-week-old bank voles, derived from a PUU virus-free colony established several years earlier with animals captured in Sweden, were inoculated subcutaneously with serial dilutions of PUU virus Kazan strains. The animals were sacrificed at 21 days postinfection. New Zealand White rabbits were inoculated with Kazan strains by the intranasal route as previously described (32).

Immunoassays. Virus stocks were endpoint titrated by the focus reduction neutralization test (FRNT) as previously described (32), and the amount of N antigen was quantified by endpoint titrations in a hantavirus antigen enzyme-linked immunosorbent assay (ELISA) (26). Virus strains were antigenically examined with a panel of 14 MAbs by immunofluorescence assay (IFA), essentially as described previously (23). Serum antibody responses against PUU virus were analyzed by IFA and FRNT. Lung samples from virus-challenged bank voles were examined for the presence of PUU virus N protein by hantavirus antigen ELISA (26).

RT-PCR, cloning, and sequencing. Reverse transcription-PCR (RT-PCR) of the entire S segment was performed as described previously (34). In short, RNA was purified from lung tissue of bank voles or from Vero E6 cells by guanidinium thiocyanate-phenol-chloroform extraction (3). cDNA was prepared by using specific oligonucleotide primer 5′-TAGTAGTAGAC-3′ and random hexamers (Pharmacia). PCR amplification was performed with primer 5′-TTCTGCAGT AGTAGTAGACTCCTTGAAAAG-3′ (48). M segments from viruses of rodent and Vero E6 origin were amplified in three parts (nt 1 to 1150, 1129 to 2828, and 2775 to 3683) (sequences of all primers are available upon request). The PCR products were purified with a Wizard PCR purification kit and cloned with the pGEM-T Vector System (Promega). Plasmids were purified with a Wizard Minipreps kit (Promega) and sequenced with Sequenase version 2.0 (United States Biochemicals). All sequences in the text and figures are given for genomic RNA.

Phylogenetic analysis. The PHYLIP program package (11) was used to make 200 bootstrap replicates of the sequence data (Seqboot). Distance matrices were calculated by using Kimura's two-parameter model (Dnadist) and analyzed by the Fitch-Margoliash tree-fitting algorithm (Fitch) with the global arrangements option set. The bootstrap support percentages of particular branching points were calculated from these trees (Consense). All calculations were made with a Macintosh Performa 6200.

For comparison, hantavirus sequence data were obtained from the Genome Sequence Database. The M-segment sequence accession numbers for the indicated viruses are as follows: PUU virus strain Sotkamo, X61034; PUU virus strain Vindeln/83-L20, Z49214; PUU virus strain Bashkiria/CG1820, M29979; PUU virus strain Paris 90-13, U22418; PUU virus strain "Vranica"/Hällnäs, U14136; TUL virus strain Moravia/5302v/95, Z69993; PH virus strain PH-1, Z55129; SN virus strain H10, L25783; New York (NY) virus strain RI-1, U36801; El Moro Canyon (ELMC) virus strain RM-97, U26828; Bayou (BAY) virus strain Louisiana, L36930; Black Creek Canal (BCC) virus, L39950; SEO virus strain SR-11, M34881; HTN virus strain 76118, M14627; DOB virus, L33685; and Thailand (THAI) virus strain 749, L08756. The S-segment sequences from the following viruses were used, with the indicated accession numbers: PUU virus strain Sotkamo, X61035; PUU virus strain P360, L11347; PUU virus strain Bashkiria/CG1820, M32750; PUU virus strain Vindeln/83-L20, Z48586; PUU virus strain Udmurtia/894Cg/91, Z21497; PUU virus strain Paris 90-13, U22423; PUU virus strain "Vranica"/Hällnäs, U14137; TUL virus strain Moravia/5286Ma/94, Z48573; TUL virus strain Tula/76Ma/87, Z30941; PH virus strain PH-1, Z49098; Isla Vista (ILV) strain MC-SB-1, U31534; SN virus strain H10, L25784; ELMC virus strain RM-97, U11427; BAY virus strain Louisiana, L36929; BCC virus, L39949; SEO virus strain SR-11, M34882; HTN virus strain 76118, M146271; and DOB virus, L41916.

Nucleotide sequence accession numbers. The nucleotide sequences of the genomic M and S segments have been deposited in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession no. Z84205 and Z84204, respectively.

RESULTS

Sequence, phylogenetic, and antigenic analyses of PUU virus strain Kazan. The PUU virus strain Kazan has not been thoroughly characterized previously, and for this study we initially investigated its genetic and antigenic characteristics. The genomic M segment was found to consist of 3,683 nt: 40 in the 5′ noncoding region (5′NCR) (in the plus-sense strand), an open reading frame (ORF) for a G1-G2 precursor of 1148 amino acids (aa), and a 3′NCR of 196 nt. The S segment contains a 5′NCR of 42 nt, an ORF for the N protein of 433 aa, and a 3′NCR of 483 nt. An additional small ORF encoding a 92-aa protein in the +1 frame was found in the S segment. All these features were similar, or even identical, to those of other PUU virus strains (1, 16, 35, 48, 51). On a phylogenetic tree based on the M segment (Fig. 1), the Kazan-wt strain was situated with other strains belonging to the PUU virus serotype and closest to strains P360 and CG1820, which originate from the neighboring region, Bashkiria (Russia), and share with them a recent ancestor. These three strains share a more ancient ancestor with the Finnish strain Sotkamo but not with the Swedish strains Vindeln/83-L20 and "Vranica"/Hällnäs or strain Paris 90-13, originating from the Ardennes. The tree calculated for the S-segment sequences showed a similar topography (data not shown). Thus, our data are consistent with the view of geographical clustering of PUU virus genetic variants (16, 34, 35) and a proposed scenario of postglacial spreading of PUU virus through Europe with separate glacial refugia of infected bank voles (18, 37).

