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Molecular Characterization of Equine Herpesvirus 1 (EHV-1) Isolated from Cattle Indicating No Specific Mutations Associated with the Interspecies Transmission

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***Abstract*: Interspecies trasmission of equine herpesvirus 1 (EHV-1) from horse to cattle was shown by Crandell et al. (1988). Specific mutations related to the transmission were studied by comparison of five EHV-1 isolates in cattle (BH1247, 3M20-3, G118, G1753, and 9BSV4) using polymerase chain reaction and restriction fragment length polymorphism analysis with added sequencing. G118 and 3M20-3 were the genome type EHV-1 P, while G1753 was the genome type EHV-1 B. BH1247 and 9BSV4 might be other genome types. We could not identify specific mutations related to the interspecies transmission.**

***Key words*: EHV-1, Interspecies transmission, RFLP**

Equine herpesvirus 1 (EHV-1), a member of the subfamily *alphaherpesvirinae*, has long been causally implicated in the occurrence of abortion, neonatal deaths, respiratory disease and neurological disorders in horses (3). Although the host range of EHV-1 seems to be restricted in the horse, EHV-1 has been isolated from other non-equine such as cattle (9), fallow deer (12, 22), antelopes (7), alpacas and llamas (17), and camels (4). Five strains of EHV-1 have been isolated from cattle from 1974 to 1986 from bovine aborted fetuses, bovine tissues and nasal secretions. They were identified as EHV-1 by a serum neutralization test using EHV-1-specific antiserum (9). The authors found differences in the restriction endonuclease digestion patterns and attributed them to alterations in the terminal repeat and unique short region of EHV-1. Two abortions of bovine fetuses were attributed to EHV-1 (6, 7, 18). Chowdhury et al. (7) also used DNA fingerprinting to show that bovine isolates were almost identical to EHV-1. These studies indicated that EHV-1 could jump the species barrier to infect ruminants. A famous example of species barrier-breaking is the jumping of feline parvovirus to dogs as the result of mutations (5). Therefore it is possible that mutations in the viral genome of EHV-1 might allow the interspecies transmission from horse to cattle.

The EHV-1 genome is 150,224 base pairs (bp) long, consisting of a long unique region (U_L) and a short unique region (U_S) which is flanked by a short inverted repeat (IR) region and a terminal repeat (TR) region (20). Allen et al. (1) described 16 electropherotypes of EHV-1 including EHV-1 P and EHV-1 B, two of which are the types reported to cause the majority of non-vaccine-related abortions in the state of Kentucky in the United States (2). Restriction fragment length polymorphism (RFLP) analysis of whole viral DNA has been used for molecular epidemiological research on herpesviruses. RFLP analysis of long DNA fragments amplified by the long and accurate polymerase chain

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Abbreviations: bp, base pair; DDBJ, DNA Data Bank of Japan; EHV-1, equine herpesvirus 1; FHK, fetal horse kidney; IR, inverted repeat; LA, long and accurate; MEM, minimum essential medium; ORF, open reading frame; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; RK, rabbit kidney; TE, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0; UL, long unique; US, short unique.

reaction (LA-PCR) has recently been developed as an alternative method for determining the molecular epidemiology of Varicella-Zoster virus (19). We recently revealed the occurrence of natural recombination between EHV-1 and EHV-4 by RFLP with LA-PCR and sequencing analyses (16).

In this study, we analyzed sequences of cattle EHV-1 isolates to see if they contain mutations that might be responsible for transmission of the virus from horse to cattle.

Five EHV-1 isolates were used in this study. All were isolated from cattle. BH1247 was isolated in 1974 (8) and 3M20-3, G1753, 9BSV4 and G118 were isolated in 1975, 1977, 1985 and 1986 (9). EHV-1 Ab4p was used as a reference, and was kindly provided by A. J. Davison (University of Glasgow, Scotland). Viruses were propagated as described previously (15). Fetal horse kidney (FHK) cells and rabbit kidney 13 (RK-13) cells were used. The cells were cultivated with Eagle's minimum essential medium (MEM) supplemented with 5% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin.

