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Restriction Endonuclease Analysis of Field Isolates of Feline Herpesvirus Type 1 and Identification of Heterogeneous Regions

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The genomic heterogeneity of 78 isolates of feline herpesvirus type 1 (FHV-1) recently isolated from cats suspected to have feline viral rhinotracheitis was analyzed by examining the digestion patterns found with restriction endonuclease *Mlu*I. The FHV-1 field isolates were classified into at least three genotypes, namely, the C7301, F2 (an attenuated vaccine strain), and C7805 types. The C7301 type seems to be a major type, since 64 of the 78 isolates belonged to this type. Eight and six isolates belonged to the F2 and C7805 types, respectively. Compared with the C7301 type, the heterogeneous region of the F2 type was localized to a 4.3-kbp *Eco*RI fragment within the U_S segment and the heterogeneous region of the C7805 type was localized to a 5.5-kbp *Xba*I fragment within the U_L segment. Northern (RNA) blot analysis revealed no differences in the products transcribed from these regions. In addition, nucleotide sequence analysis showed that the *Mlu*I sites not found in the F2 and C7805 types were located in the regions homologous to the herpes simplex virus type 1 gI and U_L5 genes, respectively.

Feline herpesvirus type 1 (FHV-1), a member of the subfamily *Alphaherpesvirinae* of the family *Herpesviridae*, causes an upper respiratory tract disease in cats known as feline viral rhinotracheitis. In particular, infections of newborn or debilitated cats with FHV-1 can result in severe generalized disease with high mortality rates (29). However, the genomic analysis of FHV-1 lags behind those of other herpesviruses, and only four genes encoding FHV-1 gB, gH, and gD (gp60) homologs and thymidine kinase have been identified (19–21, 26, 35).

To date, many serological comparisons have been made by virus-neutralizing tests among FHV-1 isolates from many parts of the world and all have shown a homogeneity among the isolates (5, 8, 18, 25, 36). The homogeneity was also confirmed by complement fixation, immunodiffusion, and hemagglutination inhibition tests (25, 38). In cleavage patterns with several restriction endonucleases, small shifts in the electrophoretic mobilities of a few fragments were observed, but no remarkable change was detected (14, 16). In spite of these facts, less virulent strains were developed and these strains have been used as vaccines without clear genomic markers (2–4, 10–12, 27, 30, 32–34, 39). Recently, we compared a total of nine FHV-1 strains including the vaccine strain of F2 (3), the prototype strain of C27 (9), and our laboratory strains of C7301 and C7805 (24) by immunoblot and restriction endonuclease analyses. The results showed that a viral structural protein with a molecular mass of 36 kDa was absent in the vaccine strain and that the cleavage patterns with restriction endonuclease *Mlu*I for the field isolates and a vaccine strain were different (17). These results indicated that the absence of the 36-kDa protein and the specific *Mlu*I cleavage pattern might be used as markers for the vaccine F2 strain. Differentiation between field isolates and a vaccine strain appears to be of crucial importance, especially for evaluation of vaccine immunity. Moreover, a comparison of the properties of the vaccine strain with those of field isolates is necessary to characterize the less virulent virus for understanding FHV-1 pathogenesis in cats.

In this study, cleavage patterns found with restriction endonuclease *Mlu*I were further compared among 78 FHV-1 field isolates which were recently isolated from cats suspected to have feline viral rhinotracheitis in Japan from November 1990 to May 1991 (37), and 4 additional strains, namely, F2, C7301, C27, and C7805, were used for the comparison. In addition, we compared the nucleotide sequences and transcriptional products of heterogeneous regions present in F2 and C7805 strains.

All isolates listed in Table 1 were propagated less than three times in Crandell feline kidney cell cultures. The cells were grown in Eagle's minimum essential medium (Nissui, Tokyo, Japan) containing 10% tryptose phosphate broth (Difco, Detroit, Mich.), 8% heat-inactivated fetal calf serum, and antibiotics. The maintenance medium for harvesting the virus was free of fetal calf serum. For extraction of DNAs, cells infected with each isolate were washed once with phosphate-buffered saline, lysed in 0.1 M Tris-HCl (pH 9.0) containing 1% sodium dodecyl sulfate, 0.1 M sodium chloride, and 1 mM EDTA, and then treated overnight with 1 mg of pronase E per ml at 37°C. DNAs were extracted with phenol, precipitated by ethanol, and then treated with the restriction endonuclease *Mlu*I. The digests were subjected to electrophoresis on a 0.5% agarose gel. Following electrophoresis, gels were stained with ethidium bromide and photographed on a UV light transilluminator.

