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The genomic diversity among equine herpesvirus-1 strains isolated in Japan

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Summary. The DNAs from nine Japanese field isolates of equine herpesvirus-1 (EHV-1) were analyzed by digestion with the restriction endonuclease Bam HI and Southern hybridization. Comparing restriction profiles among the EHV-1 strains, there was no considerable difference between isolates before and after vaccine application, but some minor variations in the mobility of *Bam* HI fragments were observed. To identify these variable fragments, all genomic DNA sequences of the Japanese prototype of EHV-1 have been cloned as *Bam* HI restriction fragments into the plasmid pUC-18. Physical maps of the virus DNA were constructed by a combination of Southern blot analysis and double enzyme digestion of the cloned fragments. By using these cloned fragments as probes in Southern blot analysis, the areas of heterogeneity observed among the field EHV-1 isolates were located in both terminals of U_L, the center of U_L, IR, U_S and TR regions of the genome.

Introduction

Equine herpesvirus-1 (EHV-1) is a causative agent of contagious virus abortion, acute upper respiratory tract infection and central nervous system disorders in horses [1, 10, 11]. The EHV-1 genome consists of a unique long (U_L) segment, an internal repeat (IR), a unique short (U_S) segment and a terminal repeat (TR) [21, 34]. The U_S segment is bracketed by the two inverted repeats IR and TR. As a consequence, two isomeric forms of EHV-1 genome exist in almost equal amounts. The architecture of the EHV-1 genome is similar to that of bovine herpesvirus-1, swine herpesvirus-1 and varicella-zoster virus (VZV) [8, 15, 23]. The complete DNA sequence was determined of a pathogenic British isolate of EHV-1 [32]. Recent characterization of EHV-1 strains by restriction endonuclease analysis of their DNA revealed some differences in electrophoretic patterns among EHV-1 isolates [5, 6, 13, 25, 28, 30, 31, 33].

In Japan, an abortion storm caused by EHV-1 occurred in 1967, originating probably from newly imported pregnant mares [22]. After that, small outbreaks of abortion due to EHV-1 have been observed almost every year. In order to control this disease, inactivated vaccine was applied to pregnant mares since 1979. This vaccine was prepared from the HH 1 strain which was isolated from an aborted fetus and was adapted to bovine kindney cells [20, 22]. However, abortions due to EHV-1 infection occurred sporadically or enzootically in vaccinated mares. In the United States, after widespread use of an inactivated vaccine, EHV-1 1 B electropherotype became the most frequently recovered isolate from aborted fetuses instead of 1 P type which was the predominant isolate before the vaccine application [1, 6].

The purpose of the present study was to investigate the electropherotype of Japanese field isolates of EHV-1 before and after vaccine use and to determine the variable regions of the viral genome among the viruses. The possibility of a relationship between the genomic alterations and antigenicity or pathogenicity is discussed.

Materials and methods

Virus and cells

The EHV-1 strains used in this paper originated from aborted fetuses in Hokkaido, Japan and are listed in Table 1.The HH 1 strain was used as the Japanese prototype of EHV-1 [22]. Strains F 1-F 8 were isolated in our laboratory. All viruses were propagated in primary horse kidney (HK) cells.

Preparation of viral DNA

Cells were infected at a multiplicity of infection of about 0.01 PFU/cell. When cells showed 80–90% cytopathic effect, they were harvested and cell debris was removed by low-speed

Table 1. Japanese EHV-1 isolates from aborted fetus examined by restriction endonucleases

Isolates	Year of isolation	No. of passage in HK cells
HH 1 (−)[a]	1967	10
F 1 (−)	1978	5
F 2 (−)	1978	5
F 3 (+)[b]	1983	3
F 4 (+)	1983	3
F 5 (+)	1983	3
F 6 (+)	1983	3
F 7 (+)	1987	3
F 8 (+)	1987	3

