2003

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VIROLOGY

Virology 306 (2003) 219–224

www.elsevier.com/locate/yviro

Rapid Communication

Rescue of Hantaan virus minigenomes

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Received 27 September 2002; returned to author for revision 8 October 2002; accepted 18 October 2002

Abstract

Hantavirus infections are a major public health concern worldwide. Their widespread geographical distribution and their ability to produce serious, often fatal, human disease underline the need for a system that allows manipulation of these viruses. We describe here the first successful establishment of a reverse genetics technology for Hantaan virus, the prototype of the genus *Hantavirus*. The system offers a unique opportunity to study the biology of hantaviruses, the pathogenesis of the diseases, and the efficacy of antiviral and prophylactic measures against hantavirus infections.

Introduction

Hemorrhagic fever with renal syndrome (HFRS), caused by viruses in the *Hantavirus* genus, family *Bunyaviridae*, is a collective term for a group of clinically similar diseases. Worldwide attention was first focused on HFRS during the Korean War (1950-1953), when more than 3000 cases of Korean hemorrhagic fever occurred among United Nations troops. Since then, four distinct hantaviruses have been shown to cause HFRS in Asia, Western Russia, Europe, and Scandinavia (Lee et al., 1999). The recognition of a second hantavirus-associated disease, termed hantavirus pulmonary syndrome (HPS), in the U.S. in 1993 led to the discovery of many additional hantaviruses throughout the Americas (Nichol et al., 1993). Today hantaviruses are considered as globally distributed pathogens with major public health concerns in many countries. The occurrence of HFRS and HPS is driven by the geographic distribution of the rodent reservoirs carrying the human pathogenic hantaviruses. Thus, HFRS is primarily found in Eurasia with more than 100,000 hospitalized cases annually and a mortality of up to 15%. HPS is restricted to the Americas and has been identified in 31 U.S. states, Canada, and throughout Central and South America. It is comparatively rare but shows a much higher mortality rate (~40%) than HFRS. One of the HPS-causing hantaviruses, Andes virus, also possesses the potential of human-to-human transmission (Wells et al., 1998), which is of considerable concern.

Currently, studies of hantavirus biology and disease pathogenesis are limited by the lack of a reverse genetics system for correlating specific gene regions with gene functions. Although introducing mutations into the genomes of RNA viruses has created powerful tools for studying the pathogenesis and the functions of essential genes/proteins (Flick and Pettersson, 2001; Flick et al., 2002; Neumann et al., 2002), and for developing attenuated vaccine candidates (Murphy and Collins, 2002; Watanabe et al., 2002), the manipulation of negative-strand RNA viruses is cumbersome using the typical RNA polymerase II transcription system that has proven to work well for positive-strand RNA viruses. In part, this is due to the absence of a 5′ cap structure and a 3′ poly(A) tail at the termini of the negative-

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doi:10.1006/S0042-6822(02)00070-3

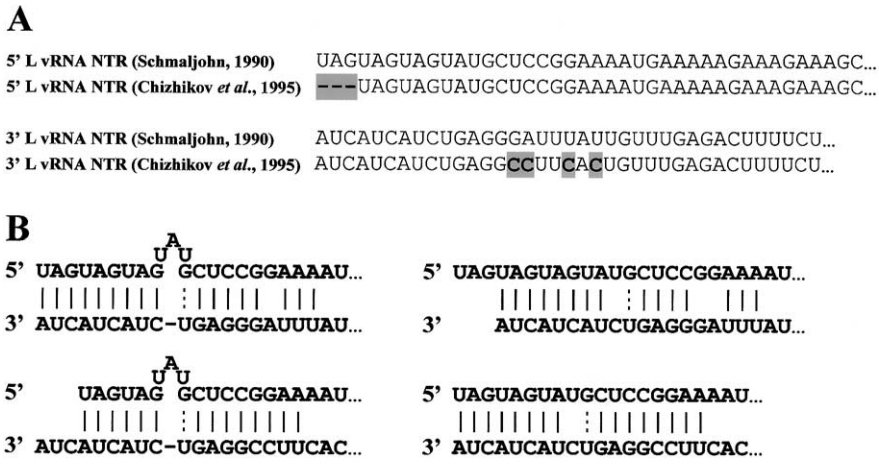

Fig. 1. Comparison of published nucleotide sequences of 5′ and 3′ termini of the Hantaan 76-118 strain. (A) The nucleotide sequences used for HTNV minigenome constructs are based on the published sequence by Schmaljohn, 1990 (Accession No. X55901), which differs in some positions to a sequence published by Chizhikov and colleagues (1995). (B) Comparison of the secondary structures predictions of the highly conserved terminal regions of the HTNV L genome segment between the sequences listed under Accession No. X55901 (Schmaljohn, 1990) (top) and determined by Chizhikov et al. (1995) (bottom).