Figure 1 shows cleavage patterns with the *Mlu*I enzyme of 15 representative samples. Data for all isolates are summarized in Table 1. The results suggested that three genotypes existed in FHV-1 isolates. The cleavage pattern of one genotype was similar to those of the C7301 and C27 strains. Both C27 and C7301 strains were very virulent against cats (9, 24). This

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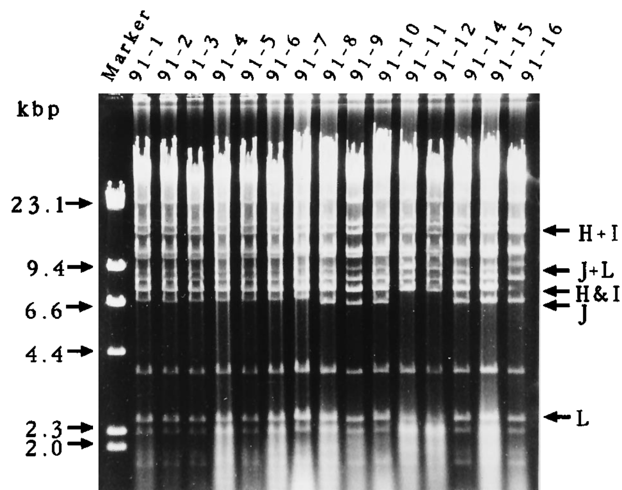

FIG. 1. Restriction endonuclease *Mlu*I cleavage patterns of 15 FHV-1 field isolates. *Hin*dIII digests (sizes shown in kilobase pairs) of λ DNA are used as size markers. Arrows and capital letters indicate the fragments that are significantly different from those of the C7301 type.

genotype (named the C7301 type) was found in 64 of the 78 field isolates. Seven of the nine strains of FHV-1 belonged to this type in the previous study (17). The second genotype, including the F2 (vaccine) strain, lost two J (6.6-kbp) and L (2.5-kbp) fragments and gained one 9.1-kbp fragment. This group (named the F2 type) included eight isolates. The third genotype includes the C7805 strain which lost two H (7.0-kbp) and I (7.0-kbp) fragments and gained one 14.0-kbp fragment. This group (named the C7805 type) has only six strains. The C7805 strain was isolated from a cat suspected of having feline viral rhinotracheitis (17). It is interesting to note that some field isolates belonging to the F2 type were obtained from both vaccinated and nonvaccinated cats. This result might imply two possibilities. The F2 strain might be recovered from either vaccinated cats or unvaccinated cats infected horizontally with

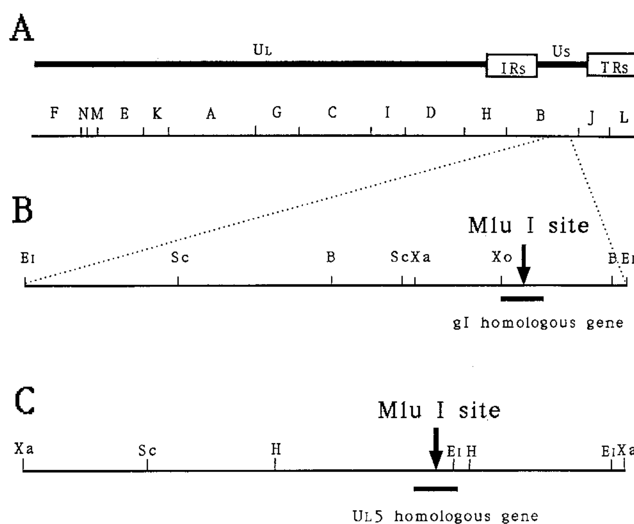

FIG. 2. Heterogeneous gene among FHV-1 field isolates. (A) Restriction endonuclease map of FHV-1 genome for *Sal*I (31). (B) Mapping of the 4.3-kbp *Eco*RI fragment. (C) Mapping of the 5.5-kbp *Xba*I fragment. The thick black lines are the regions where nucleotide sequences were analyzed in this study. EI, *Eco*RI; Sc, *Sac*I; B, *Bam*HI; Xa, *Xba*I; H, *Hin*dIII.