PUU virus has been previously reported to be serologically distinct from other hantaviruses (4, 17, 44, 49). Only minor antigenic differences between the PUU virus strains from different geographical regions have been found by using MAbs and polyclonal rabbit antiserum (23, 32). In the present study, Kazan-E6-I was analyzed by use of a panel of 14 MAbs reactive with 11 distinct epitopes in the N, G1, and G2 proteins (24). No antigenic differences compared to PUU virus strains Sotkamo (the prototype strain) and Vindeln/83-L20 (the representative of the Swedish branch of PUU virus) were found. However, a cross-FRNT comparison with rabbit antiserum raised against Kazan-wt showed 8- to 16-fold titer differences between Kazan-E6-I and the heterologous strains (Table 1). The antisera to strains Vindeln/83-L20 and Sotkamo showed two- to fourfold titer differences between the homologous strain and Kazan-E6-I. The data confirmed that the Kazan strain belongs to the PUU virus serotype but also indicated antigenic differences among the different PUU virus strains within functional (i.e., neutralizing) sites.

Adaptation of Kazan-wt to cell culture. To investigate if cell culture isolation alters the phenotype of PUU virus, a model based on the Kazan strain was established. The adaptation

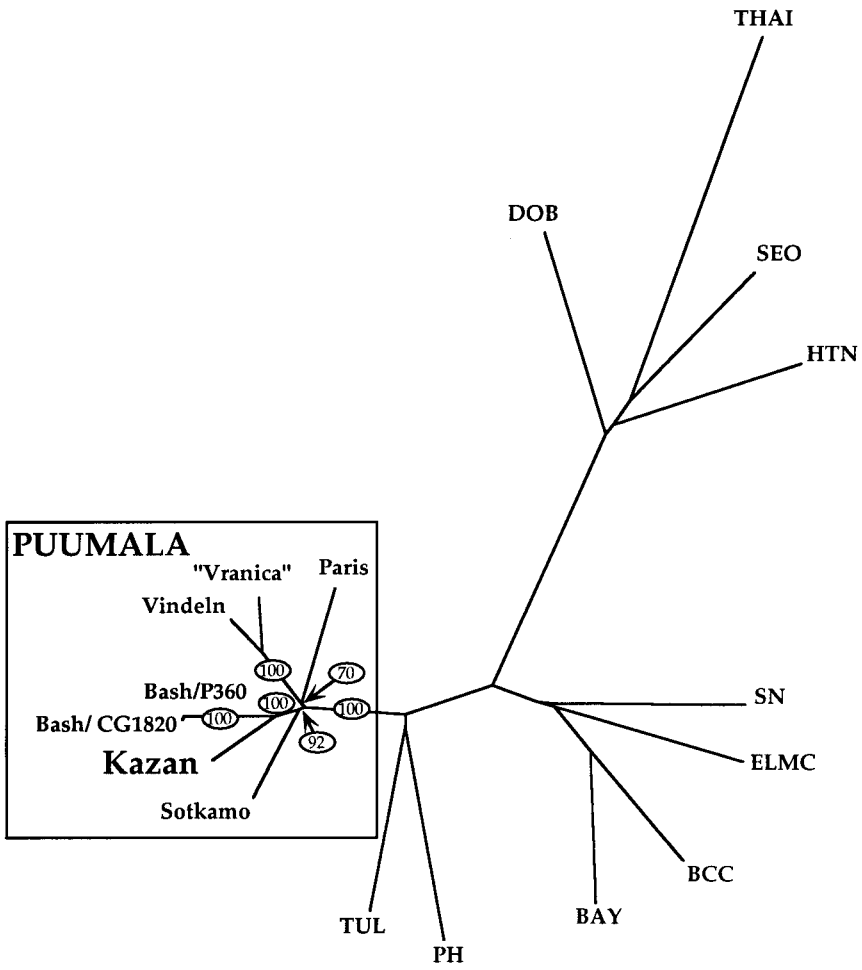

FIG. 1. Phylogenetic tree of hantaviruses based on the M-segment nucleotide sequences (coding region). Vindeln, strain Vindeln/83-L20; Bash/CG1820, strain Bashkiria/CG1820; Bash/P360, strain Bashkiria/P360; "Vranica," strain "Vranica," presumably Hällnäs B1; Paris, strain Paris 90-13; TUL, TUL virus strain Moravia/5302v/95; PH, PH virus strain PH-1; SN, SN virus strain H10 (L25784 and L25783); ELMC, ELMC virus strain RM-97; BAY, BAY virus strain Louisiana; SEO, SEO virus strain SR-11; HTN, HTN virus strain 76118; THAI, THAI virus strain 749. Bootstrap probabilities are given for PUU virus only.