strand RNA genomes, common features usually generated by RNA polymerase II transcription. It has been particularly difficult to develop reverse genetics systems for bunyaviruses (Lopez et al., 1995; Bridgen and Elliott, 1996; Flick and Pettersson, 2001) and all attempts for hantaviruses have so far failed (Marczinke et al., 2001; A. Plyusnin and R. Elliott, personal communication). Here we describe for the first time the establishment of a reverse genetics system for Hantaan virus (HTNV), the prototype of the genus *Hantavirus*.

Results and discussion

In the first step toward the establishment of a reverse genetics system, a reporter gene is usually used to demonstrate functionality. Two systems have been used to generate artificial genomes or genome segments of negative-stranded RNA viruses: the more commonly used and widely established T7 RNA polymerase-driven system (T7 system) (Fuerst et al., 1986), and, the more recently developed RNA polymerase I driven system (pol I system) (Zobel et al., 1993; Neumann et al., 1994). The pol I system, pioneered by Hobom and colleagues (Zobel et al., 1993; Neumann et al., 1994), has the advantage that the DNA-dependent RNA polymerase does not have to be provided in trans (e.g., by a recombinant vaccinia virus or plasmid cotransfection), since this enzyme is already part of all eukaryotic cells. Furthermore, the initiation and termination of pol I derived transcripts are clearly defined (Zobel et al., 1993; Flick and Hobom, 1999) and correct transcript ends can be produced without the need of genetically engineered elements, such as the hepatitis δ ribozyme. Our approach differed from those of others in that we used the pol I system and templates with the terminal nontranslated regions (NTR) of the HTNV large (L) segment rather than those of the medium (M) or small (S) segment (Fig. 1A). This strategy was based on the assumption that the L segment ends represent a stronger promoter than that of the S genomic segment to efficiently transcribe and replicate this larger RNA molecule and on our recent data obtained from the Uukuniemi virus reverse genetics system, which experimentally supports this idea (Flick and Pettersson, unpublished data). Two distinct sequences are published for the HTN NTR of the L segment (Schmaljohn, 1990; Chizhikov et al., 1995). We used the sequence determined by Schmaljohn (1990), because this sequence allowed a more stable predicted panhandle-like structure in the proximal promoter element (nine vs eight potential base pairs) (Fig. 1B).

By combining the two NTR of the HTNV L-genome segment, which are assumed to contain all regulatory elements needed for encapsidation, transcription, replication, and packaging of the genome into progeny virus particles, with the chloramphenicol acetyl transferase (CAT) reporter gene, we generated an artificial hantaviral genome segment (minigenome), which was inserted into two different pol I system vectors (pRF207, pRF240) (Flick, unpublished data). Subsequently, each plasmid was transfected into either a mixture of baby hamster kidney cells (BHK-21) and monkey kidney cells (VeroE6) (Fig. 2A) or human embryonic kidney cells (293T) (Fig. 2B) to generate CAT-containing RNA minigenomes through the activity of the pol I.

For subsequent transcription, replication, and encapsidation of these pol I generated artificial segments, the viral polymerase protein (L) and the nucleocapsid protein (N) are needed (Figs. 2A and 2B). Therefore, cells transfected with HTNV minigenome constructs were first superinfected with HTNV (strain 76-118) under biosafety level 3 conditions (Fig. 2A). To create optimal conditions, we used a combination of BHK-21 and VeroE6 cells. BHK-21 cells possess

