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Genetic Analysis of a Theiler-like Virus Isolated from Rats

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Although cardioviruses related to Theiler's murine encephalomyelitis virus (TMEV) appear to be common in mice and rats, few TMEV isolates have been obtained from rat colonies. In 1991, a cardiovirus isolate designated NGS910 was obtained from sentinel rats exposed to cage bedding previously used by adult rats that were TMEV seropositive, but had never manifested clinical signs of disease. To determine to which group and subgroup of cardiovirus this virus belongs, the sequence of the viral genome was determined. The NGS910 genome consisted of 8,021 nucleotides and the 5'-nontranslated region had a predicted secondary structure that is similar to members of the TMEV group of cardioviruses. The Leader-P3D open reading frame (L ORF) of NGS910 had strong homology with L ORFs of other TMEVs (72% identity), but lower homology with EMCV cardioviruses (55 to 56%). Phylogenetic analyses on the basis of aligned nucleotide sequences of the L ORF (6,924 b) and the internal L* ORF (471 b) supported this classification of NGS910 as a TMEV strain. However, within the TMEV group, NGS910 was sufficiently divergent from other isolates that it could not be regarded as simply a mutant strain of a known TMEV. As genetic distances between NGS910 and other TMEVs were greater than those between Mengo virus of EMCV and other EMCVs, we propose to designate the NGS910 isolate as a rat Theiler-like virus.

The *Cardiovirus* genus of the family Picornaviridae has two taxonomic clusters: Theiler's murine encephalomyelitis virus (TMEV) and related viruses, and the encephalomyocarditis viruses (EMCVs). The TMEV group consists of two subgroups that vary in their pathogenic properties in vivo: a highly neurovirulent subgroup including strains GD7 and FA (1), and a persistent subgroup composed of strains TO4, DA, WW, BeAn8386, rat encephalomyelitis virus MHG, and Vilyuisk virus (1-5). Almost all strains of TMEV have been isolated from mice; only MHG and Vilyuisk virus are exceptional, being isolated from rats in 1961 (6) and humans in Siberia in 1955 (7), respectively.

Theiler's murine encephalomyelitis virus has frequently been used as a model for virus-induced demyelination in mice and as an animal model of multiple sclerosis. Consequently, natural TMEV infections should be eliminated from breeding colonies that supply rodents for biomedical research. The virus has been reported to be common not only in mice, but in rats as well. Next to parvovirus and mouse hepatitis virus (rat coronavirus in rats), TMEV is one of the most common virus infections in rodent colonies of major biomedical research institutes (8). According to that report, the prevalence of TEMV infections was 4% in biomedical research institute colonies, 4 and 7.5% in specific-pathogen-free (SPF) mice and rats, respectively, that were supposedly TMEV free, and 34 and 19% in non-SPF mice and rats, respectively. The Federation of European Laboratory Animal Science Associations (FELASA) also found TMEV infection to be common in rat colonies (9).

In 1991, we tested rat sera, using an indirect fluorescent antibody (IFA) test with TMEV strain GD7-infected cell antigen, to determine the prevalence of related cardioviruses in rats. Of the 2,578 sera tested, 194 (7.5%) were found positive for anti-TMEV IgG. Of the 194 positive sera, 20 (10.3 %) were from rats imported from commercial breeders in the United States. Notably, all sera from SPF animals purchased from domestic (Japanese) breeders were negative for TMEV antibody (0/554). At first, we suspected that the cardiovirus infections in rats that were detected by use of serologic screening were due to infection with the rat MHG virus. However, attempts to amplify genomic sequences of a virus isolated from a seropositive rat, using generic TMEV primers, were unsuccessful. In the study reported here, we present virologic and genetic analyses of this virus that indicate that these seropositive rats are infected with a novel TMEV-like virus that is distinct from the rat MHG virus. A part of this study was published in an abstract (10).