process was followed by analysis of each cell passage for the levels of N antigen (Fig. 2). In the first adaptation attempt (Kazan-E6-I), detectable levels of N antigen were present already at the first passage (p1) (2 weeks after virus inoculation). The quantity of N antigen (i.e., optical density values) reached a plateau at p3, which indicated that a stable and maximal reproduction of this virus in Vero E6 cells was achieved after 6 weeks. In the second experiment (Kazan-E6-II), significant levels of N antigen were not detected until 8 weeks after the inoculation (Fig. 2). The adaptation reached its optimum after approximately 16 weeks (p7), in terms of high and stable levels of expressed N antigen.

TABLE 1. Cross-neutralization of PUU virus strains

Virus strain	Titer with rabbit antiserum to PUU virus strain[a]:			
	Kazan-wt	Sotkamo[b]		83-L20[c]
		Serum 1	Serum 2	
Kazan-E6-I	1,280	320	80	320
Sotkamo	160	640	160	640
83-L20[c]	80	320	40	640

[a] Reciprocal FRNT endpoint titers.
[b] Two different rabbit antisera to PUU virus strain Sotkamo were tested.
[c] 83-L20, strain Vindeln/83-L20.

To compare growth rates of the wt and Vero E6-adapted variants, serial dilutions of the Kazan-wt and Kazan-E6-I virus stocks were inoculated on monolayers of Vero E6 cells, incubated for 2 weeks, and subsequently analyzed by immunoperoxidase staining (FRNT). No visible foci could be detected in any cells inoculated with the Kazan-wt strain, while the titers of strains Kazan-E6-I (p5) and Kazan-E6-II (p7) were determined to be 4.5×10^5 and 3.0×10^5 focus-forming units/ml, respectively. Similar results were obtained when serial dilutions of the virus stocks were inoculated on Vero E6 cells, which were subsequently passaged (at dilutions of 1:4) every 2 weeks and examined for the presence of virus antigen by IFA. After three passages (6 weeks), only low percentages ($\leq$30%) of infected cells were found in the Kazan-wt-inoculated cells at a maximum initial virus dilution of 10^{-2} (Table 2), while the Kazan-E6-I-inoculated cells were infected to greater than 90% with a virus dilution of 10^{-5}. The results showed that the reproduction of Kazan-E6 strains in Vero E6 cells was much greater than that of Kazan-wt, i.e., the Kazan-E6 strains had been adapted for growth in Vero E6 cells.

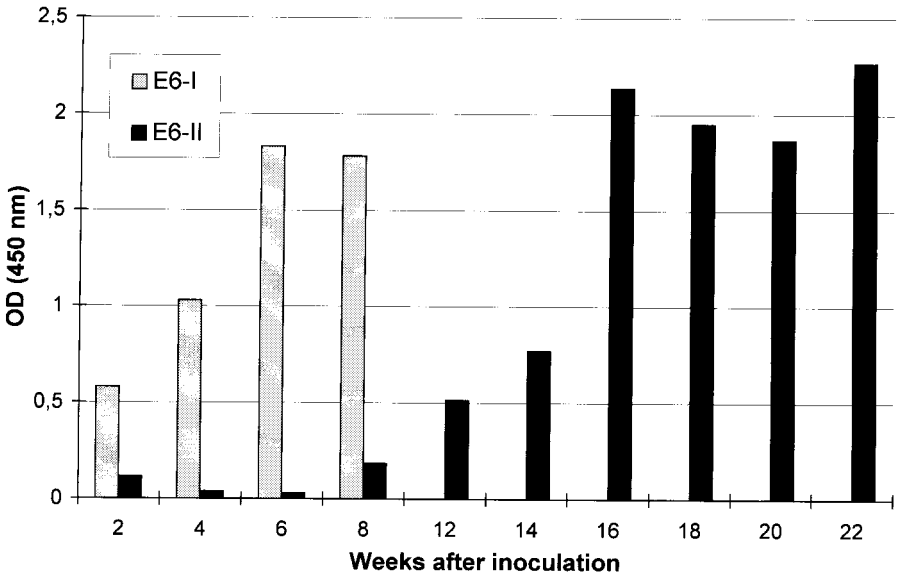

FIG. 2. Adaptation of PUU virus Kazan-wt to Vero E6 cells monitored over time. Cell extracts of each passage were analyzed for the presence of virus N antigen by hantavirus antigen ELISA. OD, optical density.

Comparison of infectivities in bank voles and rabbits. To investigate the potential differences in infectivity between the Kazan-wt and Kazan-E6 strains, colonized bank voles were inoculated with serially diluted virus stocks. All animals injected with Kazan-wt (diluted up to 10^{-6}) expressed significant levels of N antigen in the lungs 3 weeks after inoculation (Table 3). In contrast, animals injected with Kazan strain adapted to cell culture and subsequently passaged five times (Kazan-E6-I, p5) rarely expressed detectable levels of N antigen. To verify that the observed decrease in infectivity of cell culture-adapted virus was reproducible, one additional cell culture adaptation of Kazan-wt (Kazan-E6-II, p7) was analyzed as described above. Again, N antigen was only occasionally detected (Table 3).