Viral DNA was extracted as described previously (16). DNA was finally dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE). LA-PCR was examined as described previously (16). PCR products were precipitated with ethanol and dissolved in 50 μl of TE. Restriction enzymes used were *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Fba*I, *Hin*cII, *Hin*dIII, *Msp*I, *Mun*I, *Nae*I, *Nco*I, *Pst*I, *Sac*I, *Sma*I, *Ssp*I, and *Xba*I. Digestion and electrophoresis were examined as described previously (16).

The noncoding region between ORF62 and ORF63 was amplified by primers ORF62F (5'-ATGAGGGTCAGAGGTTAGATCCAAGCAACC-3'; base position 108892 to 108921 of the Ab4p genome) and ORF63R (5'-TCAGTCTGAGAGAGTGTTAATAGTCGCCGC-3'; base position 110315 to 110286 of the Ab4p genome). The other regions corresponding to bases 112756 to 114969 of the Ab4p genome were amplified by using 3 sets of primers according to virus genome types (Table 1). All amplified fragments were purified from electrophoresis gels using a QIAQuick Gel Extraction Kit (QIAGEN, Inc., U.S.A.). PCR products were ligated into pGEM-T easy vector (Promega, U.S.A.) using a Ligation Kit (TaKaRa Co., Ltd., Japan) and transformed into competent *E. coli* DH5α cells (Life Technologies, Japan). Recombinant plasmid DNA was extracted with a Flexi Prep Kit (Amersham Pharmacia, Japan).

DNA sequencing was examined with a ThermoSequenase Cycle Sequencing Kit (Amersham Pharmacia). Plasmid sequencing was examined with Cy5.5 M13 universal and reversal primers. Sequences were assembled and analyzed with Genetyx-MAC/ATSQ and GENETYX-Mac (Software Development Co., Ltd., Japan). Homology analysis was examined using the DDBJ Homology Search System and NCBI-Blast.

The genomic sequences of EHV-1 strain Ab4p (accession number M86664) (20) and EHV-4 strain NS80567 (accession number AF030027) (21) were obtained from DNA databases (NCBI and DDBJ).

The sequences reported in this paper were deposited with the DDBJ under the accession numbers AB190351 for the 9BSV4 noncoding region between ORF62 and ORF63, and AB190352 for the G1753 sequence corresponding to the bases 112756 to 114969 of the Ab4p genome.

The fingerprints of each viral genomic DNA obtained with *Bam*HI, *Bgl*II and *Eco*RI were the same as reported previously (10), indicating that no mutations occurred during virus passages in the laboratory (data not shown). The lengths of the fragments after LA-PCR amplification ranged from 5.6 kb to 14.3 kb. Amplified fragments were designated by the primers used to amplify them, e.g., 1F1R. PCR-RFLP using *Bam*HI, *Hin*cII, *Hin*dIII, *Pst*I and *Sac*I showed variation among EHV-1 cattle isolates on restriction sites and restriction fragment lengths in fragments 17F17R (Fig. 1, A-1 to A-3), 5F5R (Fig. 1, B-1 to B-3), 7F7R (data not shown), 10B10R (Fig. 1, C-1 to C-3), 11F11R (Fig. 1, C-1 to C-3), 15F15R (data not shown), 13F13R

Table 1. Primers used for PCR of ICP4 gene and downstream

Primers	Sequence (5'-3')	Strain	Position
F1	TTTCCCCCATTTTCCCCCTCCCCAACCATCTCC	Ab4p[a)]	112756–112788
F3	ACCCGCCCATCAACCCGCCCAGTAAACAAAGAC	Ab4p	113777–113809
R3	GTCCCGCGATCAGCCAGTACCACATCTA	Ab4p	114969–114942
R1	ACGCAAACTCCACCCCAATCCATCCCCAAAC	NS80567[b)]	113021–112991
F2	TTTGGGGATGGATTGGGGTGGAGTTT	NS80567	112992–113017
R2	ATGAGCTTGAGATCGACGGA	NS80567	113251–113232
F	CAGTCTAGGCGGAGTCTGCGTTGTGCTG	Ab4p	114032–114958

[a)] EHV-1 Ab4p (accession number M86664), [b)] EHV-4 NS80567 (accession number AF030027).