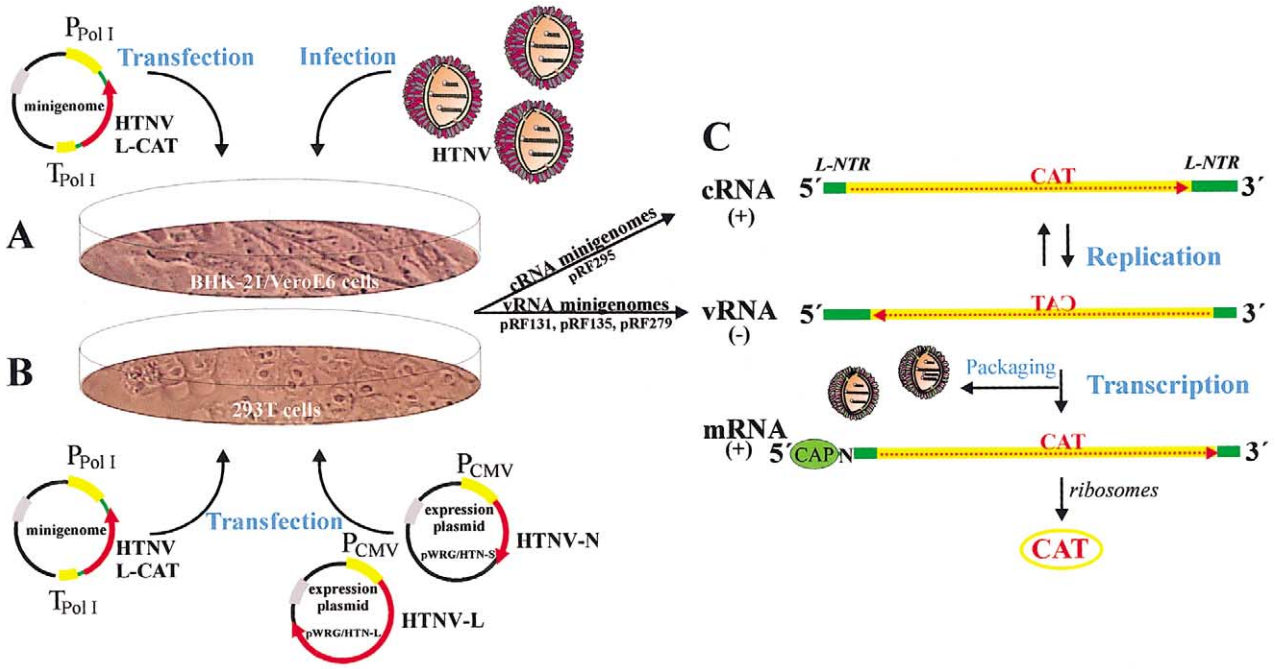

Fig. 2. Development of a pol I driven reverse genetics system for HTNV. (A) A mixture of BHK-21 and VeroE6 cells was transfected with pol I driven HTNV L-CAT minigenome constructs. Cells were superinfected with HTNV, strain 76-118, 20-24 h posttransfection. Subsequently, cells were harvested 24 h p.i. (44-48 h posttransfection) and minigenome transcription/replication was measured by detection of CAT activity. (B) 293T cells were transfected with pol I driven constructs expressing different HTNV L-CAT minigenomes. To provide the viral polymerase and nucleocapsid proteins for minigenome encapsidation, replication, and transcription, cells were cotransfected with the appropriate CMV-driven expression plasmids (pWRG/HTN-L, pWRG/HTN-S). (C) Schematic presentation of transcription and replication of pol I driven HTN L-CAT minigenomes. The constructs pRF131, pRF135, and pRF279 are transcribed into artificial vRNA segments (minigenomes), whereas pRF295 is generated as a cRNA-oriented molecule.

a higher transfection efficiency than VeroE6 cells and are suitable for the murine pol I driven HTNV minigenome constructs (pRF135, pRF279; Fig. 4) (Flick and Pettersson, 2001), whereas VeroE6 cells are better for HTNV infection and propagation. We harvested the superinfected cell cultures 24 h p.i. (44–48 h posttransfection) and measured CAT activity, an indicator for successful transcription and replication of the artificial genome segment, by thin layer chromatography using commercially available kits Fig. 3). Significant reporter gene activity could be detected in superinfected vs noninfected control cells (Fig. 3, Lane 6: 69% vs 20%, and Lane 7: 58% vs 12%). CAT expression level of superinfected cells was comparable to those that were obtained using a previously established reverse genetics system for the phlebovirus Uukuniemi (Flick and Pettersson, 2001; Flick et al., 2002) and the orthomyxovirus influenza A (Neumann et al., 1994). Our data support the notion that the viral proteins necessary for transcription and replication of the minigenomes are expressed early after infection and that the relatively slow release of virus particles is not due to a lack of replicated vRNA genome segments or an insufficient amount of viral proteins during an early stage of the infection cycle. It remains to be determined if VeroE6 cells enhanced the rescue through a more efficient propagation of minigenome-containing recombinant viral particles or if they provided specific cellular factors that favored a more efficient rescue.