Rabbits were inoculated with Kazan-wt and Kazan-E6-I by the intranasal route. Sera were drawn 10 weeks postinfection and examined for neutralizing antibodies by FRNT using the Kazan-E6-I strain. Significant differences in endpoint titers were detected (1:1,280 for the anti-wt and 1:80 and 1:20 for the anti-E6 sera), indicating a more vigorous infection by the wt strain.

To assess if the variation of the Kazan strains in their efficiency to infect bank voles and rabbits could be explained by quantitative differences, virus stocks were treated with detergent (diluted 1:2 in RIPA buffer [23]) and subsequently titrated by antigen ELISA. The results indicated that the differences in amounts of N antigen were less than fivefold and thus could not account for the observed differences in infectivity.

Taken together, the results showed that the wt virus is well adapted for reproduction in bank voles but not in cell culture and that the sometimes slow process of adaptation to cell culture growth altered the phenotype of the virus, i.e., decreased its infectivity for the natural host.

Analysis of the M and the S genome segments during the cell culture adaptation process. To compare M segments of the virus variants of rodent and Vero E6 origins, their sequences were amplified in three parts (see Materials and Methods). Three individual cDNA clones for each part were sequenced. As the very termini of the M segment (nt 1 to 25 and 3665 to 3683) belonged to the annealing sites for the amplification primers, they were not determined directly and were therefore excluded from the comparison. The sequence analysis revealed that the viral populations from both wt and Vero E6 cell-grown virus were represented by mixtures of closely related variants, i.e., quasispecies. No differences were

TABLE 2. Infectivity of PUU Kazan strains in Vero E6 cells

Virus dilution	Infectivity of virus at the indicated time postinoculation[a]			
	Kazan-wt			Kazan-E6-I, 6 wk
	2 wk	4 wk	6 wk	
10^{-1}	20	30	30	>90
10^{-2}	3	30	30	>90
10^{-3}	–	–	–	>90
10^{-4}	–	–	–	>90
10^{-5}	–	–	–	>90
10^{-6}	–	–	–	–
10^{-7}	–	–	–	–

[a] Percent infected cells as determined by IFA. –, negative result.

TABLE 3. Infectivity of PUU Kazan strains in bank voles

Virus dilution	Infectivity[a]		
	Kazan-wt	Kazan-E6-I, p5	Kazan-E6-II, p7
10^{-1}	3/3	0/3	0/3
10^{-2}	3/3	0/3	2/3
10^{-3}	3/3	0/3	1/3
10^{-4}	3/3	1/3	2/3
10^{-5}	3/3	1/3	0/3
10^{-6}	3/3	0/3	0/3
10^{-7}	2/3	0/2	1/3

[a] Number of antigen-positive animals/number of inoculated animals as determined by hantavirus antigen ELISA. Mean optical densities (450 nm) of duplicate samples of >3 times the mean value obtained with two duplicate negative-control antigen samples (from noninfected lungs) were defined as positive.