[a] Isolates before the vaccine application
[b] Isolates after the vaccine application

centrifugation. Virus particles were partially purified by centrifugation at 90,000 **g** for 90 min and the virus pellet was suspended in phosphate buffered saline, pH 7.2. Virions were lysed with 1% SDS and pronase E (1 mg/ml) in 100 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, pH 9.0 at 37 °C overnight. DNA was extracted with phenol and chloroform-isoamyl alcohol (24 : 1), precipitated with ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Restriction endonuclease digestion of DNA and agarose gel electrophoresis

DNAs were digested to completion with the desired restriction endonuclease under conditions recommended by the supplier (Boehringer Mannheim). The resultant DNA digests were electrophoresed through 0.7% agarose at 30 V for 15 h. The gels were stained with ethidium bromide (0.5 μg/ml) and visualized by transillumination at 302 nm.

Construction of recombinant plasmids

The DNAs of the HH 1 strain and pUC-18 were digested separately to completion with Bam HI. Restriction digests of HH 1 genomic DNA were ligated with T 4 DNA ligase to alkaline phosphatase-treated pUC-18 DNA overnight at 14 °C. *Escherichia coli* of the competent strain JM 109 was transformed with the ligation mixture [26].

Recombinant plasmids containing HH 1 genomic termini were constructed as follows. Whole HH 1 virion DNA was treated with DNA polymerase I, large fragment, in the presence of the four deoxynucleotide phosphates and then with T 4 DNA polymerase to make blunt ends. 5′-Phosphorylated octamer Bam HI linkers (Takara, Japan) were ligated to the virion DNA by T 4 DNA ligase. The viral DNA was digested with Bam HI and separated in preparative agarose gels. Desired fragments were recovered from the agarose gel and ligated to Bam HI sites of pUC-18 as described above. Recombinant clones were characterized by Southern hybridization and all *Bam* HI fragments of the HH 1 DNA were cloned.

Blot hybridization

DNA fragments were transferred to nitrocellulose (0.45 μm, Schleicher & Schuell) [27]. The filter was incubated in prehybridization solution containing 50% formamide, 5 × SSPE (1 × = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 10 × Denhardt's solution (1 × = 0.02% each of bovine serum albumin, polyvinylpyrolidone and Ficoll) for 2 h at 42 °C. Non-isotopic reagent, biotin-11-dUTP labelled probe which was made by random priming [18] and dextran sulfate were added to the prehybridization solution to a concentration of 10 ng/ml and 50 mg/ml, respectively. The filter was incubated for 14–16 h at 42 °C in a shaking water bath. After hybridization, the filter was washed and hybridized bands were revealed using the BlueGene non-radioactive nucleic acid detection system (Bethesda Research Laboratories).

Results

Primary restriction endonuclease patterns of HH 1 DNA

Primary restriction endonuclease digests of HH 1 DNA electrophoresed into 0.7% agarose and stained with ethidium bromide are shown in Fig. 1. In Bam HI digests, there are 20 fragments (fragments A – T). Exonuclease III digestion of the HH 1 DNA followed by digestion with Bam HI confirmed that *Bam* HI T and S fragments were located at the genomic termini of EHV-1 (data not shown).