To optimize the system and reduce background CAT activity from a potential cryptic promoter located in the pol I vector that we used, we inserted a series of stop-codons downstream of the RNA pol I terminator region (Flick and Pettersson, 2001). Transfection of BHK-21/VeroE6 cells with the resulting plasmid, pRF279 (Fig. 4), showed a notably reduced CAT activity background as compared to pRF135, without stop codons (Fig. 3, Lanes 6 vs 7). Despite background reduction of about 40%, some remaining CAT signal could still be detected after transfection of the HTNV minigenome cassette only (Fig. 3, Lane 7). This phenomenon has also been observed by other groups attempting to establish reverse genetics systems for hantaviruses (A. Plyusnin and S. Nichol, personal communication). A likely reason for this observation is the presence of a sequence located in the 3′ NTR of some of the hantaviral genome segments, which serves as a cryptic promoter site in the minigenome plasmids. Replacing the sequence preceding the CAT gene (3′ vRNA/5′ cRNA) by noncoding regions of other bunyaviruses (e.g., Uukuniemi virus, Rift Valley Fever virus, Crimean-Congo Hemorrhagic Fever virus) eliminated the background signal, confirming the role of this element as a cryptic promoter site (Flick and Pettersson, 2001 and Bouloy and Flick, unpublished data).

The ultimate aim of our work was to develop an infectious clone of HTNV. To achieve this, the viral polymerase and nucleocapsid proteins have to be provided in an alter-

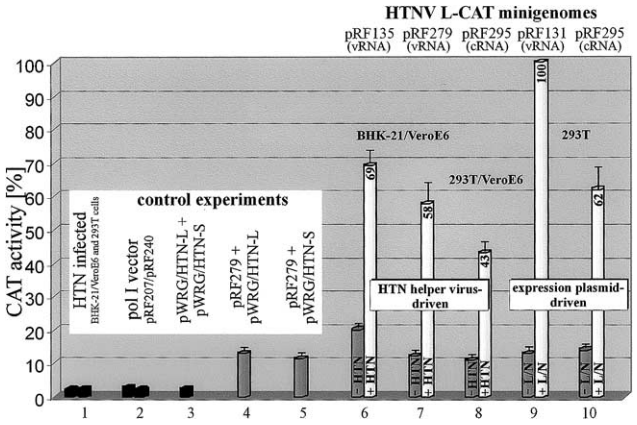

Fig. 3. Graphic representation of CAT activities from HTN minigenome rescue experiments. Lane 1 to 5: Control experiments to evaluate the reporter gene background of the HTNV reverse genetics system. CAT activity was determined after HTNV infection of BHK-21/VeroE6 cells or 293T cells (Lane 1), after transfection of two different pol I vector plasmids (Lane 2), and after transfection of HTNV specific expression plasmids (pWRG/HTN-L and pWRG/HTN-S) (Lane 3). Cell lysates were further analyzed after cotransfection of HTNV minigenome pRF279 and either HTNV-L or HTNV-S expression plasmids (Lanes 4 and 5). Lanes 6 to 8: HTNV minigenome transfections with pRF135 (Lane 6), pRF279 (Lane 7), or pRF295 (Lane 8) and HTNV superinfections were performed as illustrated in Fig. 2A and CAT activity was determined in the BHK-21/VeroE6 or 293T/VeroE6 cell lysates 44-48 h posttransfection. Lanes 9 and 10: 293T cell lysates were analyzed 24-48 h posttransfection after cotransfection of HTNV minigenome constructs pRF131 (Lane 9) or pRF295 (Lane 10) and the two HTNV expression plasmids for the L and N proteins (pWRG/HTN-L and pWRG/HTN-S) (illustrated in Fig. 2B). All transfections were done at least three times.

native way to helper virus superinfection. As a step toward this goal, we developed CMV-driven expression plasmids for the HTNV L and N proteins and cotransfected both these plasmids (pWRG/HTN-L, pWRG/HTN-S) together with the minigenome construct, pRF131 (Fig. 2B). To take advantage of the high transfection efficiency of 293T cells, we used the human pol I promoter system for this approach (Neumann et al., 1999). Cells were harvested 24-48 h posttransfection and cytoplasmic extracts were analyzed for reporter gene activity. The CAT activity in cell lysates from the cotransfected cells was higher than observed in cells transfected with only the minigenome, indicating the successful rescue of pol I driven HTNV minigenomes without helper virus superinfection (Fig. 3, Lane 9). Moreover, these results demonstrate expression of functional HTNV L- and N-proteins driven by the CMV promoter (pWRG/HTN-L, pWRG/HTN-S).