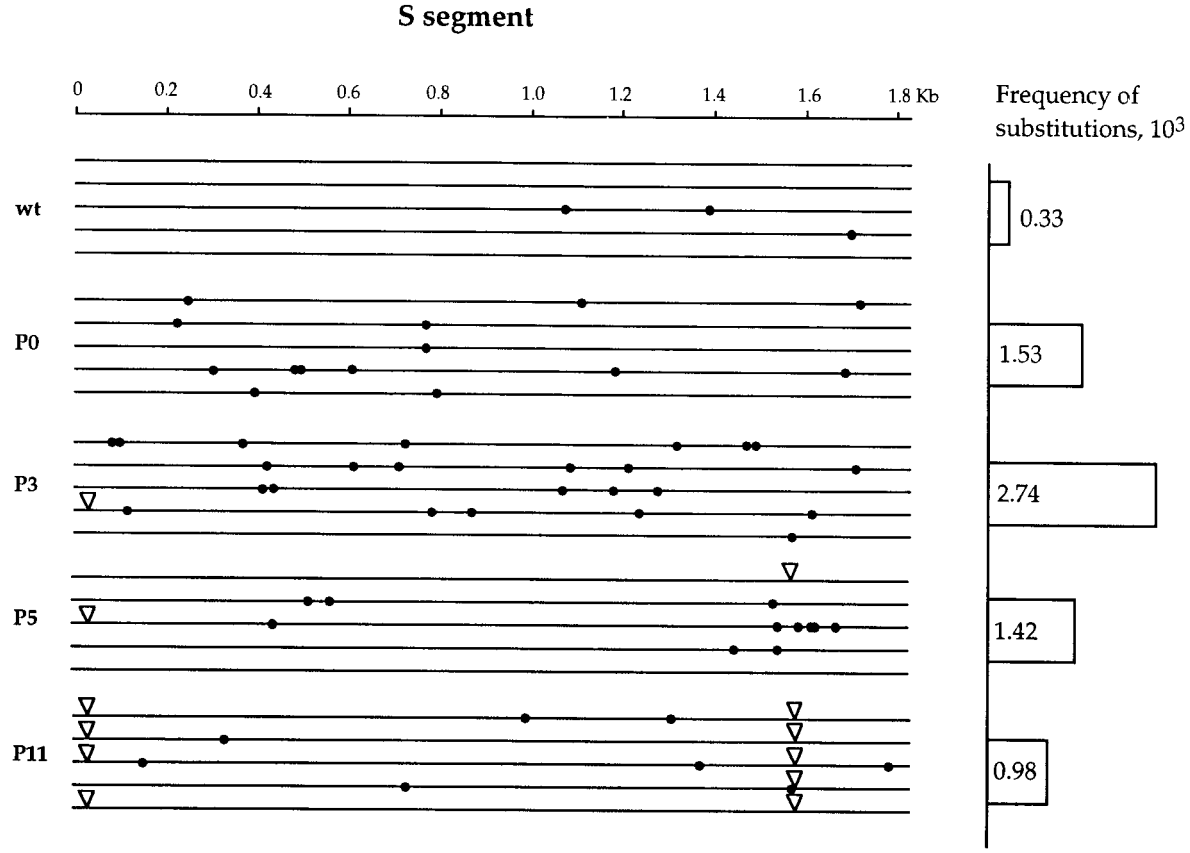

FIG. 3. Distribution and frequencies of nucleotide replacements in the S segment of PUU virus strain Kazan during adaptation to Vero E6 cells. Horizontal lines represent the S segment of individual cDNA clones prepared from the wt and p0, p3, p5, and p11 (five clones from each passage). Mutations in the noncoding region at positions 26 and 1577 (triangles) and other mutations (circles) are indicated. The frequency of nucleotide substitutions (horizontal bars) was calculated with respect to the consensus sequences deduced for each passage. The level of RT-PCR error (0.2×10^{-3} nt) was determined in the control experiment, which, in addition to reamplification and recloning of the S segment (35, 36), also included an RT step.

found in the consensus sequences of the entire M segments from the wt and Vero E6-adapted (Kazan-E6-I, p11) variants, suggesting preservation of both the noncoding regions of the gene (at least outside the first 25 nt and the last 19 nt) and the deduced sequences of G1 and G2 glycoproteins during the adaptation process.

The entire S segment was amplified in one piece with a single primer. Five individual cDNA clones containing the entire nucleotide sequence of the S segment from Kazan-wt and Kazan-E6-I, p0, p3, p5, and p11, were sequenced and, as expected, represented individual S-segment quasispecies (Fig. 3). For each set of cDNA clones, a consensus sequence of the S segment was deduced. Three clones from the initial Kazan-wt and one clone each from p5 and p11 of Kazan-E6-I were found to carry such consensus sequences. Otherwise, the individual clones differed from the consensus sequences by at least one nucleotide substitution.

Dynamics changed in the mutation frequencies calculated for the S-segment quasispecies during the adaptation (Fig. 3). After the virus was placed in cell culture, the mutation frequency increased fivefold in p0, reached its maximum in p3 (eightfold), and then decreased. The mutation frequency calculated for the proximal part of the M segment for Kazan-wt (0.68×10^{-3} nt) and Kazan-E6-I, p5 (1.15×10^{-3} nt) and p11 (0.23×10^{-3} nt), followed this pattern. The level of RT-PCR errors, estimated in a control experiment, was 0.2×10^{-3} nt and should therefore not be considered crucial for the interpretation of the data.

Comparison of the consensus sequences from the Kazan-wt strain and Kazan-E6-I, p11, revealed no differences in the coding region of the S gene, suggesting the preservation of the N protein sequence during the adaptation. However, the noncoding regions were found to be different at positions 26 and 1577. Notably, accumulation of mutants with the two substitutions located in the noncoding regions (Fig. 3) became increasingly pronounced. Whereas at p3 or p5 only one cDNA clone carried a G→U transversion at position 26, at p11 four such clones were found. Similarly, all five p11 clones carried a transversion U→G at position 1577, versus only one p5 clone and none of the wt, p0, and p3 clones. To check whether these findings were dependent on the number of cDNA clones selected, we analyzed several more clones from p5 and p11. The ratios of G to U at position 26 were 8:2 for p5 and 4:4 for p11. The ratios of U to G at position 1577 were 7:2 and 2:7 for p5 and p11, respectively. Thus, the consensus sequence, which had been identical at all passages from the original Kazan-wt to Kazan-E6-I, p5, was changed at p11, at least in position 1577.

A similar phenomenon was observed in the second adaptation; nine cDNA clones (from Kazan-E6-II, p7) were sequenced, and all carried a C→U substitution at position 1580, just 3 nt downstream from the substitution observed in the first adaptation (position 1577). Interestingly, the identical muta-

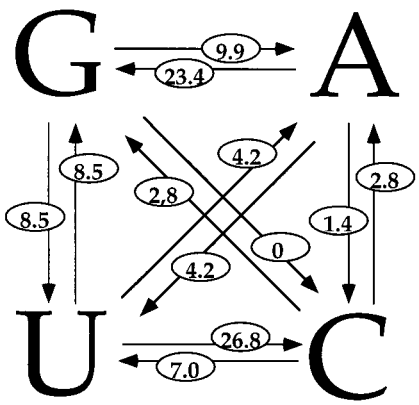

FIG. 4. Schematic illustration of the nucleotide substitutions observed during the adaptation of Kazan-wt to Vero E6 cell culture. The numbers indicate the percentages of the total number (72) of substitutions.

tion was observed in one of the p5 clones from the first set of experiments (Fig. 3), but it did not become dominant. No mutations, however, were found within the 5′NCR during the second adaptation.