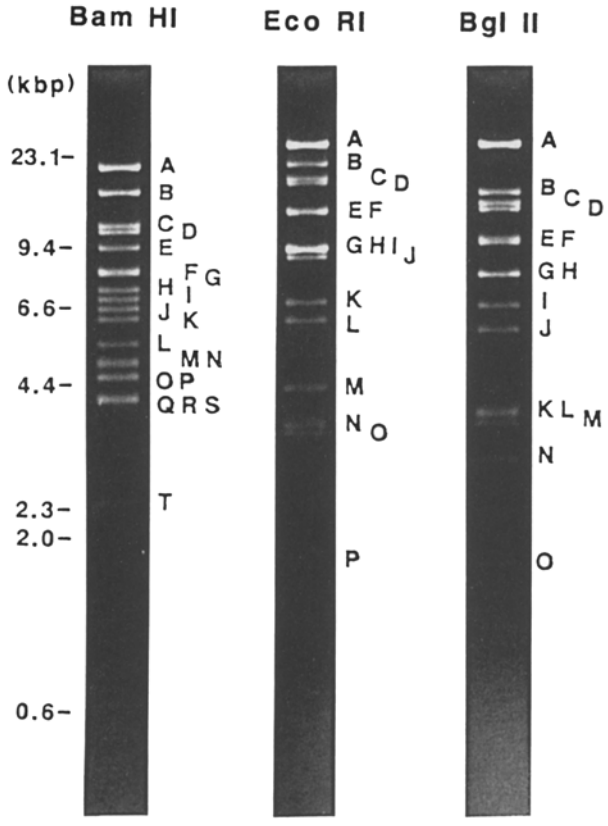

Fig. 1. Electrophoretic profiles of the HH 1 strain digested with Bam HI, Eco RI and Bgl II. The DNAs were electrophoresed in a 0.7% agarose gel. Fragment nomenclature are shown on the right. The sizes (kbp) of *Hin*dIII marker fragments from bacteriophage lambda DNA are indicated on the left

Construction of physical maps of the HH 1 strain

We cloned all *Bam*HI fragments of the HH 1 DNA as described in Materials and methods. By a combination of Southern blot analysis and double enzyme digestion of these cloned fragments, we constructed Bam HI, Eco RI and Bgl II physical maps of the HH 1 strain (Fig. 2). These maps were essentially similar to those of the HVS-25 strain [34, 35], but we found some new fragments in the Eco RI and Bgl II maps compared to those of the HVS-25 strain.

Restriction endonuclease and Southern blot analysis of field EHV-1 isolates

The DNAs of eight field EHV-1 isolates F 1-F 8 and the HH 1 strain were analyzed with Bam HI digestion (Fig. 3). HH 1, F 1 and F 2 were isolated before the vaccine application and the other six isolates were isolated after that. The electrophoretic patterns of these viruses were essentially similar to each other, but some *Bam*HI fragments exhibited electrophoretic mobility variation. The variation of *Bam*HI E and T fragments were observed clearly. Some fragments between *Bam*HI L and S fragments showed variability. In order to identify these variable fragments in the EHV-1 genome, Southern blot hybridization was carried out using molecularly cloned all *Bam*HI fragments of the HH 1