Both strategies, helper virus superinfection (Fig. 2A) and plasmid cotransfection (Fig. 2B), confirmed the ability of the artificial genome segments to serve as templates for transcription. To demonstrate replication of these minigenomes, we generated HTNV minigenome constructs, which are transcribed by the cellular RNA pol I into (+)-sense cRNA (pRF295) rather than vRNA (pRF131) molecules (Figs. 2C and 4). These HTNV cRNA minigenomes have to undergo replication (cRNA → vRNA) prior to serving as templates for the generation of viral mRNA molecules (vRNA → mRNA) (Fig. 2C). Following cotransfection of pRF295 with the HTNV-L and -N expression plasmids, CAT activity could be detected in cell extracts (Fig. 3, Lane 10). This demonstrates that the viral L protein mediates transcription and replication of pol I driven minigenomes and confirms that the L- and N-proteins expressed under the control of the CMV promoter were functional. Furthermore, the cRNA minigenome (pRF295) could also be rescued using the helper virus-driven system. However, the reporter gene expression rate was lower (Fig. 3, Lane 8) compared to vRNA transcripts (Fig. 3, Lanes 6 and 7). All results were confirmed by at least three independent experiments including a series of control experiments (Fig. 3, Lanes 1 to 5) to exclude false positive results.

This reverse genetics system is a breakthrough in hantavirus research. It is the first time that a functional recombinant hantavirus polymerase has been expressed. Furthermore, the successful rescue of HTNV minigenomes in the absence of helper virus makes it possible to use this reverse genetics system outside of a containment laboratory. Using the system we described, it will be possible to determine important regulatory elements of virus replication (Fuerst et al., 1986; Flick and Hobom, 1999; Flick et al., 2002) and, thus, should be useful for antiviral drug screening. Following further development and optimization, this system will facilitate studies of the biology of hantaviruses and the pathogenesis of HFRS. Thus, it will greatly improve the chances to develop urgently needed prophylactic measures and more feasible therapeutic interventions for these important human pathogens. Furthermore, the establishment of this reverse genetics system is an example of the usefulness of the pol I system to drive reverse genetics systems for viruses that do not replicate in the nucleus, such as the bunyaviruses.

Materials and methods

Cells and virus

BHK-21, 293T, and VeroE6 cells (American Type Culture Collection) were grown on plastic dishes in Glasgow or Eagle's minimal essential medium (GMEM/EMEM), supplemented with 7.5% fetal calf serum, 2mM L-glutamine, 100 IU penicillin/ml, and 100 μg streptomycin/ml. HTNV, strain 76-118, was used for all experiments. Cells were infected with a multiplicity of infection (m.o.i.) of approximately 0.1-1 PFU/cell.

Construction of plasmids

Plasmids designed for the transcription of HTNV vRNA or cRNA molecules by the RNA pol I carried the rDNA promoter (−251 to −1 relative to the 45S pre-rRNA start

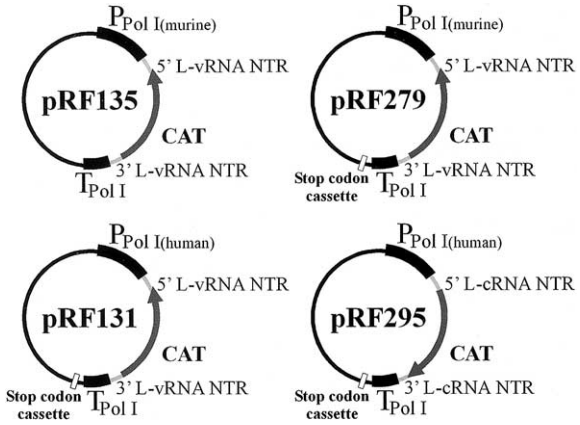

Fig. 4. Schematic diagram of different HTNV L-CAT minigenomes used for reverse genetics studies. The human and murine RNA polymerase I (pol I) promoters are designated as $P_{polI(murine)}$ and $P_{polI(human)}$. The murine pol I terminator sequence is indicated as T_{polI}. vRNA minigenome constructs pRF131, pRF135, and pRF279 contain CAT reporter genes in antisense orientation, whereas a cRNA transcription cassette in sense orientation was inserted in construct pRF295. The CAT gene is flanked by the nontranslated regions (NTR) of HTNV L segment; 40 nt represents the 5′ vRNA (or 3′ cRNA) and 37 represents the 3′ vRNA (or 5′ cRNA) part (Fig. 1) (Schmaljohn, 1990). For background reduction of CAT activity stop codons (indicated with a white box) were inserted adjacent to the pol I terminator.