Most of the 72 nucleotide substitutions observed (Fig. 4) were transitions (67%). Among them, U→C and A→G dominated, while their counterparts, C→U and G→A, as well as transversions U→G and G→U, occurred less often. Thus, all three substitutions described above, G_{26}→U, U_{1577}→G, and C_{1580}→U, belonged to neither the more prominent mutation events nor the rarest ones. The mutations were evenly distributed among codon positions, indicating that there was no selection operating at the protein level, similarly to what was described earlier for S-segment quasispecies of another hantavirus, TUL (36).

Taken together, the genetic data supported altered phenotypes for the Vero E6-adapted variants of the Kazan strain. The results further indicated (i) different impacts of the S and the M genomic segments for the adaptation process and (ii) selective advantages for the variants carrying altered noncoding sequences in the S segment.

DISCUSSION

Characterization of PUU virus strain Kazan. Hantaviruses are usually difficult to isolate and grow in cell culture (29). This has hampered the study of the viruses in the laboratory, and many questions concerning the potential genetic changes which influence the ability of hantaviruses to replicate in different hosts remain. In addition, previous experimental data have indicated important differences in the infectivity of various PUU virus strains (references 5, 23, 27, and 41, and unpublished data). There might be several possible explanations for these observations, e.g., (i) cell culture-isolated strains are less infective for animals than wt strains, (ii) PUU virus strains of different origins may vary in their infectivity, and (iii) some of the earlier results may be partially explained by different virus passage histories or the use of different rodent species, different inoculation protocols, and different systems for analysis. There have also been reports suggesting that PUU virus strains from western Russia may cause more severe clinical symptoms in humans than those from Scandinavia (45). The data for patients support the first alternative, since there are several reports of acquired PUU virus infection in people who captured or worked with infected animals, but to our knowledge, there are no reports of HFRS cases contracted from work with cell-cultured PUU virus.

In order to address these questions, a model based on the PUU virus strain Kazan was established. Strain Kazan was originally isolated in colonized bank voles (14), and the wt variant of this strain (Kazan-wt, passaged only in bank voles) has previously been shown to efficiently infect bank voles and was consequently selected for our virus challenge model (reference 27 and our unpublished data). However, PUU virus strain Kazan has not previously been characterized in detail. Our phylogenetic analysis placed the Kazan-wt strain with the other strains belonging to the PUU virus serotype and close to PUU virus strains originating from the same geographic region, pre-Ural Russia. Cross-neutralization data confirmed that Kazan belongs to the PUU virus serotype. This was further confirmed by an investigation with a panel of PUU virus MAbs in which no antigenic differences were found. It should be noted, however, that neutralization tests revealed antigenic differences between the Kazan strain and the Finnish prototype strain Sotkamo and the Swedish strain Vindeln/83-L20. Whether PUU virus strains from certain geographical regions could be regarded as distinct subtypes of PUU virus remains to be discussed. The sequence data for the S and M genome segments revealed characteristics that were similar, or even identical, to those of other PUU virus strains (1, 16, 34, 35, 48, 51). Thus, the genetic and serologic data were in good agreement.

Adaptation to Vero E6 cells attenuates PUU virus strain Kazan for reproduction in its natural host. The virologic analysis of the Kazan-wt and Kazan-E6 strains revealed major differences in their infectivity for bank voles. The Kazan-wt strain infected the animals efficiently, in terms of detectable levels of N antigen in the lungs, whereas the Vero E6-adapted strains did not. The inoculated rabbits, although limited in numbers, showed similar results. Titration experiments revealed that there were no major differences in the total amounts of N antigen between the different virus stocks used in the study, indicating that the different characteristics observed in vivo could not be explained by quantitative differences. This was further confirmed by the relatively high titers observed for the Kazan-E6 strains when used to inoculate cells (4.5×10^5 and 3.0×10^5 focus-forming units/ml, respectively) and also by the single animal infected by a 10^{-7} dilution of Kazan-E6-II.

The opposite was observed in the cell culture experiments, in which the E6 strains replicated to a much higher efficiency. The results also indicated that the cell culture adaptation process varies from one trial to another, although the same inoculum was used: in the first Vero E6 adaptation, the reproduction seemed to have reached an equilibrium already after 6 weeks, in contrast to the second adaptation, in which a plateau was not reached until 16 weeks after the first Vero E6 inoculation (Fig. 2). Taken together, the results showed that the wt virus is well adapted for growth in bank voles but not in cell culture and that the sometimes slow process of adaptation to cell culture growth altered the phenotype of the virus, i.e., decreased its infectivity for the natural host. The data are in agreement with the reports of difficulties regarding isolation of many hantaviruses (references 42 and 49 and our unpublished data). Although not yet proven, the results may further explain why there have been no reports of infections of humans, at least not with clinical symptoms, caused by cell culture-adapted hantaviruses.