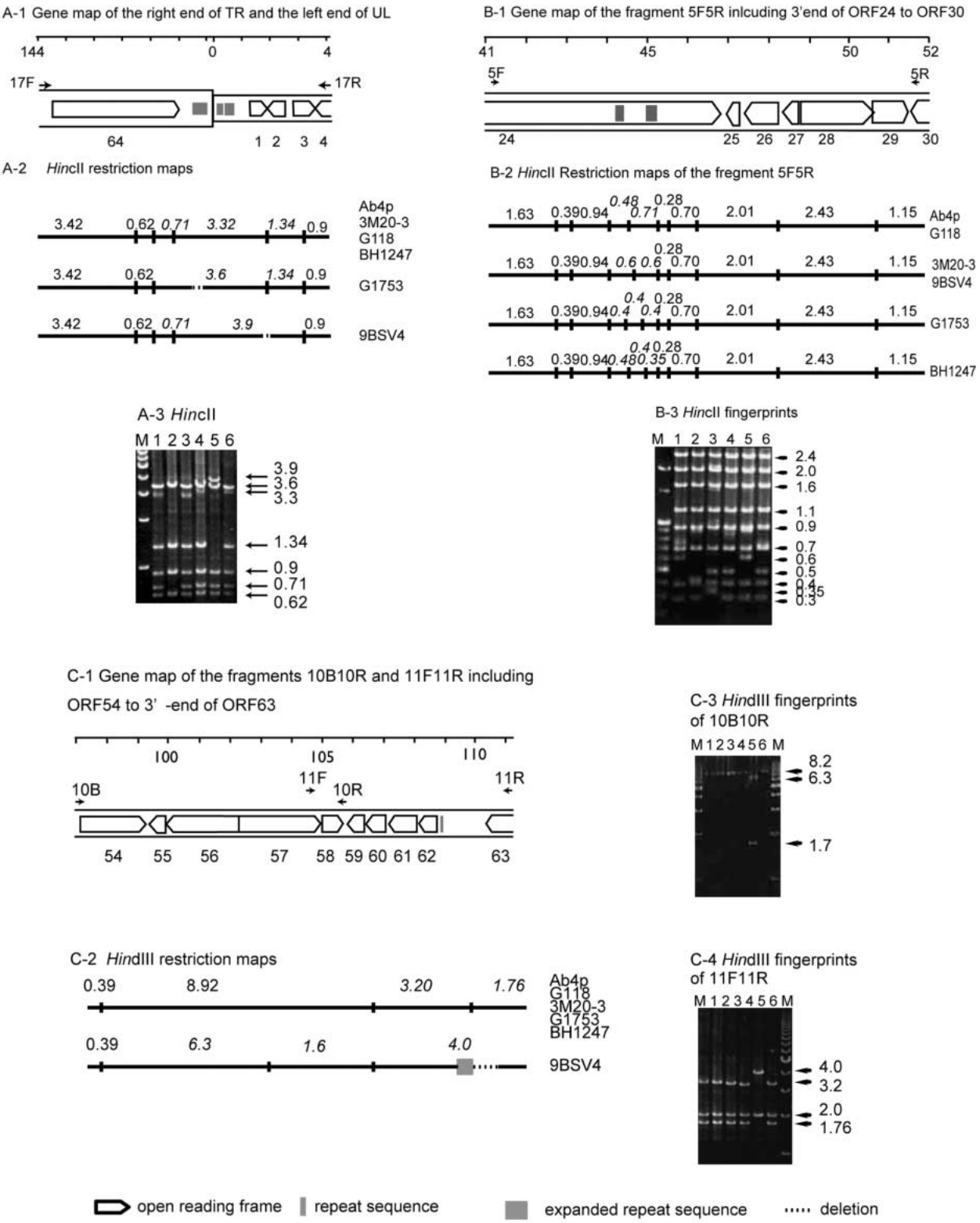


Fig. 1. PCR-RFLP with *Hinc*II and *Hind*III on fragments 17F17R (A), 5F5R (B), and 10B10R and 11F11R (C). A-1: Gene map of the right end of TR and the left end of UL. A-2: *Hinc*II restriction maps. A-3: *Hinc*II fingerprints. B-1: Gene map from ORF24 to ORF30. B-2: *Hinc*II restriction maps. B-3: *Hinc*II fingerprints. C-1: Gene map from ORF32 to ORF39. C-2: *Hinc*II restriction maps. C-3: *Hinc*II fingerprints. The scale is in kb from the leftmost end of the genome. Numbers in restriction maps are the size of restriction fragments. Varied fragment sizes are indicated in italic. M: 1 kb ladder markers; 1: 3M20-3; 2: G1753; 3: BH1247; 4: G118; 5: 9BSV4; 6: Ab4p.