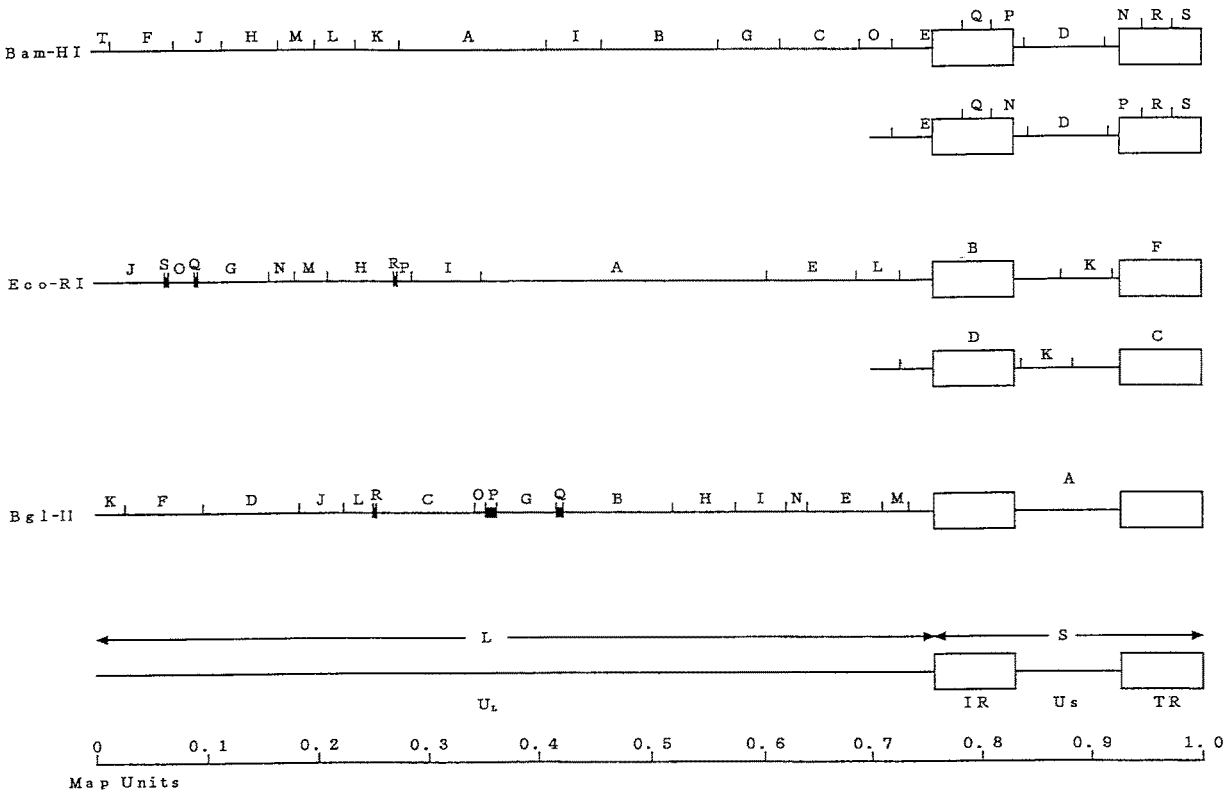

Fig. 2. Restriction endonuclease maps of the EHV-1 HH 1 strain. Newly recognized fragments in the Eco RI and Bgl II maps are indicated by the thick line

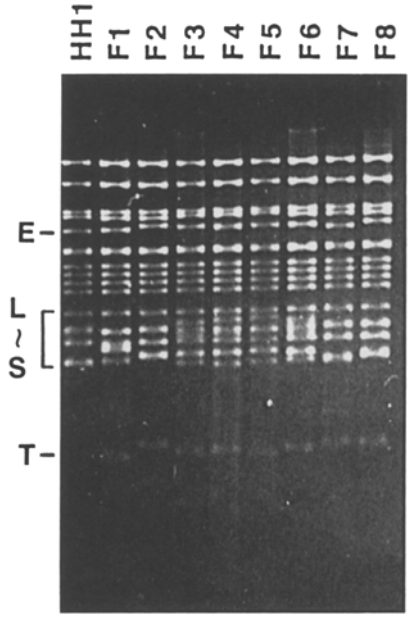

Fig. 3. Bam HI restriction profiles of the DNAs of nine EHV-1 isolated in Japan

strain as probes. *Bam* HI N, Q(R), and S probes revealed the variabilities in migration of these fragments of the nine EHV-1 isolates (Fig. 4). *Bam* HI N and S probes hybridized with *Bam* HI P and E fragments, respectively (Fig. 4 A, C), because of some homology originating from inverted repeat sequences of EHV-1 genome, and also variations of P and E fragments were detected. Hybridization pattern of *Bam* HI E probe was the same as that of S probe (data not shown). *Bam* HI T probe also revealed the variabilities consistent with the observation in Fig. 1. The other probes, A, B, C, D, F, G, H, I, J, K, L, M, and O probes did not detect any variabilities (data not shown).