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Materials and Methods

Animals. The ICR mice and Wistar rats (6-week-old males and females) that were SPF grade and especially seronegative for TMEV were purchased from Japan SLC Inc. (Hamamatsu, Japan). Virus was isolated from three 4-week-old male Wistar-Furth rats purchased from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.) in 1991. All animals were maintained in a pathogen-free environment of temperature 22°C and humidity 40 to 70%; chow (MF, Oriental Yeast, Tokyo, Japan) and autoclaved water were available ad libitum. Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee.

Virus isolation and virus properties. Three 4-week-old male Wistar-Furth rats that tested positive for TMEV antibody by use of the IFA test were housed together in a cage for one week. Two 6-week-old male Wistar rats that were certified SPF grade and nega-

tive to the four viral agents—TMEV, sialodacryladenitis virus, Sendai virus, and hantavirus—and to five other agents on the basis of results of serologic or biological tests were then exposed to the used bedding from the cage that had housed the three TMEV seropositive rats. The two rats seroconverted to TMEV-positive status during the four weeks they were housed on the old bedding. These two rats were sacrificed, the intestines were removed and rinsed in sterile phosphate-buffered saline (PBS), and a pooled 10% tissue homogenate was prepared in PBS and filtered through a 450-nm filter, then was stored at –70°C until use.

Newborn SPF mice and rats (two each) were inoculated intracerebrally with 20 µl of the 10% intestinal homogenates from the sentinel rats. These inoculated newborns were sacrificed at postinoculation day (PID)10, and 10% brain homogenates were prepared. Clinical signs of gut disorder were not observed in either the sentinel rats or the newborn mice and rats inoculated intracerebrally. Brain homogenates from the inoculated rats were then inoculated onto BHK21 cells. At PID 4, cytopathic effect (CPE) typical of cardioviruses was observed, and these cells tested positive for TMEV antigen by use of the IFA test. Testing of fresh feces and intestinal contents collected at necropsy from sentinel rats or intracerebrally inoculated mice and rats failed to produce CPE in BHK21 cells. The new rat agent was designated as isolate NGS910. Basic physiochemical properties of isolate NGS910 were analyzed as described (6). A virus similar to the NGS910 isolate was obtained from brain homogenates of mice inoculated intracerebrally.

Since the partial sequence of the three-dimensional region indicated that the NGS910 isolate obtained from the brain of suckling rats was probably a novel virus of rats, the rat NGS910 was preferentially analyzed over the isolate from mouse brains. It was suggested that the rat NGS910 was identical to the isolate from mouse brains on the basis of the sequence of the three-dimensional region and by use of immunoblot (western blot) analyses. Further detailed studies will be described only for the NGS910 isolate.

Immunoblot analysis. Virus-infected cell proteins were prepared from BHK21 cells infected with NGS910, three isolates of TMEV (MHG [ATCC VR-802], GD7 [ATCC VR-57], and YOC [a member of the persistent TMEV subgroup isolated from mice in our laboratory; 11], and EMCV [ATCC VR-129B]). Virion proteins were separated on 12.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. Details of procedures used for immunologic detection of viral proteins by use of western blot analysis have been described (11, 12). In brief, appropriate dilutions of specific antisera (anti-NGS910 rabbit serum and IFA-positive rat serum) were applied to the membrane sheets, which were then blocked with a 10% skim milk solution. After washing, the sheets were reacted with biotinylated secondary antibodies and avidin-peroxidase according to the manufacturer's protocol (Vectastain ABC kit, Vector Laboratories, Burlingame, Calif.). Bound antibody was then detected by use of the horseradish peroxidase substrate, 4-chloro-1-naphtol (4-CN substrate kit, Vector Laboratories.).

Reverse-transcripase polymerase chain reaction (RT-PCR) analysis. Differential display RT-PCR was performed, using an RNAmap Kit A (Genhunter, Nashville, Tenn.). The RNA was isolated from uninfected and NGS910-infected BHK21 cells, using a single-step isolation method (13). Reverse transcriptase reactions were performed in 1X reaction buffer with denatured RNA, 200 U of M-MuLV reverse transcriptase (Roche Diagnostics, Switzerland), 10 m*M* dithiothreitol (DTT), 1 m*M* dNTP mixture (0.25 m*M* each), poly-T_{12}MN primer, and 1 U of RNase inhibitor for one hour at 37°C. The cDNA products were purified, using a microspin column (S-300 HR, Amersham Biosciences, Mannheim, Germany), and total DNA was eluted in water.