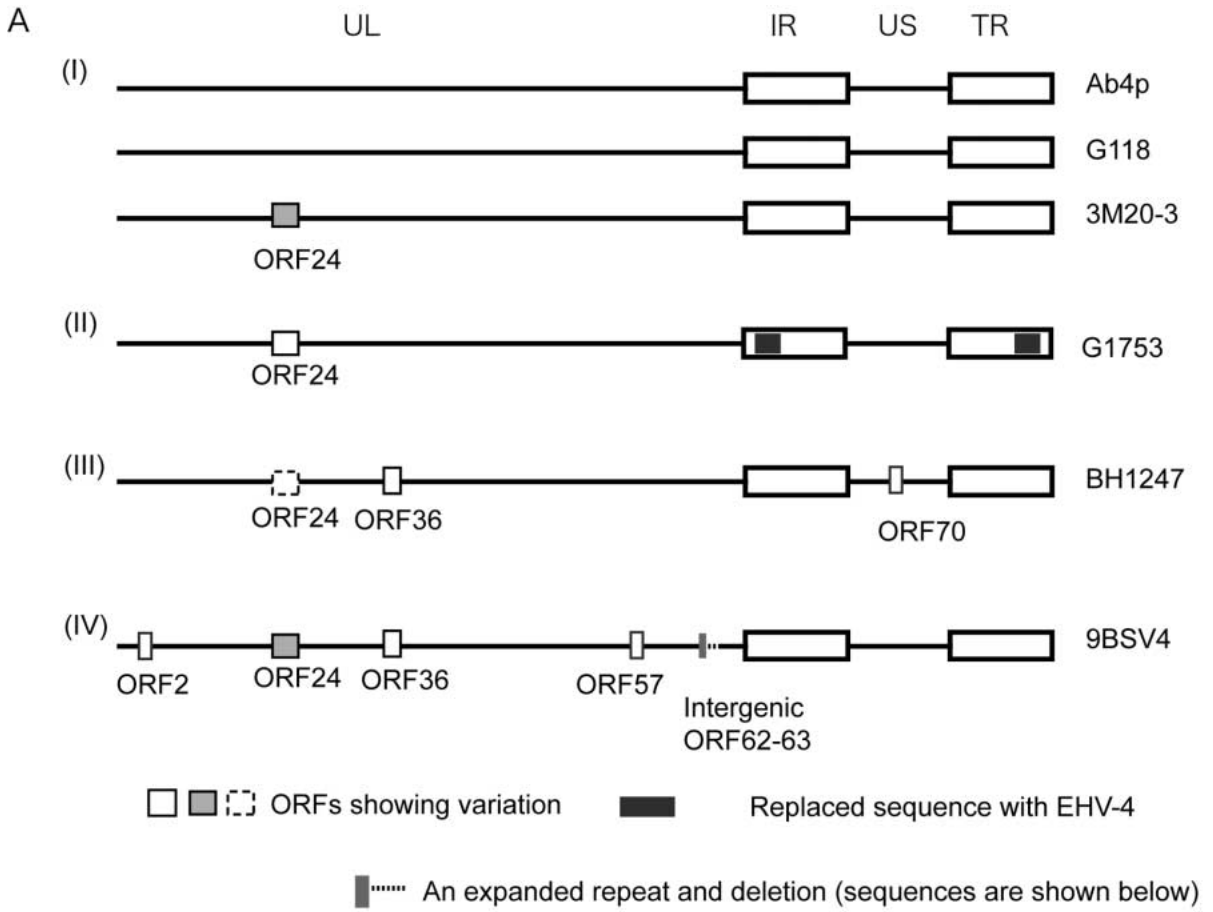
(data not shown) and 13F17F (data not shown). Variation sites were mapped in ORF2, ORF24, ORF36, ORF57, ORF64, ORF70 and the noncoding region between ORF62 and ORF63 (Fig. 2). Functions of these ORFs have been predicted by analogy with gene homologues in HSV-1 as follows except ORF2, which is belived to be unique in EHV-1 and has an unknown function (20, 21): ORF24 as UL36 encoding the large tegument protein, ORF36 as UL25 encoding a DNA-cleavage-packaging virion protein, ORF57 as UL5 encoding DNA helicase/primase complex, ORF62 as UL1 encoding glycoprotein L associated with fusion activity, ORF63 as IE110 encoding ICP0 of a transcription activator, ORF64 as IE175 encoding ICP4 of another transcription activator, and ORF70 as US4 encoding glycoprotein G with an unknown function.