TABLE 1. FHV-1 isolates or strains used in this study

Isolate or strain	Isolation place[a]	Vaccination	Presence of the *Mlu*I site in fragment:	
			4.3-kbp *Eco*RI	5.5-kbp *Xba*I
Field isolates				
91-1	Tokyo	Unknown	+	+
91-2	Tokyo	Unknown	+	+
91-3	Tokyo	Unknown	+	+
91-4	Tokyo	Unknown	+	+
91-5	Tokyo	Unknown	+	+
91-6	Tokyo	Unknown	+	+
91-7	Tokyo	Unknown	+	+
91-8	Tokyo	Unknown	+	+
91-9	Tokyo	Unknown	+	+
91-10	Tokyo	Unknown	+	+
91-11	Tokyo	Yes	−	+
91-12	Osaka	Unknown	−	+
91-14	Yamaguchi	Unknown	+	+
91-15	Yamaguchi	Unknown	+	+
91-16	Hiroshima	Unknown	+	−
91-17	Ehime	Unknown	+	+
91-18	Ehime	Unknown	+	+
91-19	Ehime	Unknown	+	+
91-20	Ehime	Unknown	+	+
91-21	Ehime	Unknown	+	+
91-22	Okayama	Unknown	+	+
91-23	Tottori	No	−	+
91-24	Tottori	No	−	+
91-25	Tottori	No	−	+
91-26	Tottori	No	+	+
91-27	Tottori	No	+	+
91-28	Tottori	No	+	+
91-29	Tottori	Yes	+	+
91-30	Tottori	Yes	+	+
91-31	Tottori	No	+	+
91-32	Tottori	Yes	+	+
91-33	Tottori	No	+	+
91-34	Tottori	No	+	+
91-35	Tottori	No	+	+
91-36	Tokyo	No	+	+
91-37	Tokyo	No	+	+
91-39	Tokyo	No	+	+
91-40	Tokyo	No	+	+
91-41	Tokyo	Yes	+	+
91-42	Tokyo	No	−	+
91-43	Tokyo	No	+	+
91-44	Tokyo	No	+	+
91-45	Tokyo	No	+	+
91-46	Tokyo	Yes	+	+
91-47	Tokyo	No	+	−
91-48	Tokyo	Yes	+	+
91-49	Saitama	Unknown	+	+
91-50	Saitama	Unknown	+	+
91-51	Saitama	No	+	−
91-52	Tokyo	Unknown	+	+
91-53	Tokyo	Unknown	+	+
91-54	Tokyo	Unknown	+	+
91-55	Tokyo	No	+	+
91-56	Tokyo	Unknown	+	+
91-57	Tokyo	Yes	−	+
91-58	Tokyo	Unknown	+	+
91-59	Tokyo	No	+	+

Continued on following page

TABLE 1—*Continued*

Isolate or strain	Isolation place[a]	Vaccination	Presence of the *Mlu*I site in fragment:	
			4.3-kbp *Eco*RI	5.5-kbp *Xba*I
91-60	Tokyo	No	+	+
91-61	Tokyo	No	+	+
91-62	Yamagata	Unknown	+	+
91-63	Yamagata	Unknown	+	+
91-64	Yamagata	Unknown	+	+
91-65	Yamagata	Unknown	+	+
91-66	Yamagata	Unknown	+	+
91-67	Yamagata	Unknown	+	+
91-68	Yamagata	Unknown	+	+
91-69	Yamagata	Unknown	+	+
91-70	Yamagata	Unknown	+	+
91-71	Yamagata	Unknown	+	−
91-72	Yamagata	Unknown	+	−
91-73	Yamagata	No	+	−
91-74	Fukuoka	Unknown	+	+
91-75	Yamaguchi	Unknown	+	+
91-76	Yamaguchi	Unknown	+	+
91-77	Tokyo	No	+	+
91-78	Tokyo	No	+	+
91-79	Tokyo	No	+	+
91-80	Tokyo	No	−	+
Laboratory strains				
F2	United States		−	+
C7301	Tokyo		+	+
C27	United States		+	+
C7805	Tokyo		+	−