point) and terminator region (+571 to +745 relative to the 3′ end of the 28S rDNA) of the murine rDNA (Zobel et al., 1993). Between these two elements, the 5′ and 3′ NTR of the HTNV L segment (Schmaljohn, 1990) (Accession No. X55901) (Fig. 1A) were precisely inserted in either antisense (primers RF132/RF133 or RF132/RF131; RF131: 5′-AAT**GAAGAC**GTTATTTAGTAGTA-GTATGCTCCGG-AAAATGAAAAAGAAA-GAAAGC*TTACGCCCCGCCCTGCCACTC*, RF132: 5′-AAT**GAAGAC**GGGGGTAGTAGTAGACTCCCTAAA-TAACAAACTCTGAAAAGA*ATGGAGAAAAAAATCACT-GGATATACC*, RF133: 5′-ATT**GAAGAC**GTAG-GTTAGTAGTAGTATGCTCCGGAAAATG) or in sense orientation (primers RF302/RF303; RF302: 5′-AAT**GAAGA-C**GGTATTTAGTAGTAGACTCCCTAAATAACAAACTCT-GAAAAGA*ATGGAGAAAAAAATCACTGGATATACC*, RF303: 5′-**AATGAAGAC**GGGGGTAGTAGTAGTATGCTCCGGA-AAATGAAAAAGAAAGAAAGC*TTACGCCCCGCCCTGC-CACTCATCGC*) for vRNA or cRNA transcription, respectively [cleavage sites are marked in bold; CAT reporter gene sequences are marked in italics, and HTNV L-NTRs are underlined]. For sensitive detection of protein expression following transcription and replication, the open reading frame of the CAT gene was inserted. Using this strategy, we generated two vRNA [pRF131 (human pol I promoter) and pRF135 (murine pol I promoter)], and a single cRNA construct [pRF295 (human pol I promoter)] (Fig. 4), which following transfection into cells (BHK-21, 293T, and VeroE6) result in transcripts lacking 5′ and 3′ modifications [e.g., cap structure, poly(A) tail] (Fig. 2C) (Zobel et al., 1993; Flick and Hobom, 1999). All constructs were verified by dideoxy-sequencing using an ABI PRISM310 sequencer.

For the expression of HTNV-specific proteins, plasmids containing the HTNV L gene or N gene controlled by a CMV promoter were constructed. The plasmid containing the HTNV L gene, designated pWRG/HTN-L(UU5), was constructed as follows: the L gene was excised from plasmid puc18HTN-L(PP3) using *Bsp*EI (blunted), gel purified, and then ligated into the *Not*I site (blunted) of a CMV promoter-containing expression vector, pWRG7077. pWRG7077 has been described previously (Schmaljohn et al., 1997). The source of the L gene, puc18HTN-L(PP3), was constructed by RT-PCR amplification of the full-length L gene from HTNV 76-118 RNA in two parts, followed by ligation into a puc18-based vector. The HTNV N gene was subcloned from a previously described plasmid (Schmaljohn et al., 1986) using forward primer HTNS20-39F (5′-CG**AGATCT**AGAGCTACTAGAACAACG) and reverse primer HTNS15527-1535R (5′-CG**AGATCT**GATCAA-ATGAAATCTAC). The PCR product was cut with *Bgl*II (in bold) and ligated into the *Bgl*II site of pWRG7077 to give pWRG/HTN-S.

Transfection and CAT assays

Transfection and CAT assays were performed as described previously (Flick and Pettersson, 2001; Flick et al., 2002).

Acknowledgments

The work was supported by a grant awarded to Heinz Feldmann by the Canadian Institutes of Health Research (CIHR) and the Manitoba Health Research Council (MHRC) (partnership program). Kirsten Flick was awarded a fellowship from the Natural Sciences and Engineering Research Council (NSERC) of Canada. The authors thank Daryl Dick, Michael Garbutt, and Steven Theriault for their support in containment work.

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