Restriction maps of isolates G118 and 3M20-3 were identical to that of Ab4p, electropherotype EHV-1 P except *Hin*cII fragment length variation in ORF24 of 3M20-3 (Figs. 1, 2). Variations of G1753 included gains and losses of *Hin*cII sites in ORF24 (Figs. 1, 2) and large variations in ORF64 (data is shown below). The restriction map of G1753 was identical to that of electropherotype EHV-1 B (Fig. 2). Variations of BH1247 were shown by the absence of *Hin*cII sites in ORF24 and ORF36, and by the presence of a *Bam*HI site in ORF70 (Fig. 2). Restriction mapping of 9BSV4 showed the absence of *Hin*cII sites in ORF2, ORF24 and ORF36 and the presence of a *Hin*dIII site in ORF57 (Fig. 2). Moreover, the fragments 11F11R and 17F17R of 9BSV4 were 0.9 kb and 0.3 kb shorter than those of Ab4p, respectively.

The isolates 9BSV4 had much more variability than the other 4 cattle isolates. One of the variable regions in 9BSV4 is a shorter region in which fragments 11F11R and 12F12R overlap. This shorter region in 9BSV4 was examined in detail. First the fragment 12F12R of 9BSV4 was digested by restriction enzymes of *Eco*RV, *Dra*I, *Msp*I, *Fba*I, *Nco*I, *Ssp*I, *Sma*I, *Nae*I, *Mun*I and *Xba*I for fine restriction mapping. The isolate 9BSV4 lost these restriction sites, which should be located in the noncoding region between ORF62 and ORF63. Therefore, we determined the nucleotide sequence between ORF62 and ORF63 of 9BSV4, which should correspond to the 1,424 bp sequence from nucleotide 108892 to 110315 in the Ab4p genome (Fig. 2). The sequence of 9BSV4 was 500 bp, which was 924 bp shorter than that of Ab4p. The 500 bp sequence consisted of a 72 bp unique sequence (nucleotide 1 to 72), a 352 bp repeat sequence (nucleotide 73 to 424), which is 19 complete copies and one partial (10 bp) copy of an 18 bp unit sequence of 5'-GCTAGCGCTAACGCTAGG-3', and a 76 bp unique sequence (nucleotide 425 to 500). The two unique sequences were identical to those of EHV-1 Ab4p. Thus a sequence of 1,222 bp corresponding to nucleotides 109019 to 110240 of Ab4p was deleted and replaced by a repeat sequence of 298 bp, which consists of 16 complete and one partial copy of the 18 bp unit sequences in 9BSV4.

The other variable region was observed in ORF64 of G1753, which encodes the infected cell protein 4 (ICP4) and the downstream region of the ICP4 gene. This region was characterized by the presence of a *Pst*I restriction site and the absence of *Hin*cII (Fig. 1), *Bam*HI and *Sac*I sites (data not shown). The ICP4 gene is included in each IR and TR. The identity of IR and TR in G1753 was confirmed by LA-PCR-RFLP on fragments 12F17F including IR and 17F17R including TR (data not shown). The nucleotide sequence of G1753, which should correspond to nucleotides 112756 to 114969 in the Ab4p genome was determined in order to find the difference between G1753 and Ab4p. The sequence of G1753 determined was 1,812 bp, which was 401 bp shorter than that of Ab4p. A BLAST search indicated that the sequence of G1753 was identical to that of EHV-1 97c7 with some point mutations (1806/1812 identity). EHV-1 97c7 is an EHV-1 B type strain isolated in Japan and is considered to be a natural recombinant between the progenitor of EHV-1 P and EHV-4 in our previous study (16).