The PCR reactions contained 1X PCR buffer, 2 m*M* $MgCl_2$, 0.2 m*M* each dNTP, 200 n*M* each primer, and cDNA template. Reaction mixtures were initially incubated for three minutes at 94°C, followed by the addition of 1 U of Ex *Taq* polymerase (Takara Co., Kyoto, Japan). Reactions were then run through 40 cycles of 15 sec at 94°C, five minutes at 45°C, and 30 sec at 72°C, with a final elongation step of five minutes at 72°C. The primer sets of the PCR analysis were the combination of poly-T_{12}MN primer and one of five 10-nucleotide primers (AP3: 5'-AGGTGACCGT-3' or AP5: 5'-GTTGCCATCC-3'). The PCR products of 400 to 800 base pairs in size were amplified in the first PCR analysis, then were re-amplified for the sequencing template by the second PCR analysis, using the same primer set.

Sequencing of DNA sequence, rapid amplification of cDNA ends (RACE) and analyses. The PCR products amplified specifically from infected cells were purified, using a gel extraction kit (QiaexII, Qiagen, Helden, Germany) and were directly sequenced by use of a DNA analyzer (ABI 377, Applied Biosystems, Foster City, Calif.). Ten 400- to 800-base pair PCR products were sequenced and compared with sequences of other TMEVs to map their location in the NGS910 genome. Remaining gaps in the NGS910 genomic sequence were then filled, using PCR amplification, with newly designed primers located in known sequences flanking the gaps. End sequences of the virus genomic RNA were determined, using a 5'/3' RACE kit (Roche Diagnostics) and TA cloning (Invitrogen, Carlsbad, Calif.) following the manufacturers' protocols.

Phylogenetic and molecular evolutionary analyses were conducted, using MEGA version 2.1 (14). Distances were estimated by use of the Tamura-Nei method with pairwise gap deletion, and phylogenetic trees were constructed by use of the neighbor-joining method. The genomic RNA sequence of NGS910 has been deposited into the GenBank database (AB090161).

Results

Virus was isolated from brain homogenates of two suckling rats that were inoculated intracerebrally with intestinal homogenates from two sentinel rats that had sero-converted after being exposed to cage bedding used by rats that were seropositive for TMEV. Another isolate was obtained from two suckling mice inoculated intracerebrally with the same sentinel rat intestinal homogenates. These viral isolates were propagated in BHK21 cells. The rat isolate designated NGS910 was passaged once though a mouse brain and twice in BHK21 cells. The basic physiochemical properties of the NGS910 isolate were analyzed, and the virus was found to be: resistant to ether (4°C for 18 h), chloroform (room temperature for 15 min), and heat (loss of three logarithmic units in virus titer at 55°C by use of the plaque assay); acid stable at pH of 3.0 (room temperature for three hours); 10- to 50-nm in size; particle gravity of 1.27 to 1.30; a genome consisting of single-stranded RNA; and ability to agglutinate human type-O and guinea pig red blood cells. These properties are all consistent with those of cardioviruses.