[a] All cities and prefectures in Japan unless indicated otherwise.

the vaccine strain under various conditions. Alternatively, virulent field strains belonging to the F2 type could have been present under natural conditions. Further study is required to ascertain which of these possibilities is the case.

To identify the regions of these heterogeneous *Mlu*I sites, purified DNA of the FHV-1 C7301 strain was extracted from virus particles, digested completely with *Mlu*I, blunted by T4 DNA polymerase, and then inserted into the *Eco*RV site of plasmid Bluescript SK+ by T4 DNA ligase. We cloned several *Mlu*I fragments including the H, I, and J fragments. Next, we carried out Southern blot analysis by using these three fragments labeled with [^{32}P]dCTP as probes to identify their positions on the FHV-1 genome (data not shown). The results showed that the heterogeneous *Mlu*I sites absent in the F2 and C7805 types were located in a 4.3-kbp *Eco*RI fragment within the *Sal*I-B fragment (Fig. 2A and B) and a 5.5-kbp *Xba*I fragment within the 13.0-kbp *Sal*I fragment (Fig. 2C), respectively. However, we could not clearly identify the region of the 5.5-kbp *Xba*I fragment on the restriction map of the FHV-1 genome (31).

Southern blot analysis was carried out to confirm the heterogeneity of the *Mlu*I sites within these fragments. Purified viral DNAs of F2, C7301, and C7805 strains were extracted from virus particles and digested with *Mlu*I. Figure 3 shows the electrophoretic pattern of the digested DNAs. After these DNAs were transferred to nylon sheets, hybridization was done by using ^{32}P-labeled 4.3-kbp *Eco*RI (Fig. 3A) and 5.5-kbp *Xba*I fragments (Fig. 3B) as the probes. The results confirmed that the two J and L fragments were lost, and only one band was detected in the F2 strain (Fig. 3A). In the

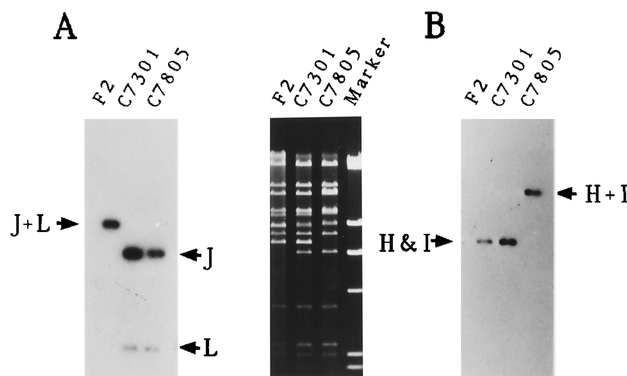

FIG. 3. Southern blot analysis. (A) The 4.3-kbp *Eco*RI fragment was used as a probe. (B) The 5.5-kbp *Xba*I fragment was used as a probe. The middle gel shows the results of restriction endonuclease analysis with *Mlu*I. Arrows and capital letters show different fragments among the three genotypes. In the marker lane, the bands from top to bottom indicate molecular sizes of 23.1, 9.6, 6.6, 4.3, 2.3, and 2.0 kbp.

C7805 strain, the two H and I fragments were lost, and only one band was detected (Fig. 3B). These results suggested that these fragments contained the heterogeneous *Mlu*I sites.

Northern (RNA) blot analysis was conducted to identify possible changes of products transcribed from these heterogeneous regions. Total RNAs were extracted from F2-, C7301-, C7805-, and mock-infected cells at 36 h postinfection by the method described previously (7). Hybridization was done with the same probes used in Fig. 3. However, no change was observed in mRNA transcribed from these heterogeneous regions (Fig. 4).