Virulence of each cattle-derived EHV-1 was evaluated by using experimental infection in hamsters. Methods of the experimental infection in hamsters were described previously (10, 16). All experiments were conducted under the guidelines for animal experiments at Gifu University with approval by the Committee of Animal Care and Welfare in the Faculty of Applied Biological Sciences, Gifu University. In brief, 3-week old male specific pathogen-free Syrian hamsters (SLC, Shizouka, Japan) kept in specific pathogen-free housing were inoculated intranasally under anesthesia by delivering 25 μl of viral suspension containing 10^3 pfu/g of body weight into each nasal cavity. Control animals were inoculated with the same volume of MEM. Four hamsters were inoculated with each sample. The hamsters were observed for 12 days after inoculation. Hamsters inoculated with G1753 isolate did not show any apparent signs of disease during the observation period. Hamsters inoculated with the other 4 out of 5 bovine isolates developed a loss of body weight and neurological signs at 4–5 days post infection (data not shown). The neurological signs included restless behavior, salivation, nasal and ocular discharge and crouching posture at the onset of disease followed by sensitive reaction to external stimulation, epileptic signs and incoordination of limbs+the climax, and then



B

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9BSV4        1 ATGAGGGTCAGAGGTTAGATCCAAGCAACCCTTTGTTTCACAATTCAATAGAATCATAAATTTAACTTTGGCGCTAGCGC
Ab4p    118892 ................................................................................

9BSV4       81 TAACGCTAGGGCTAGCGCTAACGCTAGGGCTAGCGCTAACGCTAGGGCTAGCGCTAACGCTAGGGCTAGCGCTAACGCTA
Ab4p    118972 ..................................................----------------------------

9BSV4      161 GGGCTAGCGCTAACGCTAGGGCTAGCGCTAACGCTAGGGCTAGCGCTAACGCTAGGGCTAGCGCTAACGCTAGGGCTAGC
Ab4p    119024 --------------------------------------------------------------------------------

                          (abbreviated from nucletide numbered 241 to 400)

9BSV4      401 GCGCTAACGCTAGGGCTAGCGCTACCCAC--------------------------------------------------
ab4p    119025 ----------------------------AATGAGGCTGGCCACCAGCACCGGAAGCTTGTCATATTTGTGAGCCTGGAG

9BSV4      429 --------------------------------------------------------------------------------
Ab4p    119076 CAGCCATTTTCCAAAATCTGTACTGTCATGTTTCTTGACCTTTGGATGTCATATCTGTGGACTGGAGGCAGCCATTTTCC

9BASV4     430 ------------------------------------------------ACCCCTGCATAGGTTTTTGTAATAGGTTCC
Ab4p    110196 CAAAGATATCTATTTTTAAAGCTCCCCCTTTCGCGGTTGCCCCCACCCAC..............................

9BSV4      459 ATATACCCAGGGCGGCGACTATTAACACTCTCTCAGACTGA
Ab4p    110276 ....................................
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Fig. 2. Summarized scheme of genomic DNA comparison among EHV-1 isolated from cattle and horses. Differences between the Ab4p and each cattle EHV-1 isolate are shown in the figure. A: Genome restriction maps of isolates G118 and 3M20-3 were almost identical to that of Ab4p which is an electropherotype 1 P (I). A genome restriction map of G1753 was identical to that of the electropherotype 1B (15) (II). Genome restriction map of BH1247 included three restriction site variations in ORF24, ORF36 and ORF70 (III). Genome restriction map of 9BSV4 included four restriction site variations and an expanded repeat sequence and deletion (IV). Variation in ORF24 in 3M20-3 was the same as that in 9BSV4 (indicated as gray rectangles). Variation in ORF36 in BH1247 was the same as that in 9BSV4 (indicated as open rectangles). B: Alignment of nucleotide sequences of noncoding region between ORF62 and ORF63 of isolate 9BSV4 and Ab4p. We sequenced a 500 bp region of 9BSV4. A sequence of 1,222 bp corresponding to nucleotides 109019 to 110240 of Ab4p was deleted and replaced by a repeat sequence of 298 bp which consists of 16 complete copies and one partial copy of the 18 bp unit sequence in 9BSV4. Dashes indicate the absence of corresponding nucleotides. Dots indicate nucleotides that are identical to those of the Ab4p sequence.

coma. The signs disappeared on days 6 to 8 post infection. The histologic features of hamsters inoculated with BH1247, G118, 3M20-3, and 9BSV4 isolates included degeneration and necrosis of neurons containing intranuclear inclusion bodies in the olfactory bulbs, cerebral cortex and hippocampus, with severe perivascular infiltration of mononuclear cells and diffuse glial proliferation (data not shown). The histopathology of the lungs included an infiltration of neutrophils and monocytes. Hamsters inoculated with G1753 isolate showed only a mild degeneration in the hippocampus.