Western blot analysis revealed the antigenic relatedness of

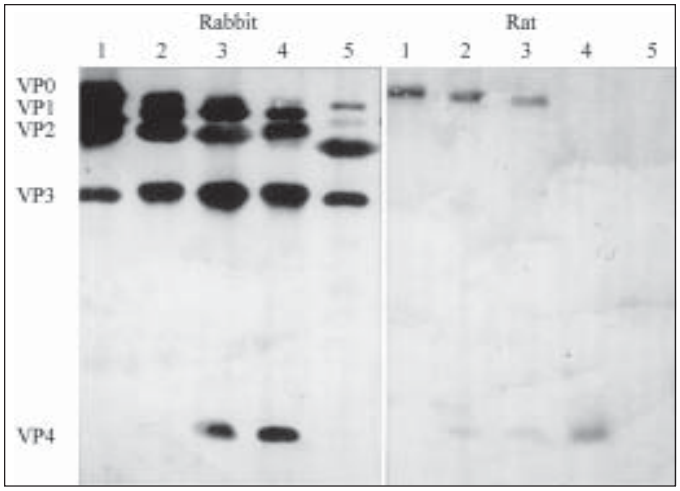

Figure 1. Immunoblot analysis of four Theiler's murine encephalomyelitis virus (TMEV) strains. Lanes: 1, NGS910; 2, MHG; 3, GD7; 4, YOC and 5, encephalomyocarditis virus (EMCV: ATCC VR-129B). Left sheet: detection by use of rabbit antisera raised to NGS910. Right sheet: detection by staining with a TMEV-positive rat serum. Rabbit antisera were similarly reacted with VP0, VP1, VP2 and VP3 of four TMEVs, and seropositive rat serum recognized NGS910 VP0 as well as VP0 of rat encephalomyelitis virus MHG and GD7 isolates.

NGS910 polypeptides to those of other TMEVs (Fig. 1). Consistent with the reactivity of TMEV-positive rat sera in the IFA test, an IFA-positive rat serum recognized NGS910 VP0 (precursor of virion proteins 2 and 4 encoded by the 1B and 1A regions, respectively) as well as VP0 of MHG and GD7. Rabbit antiserum raised to NGS910 similarly reacted with VP0, VP1 (from 1D region), VP2, and VP3 (from 1C) of other TMEV isolates. Thus, virion proteins of NGS910 are antigenically related to those of other TMEVs, suggesting that NGS910 is a member of the TMEV group of cardioviruses. Results of western blot analysis also suggested that NGS910 was not identical in size of the VP0 or reactivity of the VP4 to any of the other strains of TMEV tested.

The complete sequence of the NGS910 genome was determined by assembling sequences derived from various PCR products. The sequence of each PCR fragment was confirmed by sequencing at least twice. The NGS910 genome was 8,021 nucleotides in length, close to the size of other sequenced TMEV genomes (8,093 to 8,101 nucleotides). A large 6,924-base open reading frame (ORF) corresponding to the Leader-P3D (L) ORF of other TMEVs was present in the NGS910 genome. As indicated (Table 1), the NGS910 L ORF had strong homology with L ORFs of TMEV strains BeAn8386, DA and GD7 (72% identity), but lower homology with EMCVs (55-56%). Similarly, the amino acid sequence of the predicted polyprotein from the NGS910 L ORF (2,308 amino acids) was 87% similar and 78 to 79% identical to L proteins of TMEVs, 64 to 65% similar and 51 to 52% identical to EMCV L proteins, and considerably less related (22 to 25%) to equine rhinitis A virus (ERAV) and foot-and-mouth disease virus (FMDV), two members of the aphthovirus genus, viruses that are genetically close relatives of cardioviruses.

Similar to the L ORF of other TMEVs, an alternative initiation codon for an internal ORF that would encode an L* protein homologue, composed of 156 amino acids, was present 16 nucleotides downstream from the primary L ORF initiation codon in

Table 1. Homology of NGS910, Theiler's murine encephalomyelitis viruses (TMEVs), and encephalomyocarditis viruses (EMCVs) of the Leader-P3 (L) open reading frame (ORF)

	NGS910	BeAn	DA	GD7	EMC-B
Nucleotide homology (%)					
BeAn	72				
DA	72	88			
GD7	72	90	88		
EMC-B	56	57	56	56	
Mengo	55	56	56	55	79
Amino acid homology (% identity/similarity)					
BeAn	79/87				
DA	79/87	96/98			
GD7	78/87	96/98	95/97		
EMC-B	52/65	52/65	52/65	51/65	
Mengo	51/64	52/64	52/64	51/64	94/96