Next, sequence analysis of the C7301 strain was carried out to determine the nucleotide sequences within these heterogeneous regions. At first, we mapped these *Eco*RI and *Xba*I fragments by using various restriction enzymes including *Mlu*I (Fig. 2). The nucleotide sequence was extended by using Ampli *Taq* polymerase cycle sequencing kit (ABI, Foster City, Calif.) and then analyzed with a model 370A ABI autosequencer.

Figure 5 shows the nucleotide sequences around the heterogeneous *Mlu*I site located in the *Eco*RI fragment (A) or *Xba*I fragment (B). The nucleotide sequence around the *Mlu*I site within the 4.3-kbp *Eco*RI fragment was homologous to the gI

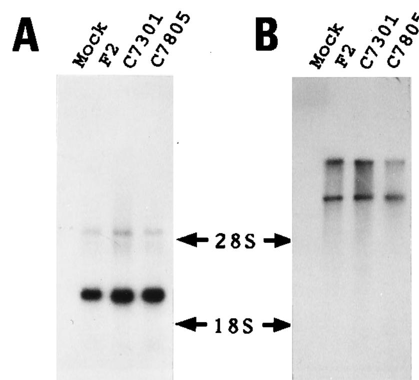

FIG. 4. Northern blot analysis. (A) The 4.3-kbp *Eco*RI fragment was used as a probe. (B) The 5.5-kbp *Xba*I fragment was used as a probe. 28S and 18S rRNAs were used as size standards.

A

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C7301  ATAATGGAACCCTCGAGATTATACATTACAACCATCACTCTTCTTGCTATAAAATCGTTCAAGTAATAGAATATTCATCATGTCCACGTG

                                Primer gI-1
C7301  TACGCAATAATGCTTTCCGGTCCTG[TCTCCACAAGACCTCTATGC]ACCAATACGATCAGCTTTCCATAAACACATCCGTTGAAACGGGGA
F2                                                   ---------------------------------------------

C7301  TGTTATTGACAATAACATCTCCGAAAATGGAAGATGGTGGAATCTACGCACTGCGGGTAAGATTTAACCATAATAACAAAGCTGATGTAT
F2     ------------------------------------------------------------------------------------------

                                        MluI
C7301  TTGGCCTTTCGGTGTTTGTTACTCATTCGAT[ACGCGT]GGTCATCGACATCATGCGGACGAAAATTTGAATGGTGAAATTCTTACTA[CTC
F2     --------------------------------T---------------------------------------------------------
                                   Thr→Met
  Primer gI-2
C7301  CATCACCGATGGAAACA]TATGTTAAAGTTAACACACCAATATATGATCATATGGTGACAACTCAAACAACTTCTAATAAATCGATGGAGT
F2

C7301  CTGAACCATCAAATACATCAATATCATGCCATACATTTCAAAATGACCCGAATGAGGGTGAGACTTTATATACACACTTATTGAACATCG

C7301  CTGGAAATATAACATATGATGACATGGTTATGGATGGCACCACATTGAAACCCAGATTAA
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B

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C7301  CAACTTGTAAATTATCGAGAGATCCGACTTTATCGATGCAGGGTTCCATTTTCAGCCCAGCTCGCGCATCCATTAACAATCCAGACTTGA

C7301  CACCTGTCGGGCGCATGCACTCTACATTGACGTCACGCATATAGGCAAATATGTATTGGTCTGTAGATTGTTAAAGTTCGGGCATA[AAGA

Primer UL5-1
C7301  GACGTCACCTCCTGAT]AGAGGAGAAATGGCAAGTACCGATCTCACCGGAGACTATTATAATGACCAAGCTGATTTTTCGAATTCGATATA
C7805                  --------------------------------------------------------------------------

                                             MluI
C7301  TCTTAATTTTACTTCAATGCATGGCATTCGGAATATAGTC[ACGCGT]ATAAAGGACCTAGCGGGATTATCAGTTTCAAAGGAAAAGATCCC
C7805  -----------------------------------------A------------------------------------------------