In conclusion, our restriction mapping and sequencing indicated that two of 5 isolates from cattle, G118 and 3M20-3, were the electropherotype EHV-1 P and that G1753 was EHV-1 B. The other two isolates, BH1247 and 9BSV4, were other genotypes of EHV-1 (Fig. 2). EHV-1 infections are normally restricted to horses in nature, although some EHV-1s were sometimes isolated from none-equids. The previous fingerprinting analysis indicated interspecies transmission of EHV-1 from horses to cattle, but the original sources of EHV-1 isolated from cattle have not been identified (9). One of the five cattle isolates, 9BSV4, was isolated from a 26-day-old calf that seemed to have had contact with horses, because this calf was born and kept in a closed research cow herd within 50 yards of a horse pasture. Three years earlier, the horses were used to work the cattle (9). The results presented here suggest that at least three of the cattle isolates (G118, 3M20-3, and G1753) were directly transmitted from horses to cattle. Hence these results confirmed the interspecies transmission from horse to cattle and indicated that the genome structure was not changed in the interspecies transmission.

We have made detailed restriction maps of the EHV-1 genome isolated from cattle. The previous analysis only compared fingerprinting patterns on gels and did not show precise mapping of variations in the viral genome. We could identify which sites were varied among the EHV-1 strains isolated from cattle. However, the EHV-1 strains isolated from cattle had no common characteristics that distinguished them from EHV-1 strains from horses. EHV-1 strains isolated from cattle have been assigned to different electropherotypes, such as EHV-1 P and EHV-1 B (2). We found that G1753 could be regarded as EHV-1 B, which we previously showed as a recombinant between the progenitor of EHV-1 P and EHV-4 (16). Pagamjav et al. (16) suggested that the intertypic recombination could cause some alteration of virulence and pathogenicity in EHV-1. However, the intertypic recombination might not to be a prerequisite for interspecies transmission according to the present data.

The EHV-1 genome contains some complex repeat sequences (20). One of the repeats is in ORF24 and consists of a 63-bp sequence repeated 3 times, a 33-bp GC-rich motif repeated 5 times and a 12-bp sequence repeated 6.5 times. Repeat sequences often give rise to variation due to changes in the number of repeats. Kirisawa et al. (13) detected 150 bp nucleotide deletion of ORF24 in attenuated EHV-1 HH1 strains and suggested that genetic alteration of the EHV-1 ORF24 may influence the replication cycle or the structural conformation of EHV-1. McCann et al. (14) also detected variations in the repeat sequences region of ORF24. However, the virulence of each cattle-derived EHV-1 in experimental hamster infection did not correlate with the ORF24 variation. Thus we could not find any biological differences among the five cattle-derived EHV-1 related to variations of reiteration sequences in ORF24.

We found the 924 bp deletions between ORF62 and ORF63 (corresponding to nucleotides 109317–110240 of the Ab4p genome) of the isolate 9BSV-4 in this study. Actually the 924 bp deletion might be caused by deletion of 1,222 bp corresponding the nucleotides 109019 to 110240 of Ab4p and might be partially replaced by an increase of copy number in the repeat sequence. In our previous study (11), we constructed a recombinant virus in which the green fluorescence protein gene was inserted at 110055, which is located in the replaced region. This virus was less virulent than the wild type, suggesting that a part of the ORF62–63 intergenic region might have a role in virus growth. Kirisawa et al. (13) found a 1.4 kb deletion in an attenuated Japanese EHV-1 strain between the *Eco*RI site (at 108739 in Ab4p) and the *Dra*I site (at 110347 in Ab4p). The deletion was located in the intergenic region between ORF62 and 63 (R. Kirisawa, personal communication). However we have no evidence that these mutations were involved in the host species tropism change in EHV-1.

In summary, we found several variations in the genomes of EHV-1 isolated from cattle, but these variations were also present in EHV-1 strains isolated from horses. Therefore we could not find any specific mutations in EHV-1 isolated from cattle. The results presented here suggest that the EHV-1 has the potential to infect cattle, and fewer mutations might be needed for interspecies transmission of EHV-1.

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