Table 2. Homology of NGS910 and TMEVs of the L* ORF

	NGS910	BeAn	DA	Vilyuisk	MHG
Nucleotide homology (%)					
BeAn	78				
DA	74	90			
GD7	61	69	68		
EMC-B	61	73	68	92	
Mengo	59	71	66	91	96
Amino acid homology (% identity)					
BeAn	65				
DA	61	84			
Vilyuisk	64	86	85		
MHG	64	96	84	86	
GD7[a]	58	88	78	82	93

[a]GD7 is not considered to have an L* ORF by mutation of initiation codon (AUG to ACG).

NGS910. As indicated (Table 2), the amino acid sequences of the L* proteins of TMEV strains BeAn8386, DA, Vilyuisk, MHG, and GD7 (in provisional sequence) had high homology with each other (78-96%). In contrast, the L* sequence of NGS910 was only 58 to 65% identical with L* proteins of other TMEVs. Alignment of L* protein sequences indicated that, despite this low level of sequence identity, the N-terminal 70% of the L* protein is fairly conserved (Fig. 2).

The 5' and 3' non-translated regions (NTR)of the cardiovirus genome are involved in transcriptional regulation, with secondary or three-dimensional structure being more relevant than primary sequence (20, 21). To examine the extent of conservation in this region of the genome, the 977-nucleotide 5'-NTR of NGS910 was aligned with the 5'-NTRs of five other TMEV strains (Fig. 3). Although the 5'-NTR of the three mouse cardioviruses were close in size, the 5'-NTRs of NGS910, Vilyuisk and MHG varied in size, principally at the 5' terminus. A poly(C) tract that has been reported in EMCVs but not in TMEVs was not present in the 5'-NTR of NGS910.

Phylogenetic analyses on the basis of aligned nucleotide sequences of the L and L* proteins are shown in Fig. 4. It was clear from these analyses that the NGS910 virus is most closely related to other TMEV viruses (strains BeAn8386, DA, GD7, MHG and Vilyuisk) and less related to EMCV viruses (B, Mengo). However, the branch length separating NGS910 from other TMEVs is similar to that separating Mengo from other EMCVs. These analyses thus indicate that NGS910 is quite divergent from other TMEVs, including the other virus isolated from rats (MHG).