As high molecular weight bands (*Bam* HI A, B, C and D fragments) have the same electrophoretic mobility among the isolates, double digests of the DNAs with Bam HI and Eco RI or Bgl II were analyzed by Southern blot hybridization. The variability of A and D fragments was newly detected (Fig. 5) but B and C fragments were not (data not shown). *Bam* HI A fragment was cleaved to three fragments by Eco RI digestion, designated A 1, A 2, and A 3 fragments according to their molecular weights and A 2 fragment showed variation (Fig. 5 A, C). The variation of A 2 fragment was also confirmed in an

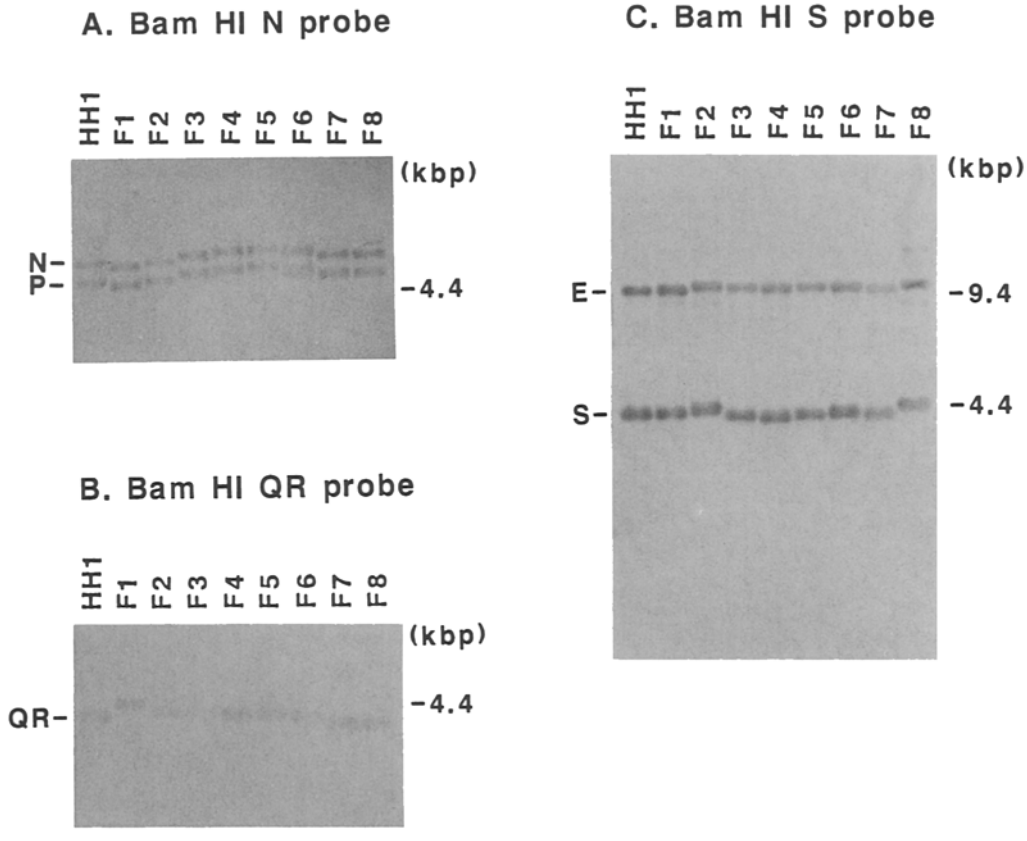

Fig. 4. Hybridization patterns of Bam HI digests of nine EHV-1 DNAs probed with molecularly cloned *Bam* HI N (**A**), Q-R (**B**), and S (**C**) fragments of the HH 1 strain