Genetic marker(s) of the adaptation. The sequence analysis revealed, as expected, that the viral populations from both wt and Vero E6-growing virus were represented by quasispecies. Such a structure, in general, allows for a rapid evolution based

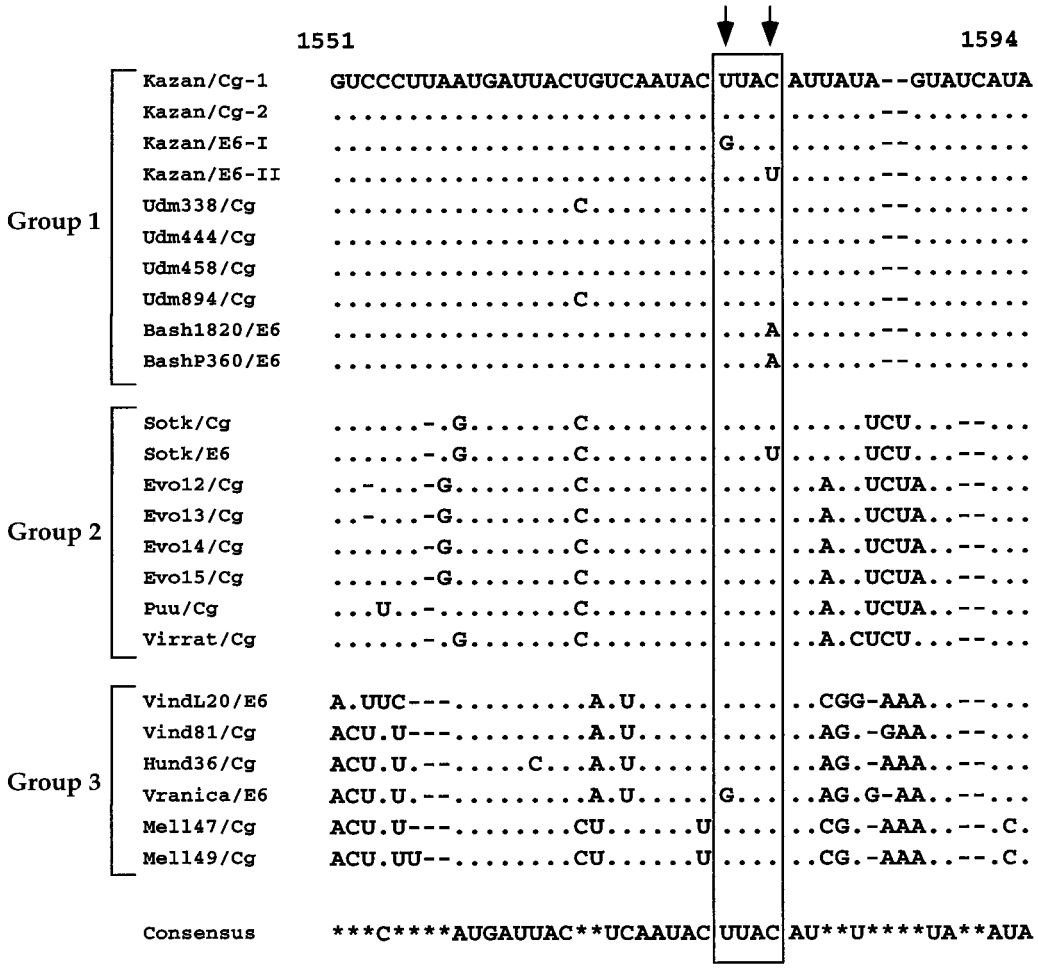

FIG. 5. Multiple alignment of the S-segment sequences of PUU virus strains (nt 1551 to 1594 in the Kazan strain sequence). ., identical nucleotide; −, deletion. Positions of base substitutions that accompanied adaptations of bank vole-originated viruses to Vero E6 cells are indicated (arrows). Group 1, strains originated from pre-Ural Russia; group 2, strains from Finland; group 3, strains from Sweden. The strains are as follows: Kazan/Cg-1, strain Kazan-wt (passaged in *C. glareolus* in Stockholm; GenBank accession no. Z84204); Kazan/Cg-2, strain Kazan, passaged in *C. glareolus* in Moscow by Irina Gavrilovskaya (our unpublished data); Kazan/E6-I, strain Kazan-E6-I, p11 (Vero E6), from the first adaptation attempt; Kazan/E6-II, strain Kazan-E6-II, p7 (Vero E6), from the second adaptation attempt; Udm338/Cg to Udm894/Cg, strains Udmurtia/338Cg/92, -444Cg/88, -458Cg/88, and -894Cg/91, originated from trapped bank voles (34, 35) (accession no. Z21497 and Z30706 to 30708); Bash1820/E6, strain Bashkiria/CG1820 (46) (accession no. M32750); BashP360/E6, strain Bashkiria/P360 (42a); Sotk/Cg, strain Sotkamo, wt (our unpublished data); Sotk/E6, strain Sotkamo passaged in Vero E6 cells (48) (accession no. X61035); Evo12/Cg to Evo15/Cg, strains Evo12/Cg/93, -13Cg/93, -14Cg/93, and -15Cg/93, originated from trapped bank voles (35) (accession no. Z30702 to 30705); Puu/Cg, strain Puumala/1324Cg/79, originated from a trapped bank vole (35) (accession no. Z46942); Virrat, strain Virrat/25Cg/95, originated from a trapped bank vole (38) (accession no. Z69985); VindL20/E6, strain Vindeln/83-L20 passaged in Vero E6 cells (16) (accession no. Z48586); Vind81, Hund36/Cg, Mell47, and Mell/49, strains Vindeln/81Cg/94, Hundberg/36Cg/94, Mellansel/47Cg/94, and Mellansel/49Cg/94, respectively, all originated from trapped bank voles (our unpublished data); and Vranica/E6, strain "Vranica"/Hällnäs passaged in Vero E6 cells (39) (accession no. U14137).