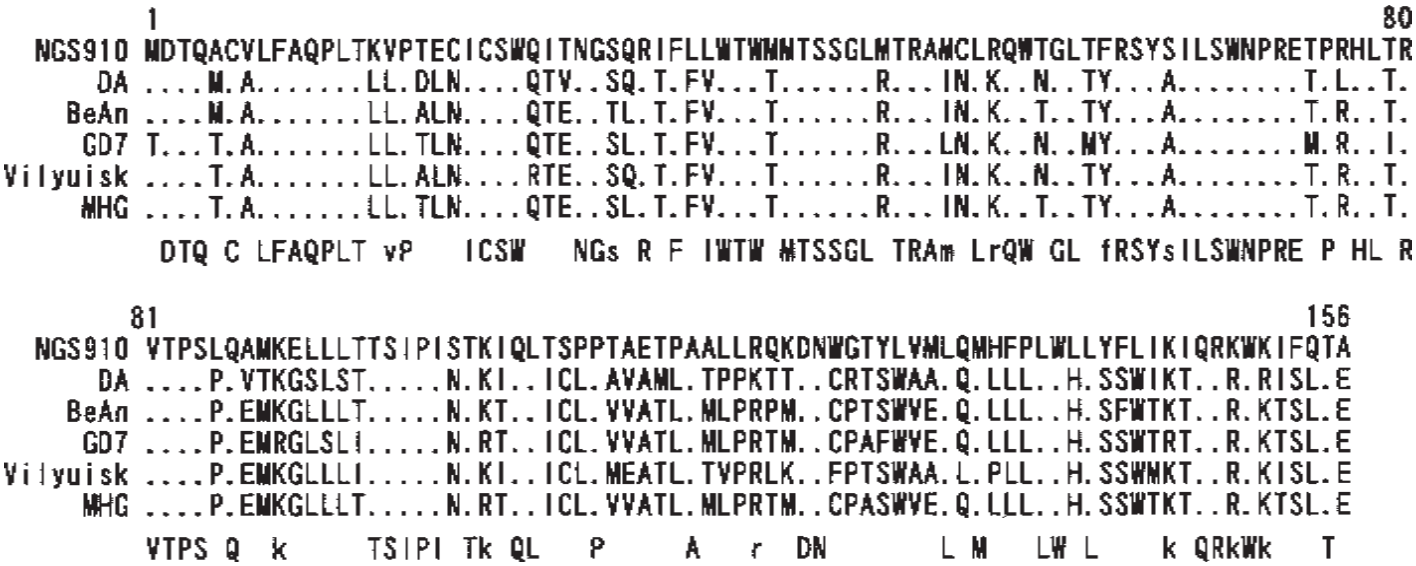

Figure 2. Alignment of predicted amino acid sequences of alternative L* open reading frame (ORF) of NGS910 and four TMEV isolates (DA, BeAn8386, Vilyuisk, and MHG). As L* ORF of TMEV GD7 is not considered to transcripted, it is aligned with a provisional sequence. All sequences are shown referenced to the NGS910, with identical amino acids indicated by dots (.). Identical (upper case) and similar (lower case) residues are shown in bottom lines.

Discussion

When anti-TMEV antibody was assayed in research rat colonies from 1983 through 1991, it was apparent that these animals had experienced infection by a cardiovirus that was antigenically related to the TMEV GD7 strain used for detection of antibody. However, GD7 and closely related TMEVs are highly neurovirulent, and clinical signs typical of infection with these cardioviruses were not observed in any test-positive rats. Rat encephalomyelitis virus MHG, the only known rat TMEV, is not a highly neurovirulent virus. Thus, it was possible that the seropositive rats had either been infected with an MHG-like virus or an attenuated TMEV more related to GD7. As reported here, a virus isolate designated NGS910 was isolated from nonclinical sign-manifesting, seropositive rats in 1991. Preliminary experiments failed to induce clinical signs of disease when the virus was inoculated into rodents, and PCR analysis using primers designed from highly conserved regions of aligned TMEV and EMCV sequences failed to amplify products from NGS910. These two results—low virulence and low similarity in genome sequence—impressed on us the fact that NGS910 might represent a novel group or subgroup of cardiovirus.

To our knowledge, viruses belonging to genus cardiovirus that have been isolated from rat colonies are few, compared with those isolated from mice. The MHG strain was isolated from three adult rats (SD strain) with clinical signs of encephalitis in 1961 (6), and it is antigenically related to murine TMEV strains GD7, TO, and FA (15). Partial nucleotide sequences of the MHG genome deposited in Genbank and our own sequence data for the MHG L* ORF (unpublished; shown in Fig. 4B) indicate that MHG is also a member of the TMEV group of viruses and is closely related to GD7 and Vilyuisk strains. Similar to GD7 and MHG, Vilyuisk is also neurovirulent in rats (7, 16). Since the Vilyuisk isolate was obtained from a human, all cardioviruses including the NGS910 isolate might be worthy of concern as potential agents of zoonotic infection or may be reverse zoonosis agents.