C7301  ACCCGTAAGTTGGTTTAAGGACTTGACGTCCCTCGAAACACCGCTTGATATAAATCCACGTGAATTACCATTTTCCGTATATCTCATTAG
C7805  ------------------------------------------------------------------------------------------

                                                Primer UL5-2
C7301  CGGTAATGCTGGTTCCGGAAAAAGTACCTGTA[TCCAGACTCTCAACGAAACC]CTGGAATGTGTTATAACAGGCGCCACCCGCGTCGCTTC
C7805  --------------------------------G
                                 Ile→Val

C7301  CCAGAATGTATATCAAAAACTCGCGTCTTCATACTCGAGCAGACCCGTCAATACTATTTTTCAAGAGTTCGGTTTCCGCGGAAATCATGT

C7301  GCAGGCACAACTCGGTAAATATCAATATGTATGTCCTTCTAACCCACC
```

FIG. 5. Comparison of nucleotide sequences between the C7301 strain and the F2 or C7805 strain. (A) Nucleotide sequence around the *Mlu*I site in the 4.3-kbp *Eco*RI fragment of C7301 and F2 strains. (B) Nucleotide sequence around the *Mlu*I site in the 5.5-kbp *Xba*I fragment of the C7301 and C7805 strains. Nucleotide and amino acid sequences different from those of the C7301 strain are indicated. The *Mlu*I sites are shown by the boxes. Four primers designed for PCR are also shown by boxes.

counterparts of alphaherpesviruses (Fig. 5A). The partial identities with the equine herpesvirus type 1 gI (13) and herpes simplex virus type 1 (HSV-1) gI (23) were 49.3 and 36.0%, respectively. To identify a mutation at the *Mlu*I site in the F2 strain, we designed two primers, 5′-TCTCCACAAGACCTCTATGC-3′ and 5′-TCTTTCCATCGGTGATGGAG-3′, and then carried out PCR with these primers. The amplified fragment was cloned with the TA cloning kit (Invitrogen, San Diego, Calif.) and sequenced. This experiment was repeated three times to obtain exact sequence data for these PCR products. The result showed that only one mutation in the *Mlu*I site existed in the PCR products and generated a change from threonine to methionine in the predicted translation product. Although it is unknown whether this change influences virulence of the vaccine strain of F2, in vaccine strains of pseudorabies virus (PRV), the Bartha strain lacks gI (gE homolog) and gp63 (gI homolog) and the Norden strain lacks gI and has a partial deletion of gp63 (28). It was reported that this gI counterpart interacts with the gE homologs in HSV-1 and PRV and that the interaction of PRV was thought to be necessary for the full expression of virulence (40). Furthermore, Card et al. (6) demonstrated that the interaction in PRV influenced neurovirulence. Therefore, the mutation of the *Mlu*I site of the F2 type might be associated with the decreased virulence of FHV-1.

On the other hand, the nucleotide and predicted amino acid

sequences around the *Mlu*I site within the *Xba*I fragment were highly homologous to U_L5 of HSV-1 (Fig. 5B). The partial identity with the HSV-1 U_L5 gene was 52.0%. The translational product of the HSV-1 U_L5 gene was reported to be associated with viral DNA replication (22). As for the F2 strain, we designed two primers, 5′-AAGAGACGTCACCTC CTGAT-3′ and 5′-GGTTTCGTTGAGAGTCTGGA-3′, and sequenced this region of the C7805 strain. The result showed that two mutations in the amplified fragment of C7805 strain existed. However, it is unknown whether these changes of the C7805 strain are nonsense mutations, because there is no significant difference between the C7301 and C7805 strains in biological nature to our knowledge (17, 24, 25). Further comparative analysis for translation products of the C7301 strain and the F2 or C7805 strain will be required.

So far, there has been no method that differentiates FHV-1 strains including the laboratory, field, and vaccine strains. However, it became possible to classify FHV-1 strains into at least three genotypes in this study. These genotypes might be useful for the epidemiological studies of FHV-1 infection which had been thought to be difficult to carry out. Furthermore, since it is possible to design primers to amplify these heterogeneous regions by the PCR method, it should be easier to differentiate FHV-1 field isolates by digesting the product with the *Mlu*I enzyme as previously reported for PRV (1, 15).

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