on the selection of variants preexisting in a mutant spectrum (for reviews, see references 8 and 15). This mechanism has been demonstrated to be involved in the formation of antibody escape (28) or drug-resistant (40) virus mutants as well as variants with altered virulence (10) or cell tropism (7, 33).

Only a limited number of variants was observed within the viral population of bank vole origin (Fig. 3). In contrast, an increased genetic heterogeneity of the viral population during the first phase of the adaptation process (p0 to p3) was observed with respect to both of the studied genes, the M and S genes. The data most probably reflected a change in the selective pressure when the virus suddenly was forced to reproduce in a new host, Vero E6 cells, which also allowed more variants to evolve.

No changes in the consensus sequences within the coding regions of either the Kazan S or M gene were observed during our adaptation experiments. In contrast to the S gene, no mutations were found in the noncoding regions of the M segment. There might be several explanations for this difference; e.g., any change in the M segment could result in nonviable variants, or the regulation of the two genes could be operating via different mechanisms, as previously suggested for SN hantavirus (2, 19). It should be stressed that the very termini, nt 1 to 25 and 3665 to 3683 of the M segment and nt 1 to 22 of the S segment, escaped our analysis. Also, variations in the L segment, which were not studied, could be important for adaptation.

Variants which carried mutations in the 5′NCR of the S gene were observed only in the first adaptation experiment, which was more efficient than the second in terms of the high level of stable expression of viral N antigen obtained earlier (Fig. 2). The mutated allele was even with the original allele

(ratio, 4:4) but did not become dominant, at least not by p11. The data indicated that during the adaptation, there was a selection for the variants with a changed 5′NCR sequence(s) (which probably carry regulatory elements, including a putative promoter) and suggest that changes in the region might be valuable for an efficient adaptation. This conclusion was further supported by the finding of more mutations within this region among the mutants from p5 and p11. For these two passages, we were able to amplify the entire S segment, using a shorter primer which contained only 18 terminal nt. One of ten cDNA clones from p5 and two of eight clones from p11 carried identical transitions (U→C) at position 20.

Different kinetics for the hantaviral N, GPC, and L protein mRNA accumulation in Vero E6 cells have been demonstrated, and different mechanisms for transcription termination for each of the three viral RNAs have been proposed (19). Also, by analogy to vesicular stomatitis virus, it was proposed that the sequence peculiarities of the noncoding regions of hantavirus RNA templates could determine the differential rates of RNA segment transcription or replication (2). If so, our findings could be interpreted in terms of a key position of the S segment in the regulation of PUU virus genome expression. This hypothesis is supported by the observation, during two independent adaptation attempts, of two closely located mutations in the 3′NCR of the S segment. The observed mutations located at positions 1577 and 1580 might belong to such a motif. Multiple alignment of the surrounding sequences (Fig. 5) revealed a remarkably conserved stretch of 23 bases, of which 21 were identical in most of the PUU virus strains. Notably, all bank vole-derived sequences carry U at position 1577 and C at position 1580 (Fig. 5). In six of seven Vero E6-derived sequences, one of these nucleotides is altered. Moreover, identical C→U mutations were found in two pairs of sequences, Kazan-wt versus Kazan-E6-II and wt Sotkamo versus Sotkamo passaged in Vero E6 cells. Taken together, these findings strongly support the idea of a functional nature of the observed C→U mutations. However, the absence of either of these substitutions in the Vind/L20/E6 sequence (Fig. 5) indicates that there might also be other mutations responsible for the adaptation to Vero E6 cells. This explanation is in line with the results observed for other RNA viruses suggesting that an altered phenotype of a given mutation may vary substantially depending on the particular genetic background in which it arises (30).

Our findings of phenotypic changes and the S-gene 3′NCR nucleotide substitutions in PUU virus are somehow contradictory to the previously published data of Chizhikov et al. (2) suggesting that no genetic selection and adaptation took place during growth of SN virus, first in experimentally infected deer mice and subsequently in Vero E6 cells. The difficulty of isolating SN virus in cell culture was explained by low concentrations of infectious virus in the starting clinical or rodent-infected material. Further nucleotide sequence analysis of paired hantavirus isolates and their original wt counterparts will be needed to determine whether the observed adaptation changes are restricted to PUU virus.

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