Experiments described here to more fully characterize the NGS910 rat virus isolate clearly indicate that although it is a TMEV, it is distinct from other known TMEVs. Western blot analysis, using anti-NGS910 sera, revealed reaction with viral proteins of other TMEVs, but these sera reacted more strongly with NGS910 proteins and reacted poorly with VP4 of other virus (MHG of TMEVs and EMCV). Also, we determined the entire sequence of the NGS910 genomic RNA by use of a combination of differential display and PCR cloning methods. On the basis of similarity of analyses of nucleotide and predicted amino acid sequences, NGS910 was clearly a member of the TMEV group of cardioviruses. However, within the TMEV group, NGS910 was sufficiently divergent from other isolates that it could not be regarded as simply a mutant strain of a known TMEV. Despite this sequence divergence, secondary structure analyses of 5' and 3'-NTRs suggested that these regions of the genome of NGS910 probably assume a confirmation and function similar to that of other TMEVs. Phylogenetic trees with Leader-P3D (L) and L* ORF also strongly supported this relatedness. The L* Protein of 18 kDa has been reported to be translated in the DA strain of the TMEVs-persistent subgroup (17), and is essential for virus persistence, induction of demyelination in mice, and impairment of virus growth in macrophage-like cell lines (18-20). Although the presence of an L* ORF and the avirulence of NGS910 in mice and rats are typical of viruses in the TMEV-persistent subgroup, genome sequence data and phylogenetic analyses do not support this association.

The genetic distance between NGS910 and other TMEVs was comparable to that between Mengo virus and other EMCVs. Thus, it is possible that the NGS910 rat virus isolate might well prove to represent a third independent virus group within the cardiovirus genus. However, until additional isolations are made to support this, we propose to tentatively designate the NGS910 isolate as a rat Theiler-like virus. We believe this is justified since, at present, NGS910 is only isolate having its unique properties, and it does serologically cross-react with other TMEVs, and so cannot be unequivocally recognized as new serotype.

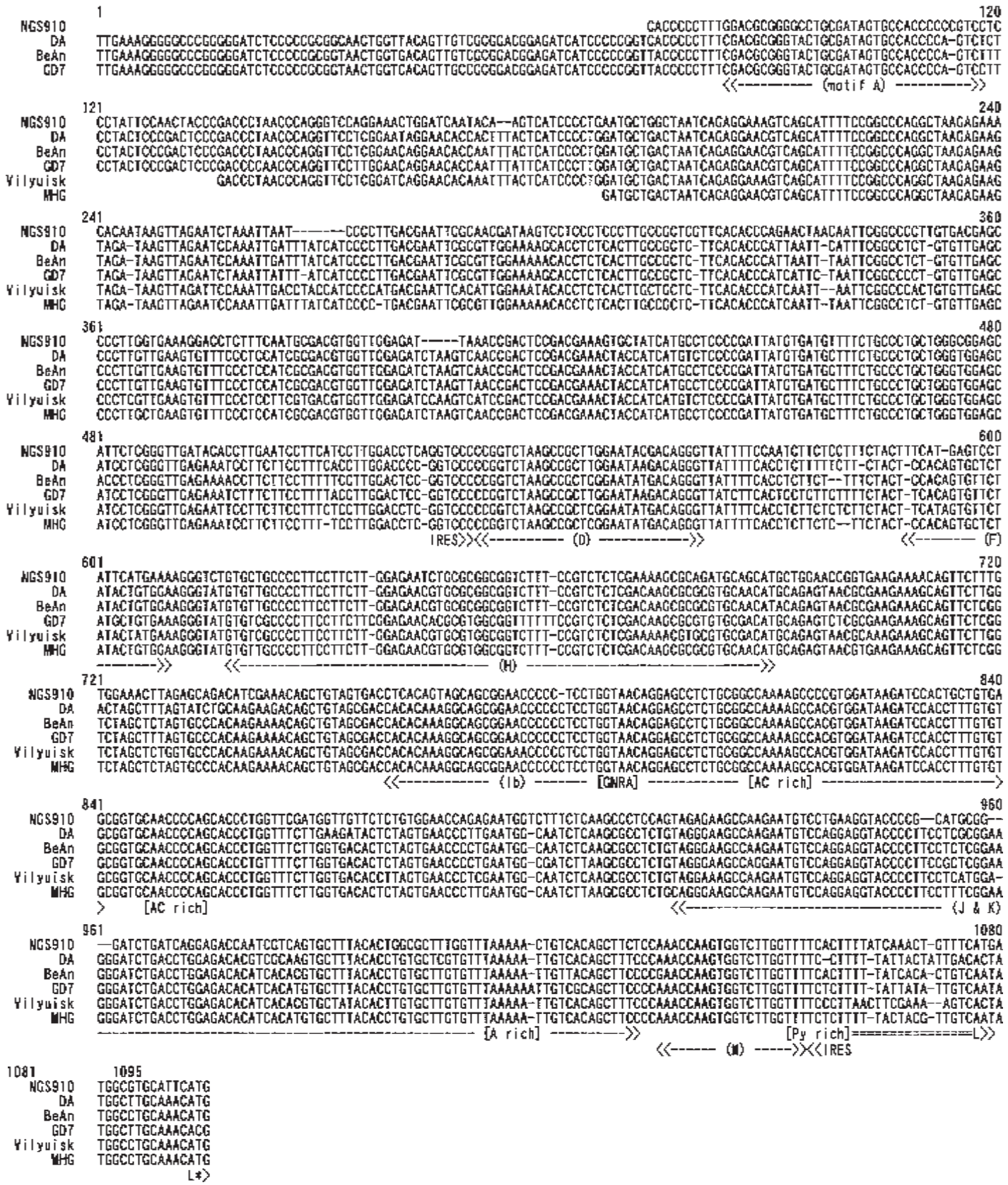

Figure 3. Alignment of 5'non-translated region (NTR) nucleotide sequences of NGS910 and five TMEV isolates (DA, BeAn8386, GD7, Vilyuisk, and MHG). Putative second structural elements and motifs (A, D, F, H, Ib, J, and K and M motifs, GNRA [guanine-N-adenine/ guanine-adenine] loop, and AC [adenine/cytosine]-rich, A [adenine]-rich, and Py [pyrimidine]-rich regions) based on NGS910 are shown in the bottom lines. Reference 21 was consulted for the notation.

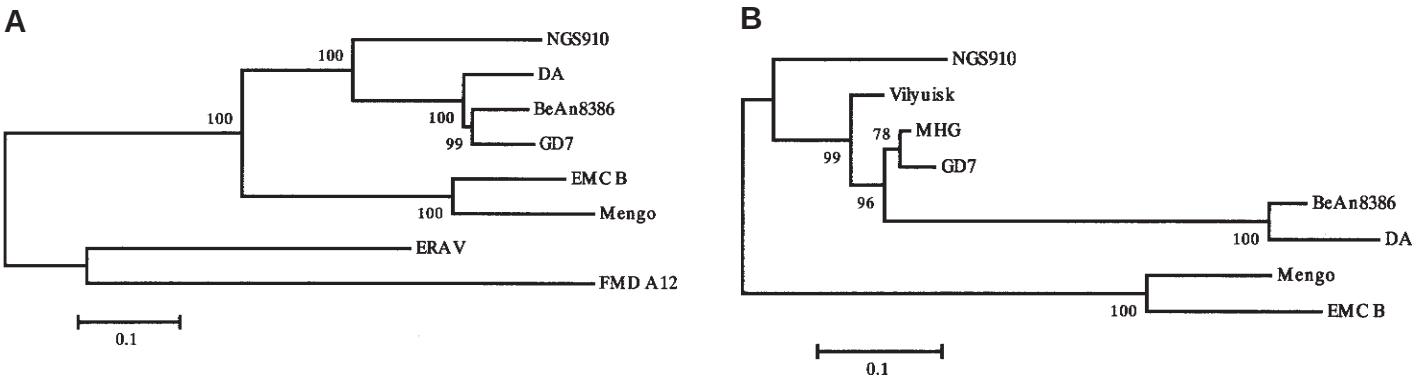

Figure 4. Phylogenetic analysis of NGS910-RNA sequences. Analyses were performed, using Tamura-Nei distances calculated by use of pairwise gap deletion and Neighbor-Joining tree construction programs. Trees were generated for 6,924 base pairs of Leader-P3D ORF (A) and 471 base pairs of L* ORF (B). Both trees indicate that the NGS910 sequence is a member of novel rat Theiler-like virus. Values at branch points represent bootstrap values generated using 500 repetitions.

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