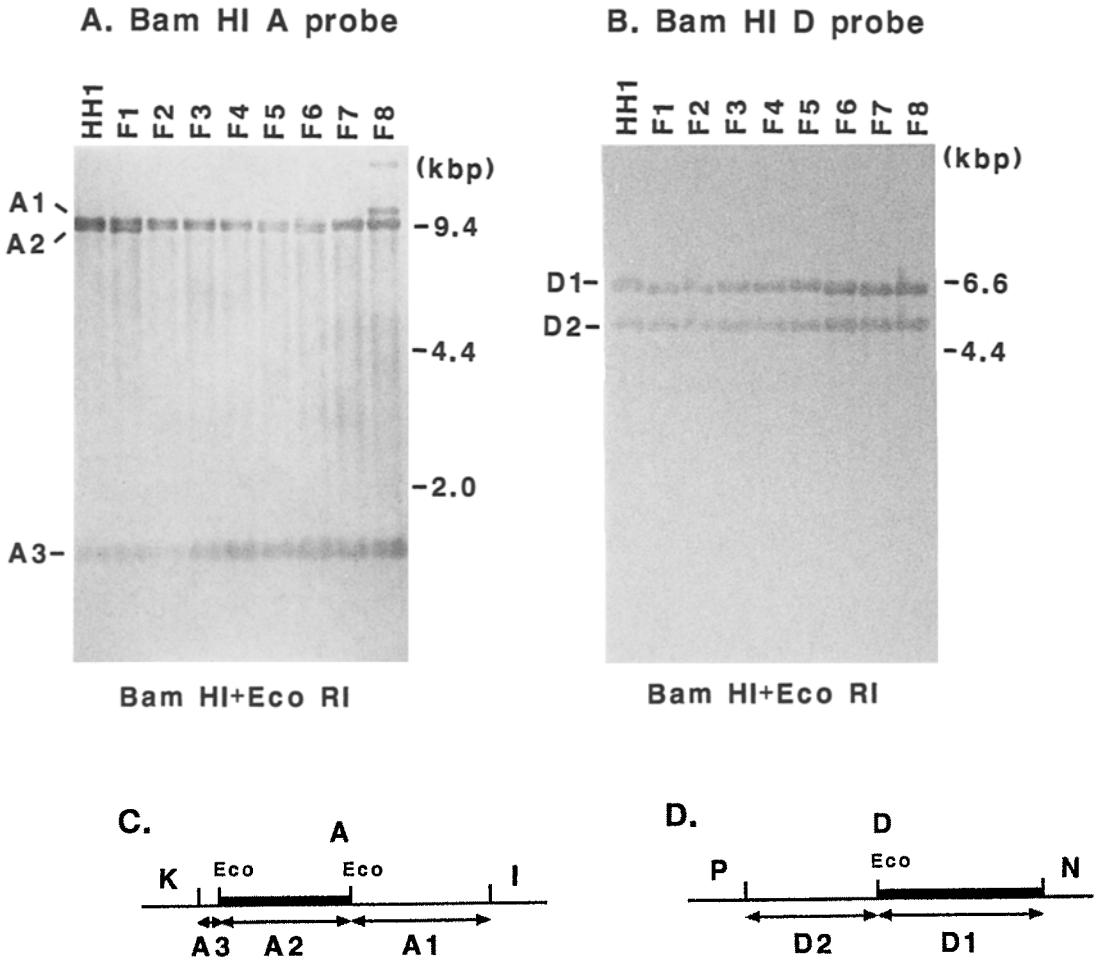

Fig. 5. Hybridization patterns of double digests of the nine EHV-1 DNAs, with Bam HI and Eco RI probed with molecularly cloned *Bam* HI A (**A**) and D (**B**) fragments and restriction maps of *Bam* HI A (**C**) and D (**D**) fragments. A 1, A 2, and A 3 fragments were generated from *Bam* HI A fragment by Eco RI digestion. D 1 and D 2 fragments were generated from *Bam* HI D fragment by Eco RI digestion. Variable fragments observed in **A** and **B** are indicated by the thick line in **C** and **D**, respectively. *Eco* Eco RI

electrophoretic profile (data not shown). *Bam* HI D fragment was cleaved to two fragments by Eco RI digestion, designated D 1 and D 2 fragments, and D 1 fragment showed variability (Fig. 5 B, D). Further, double digests revealed variability in the right terminus of the U_L region within *Bam* HI E fragment (Fig. 6). *Bam* HI E fragment was cleaved to two fragments by Eco RI digestion, designated E 1 and E 2 fragments, and also cleaved to two fragments by Bgl II digestion, designated B 1 and B 2 fragments. E 1, B 1, and B 2 fragments showed the variations (Fig. 6 A, B). The variation of B 1 fragment was due to the variation of *Bam* HI S fragment, because the variation pattern was similar

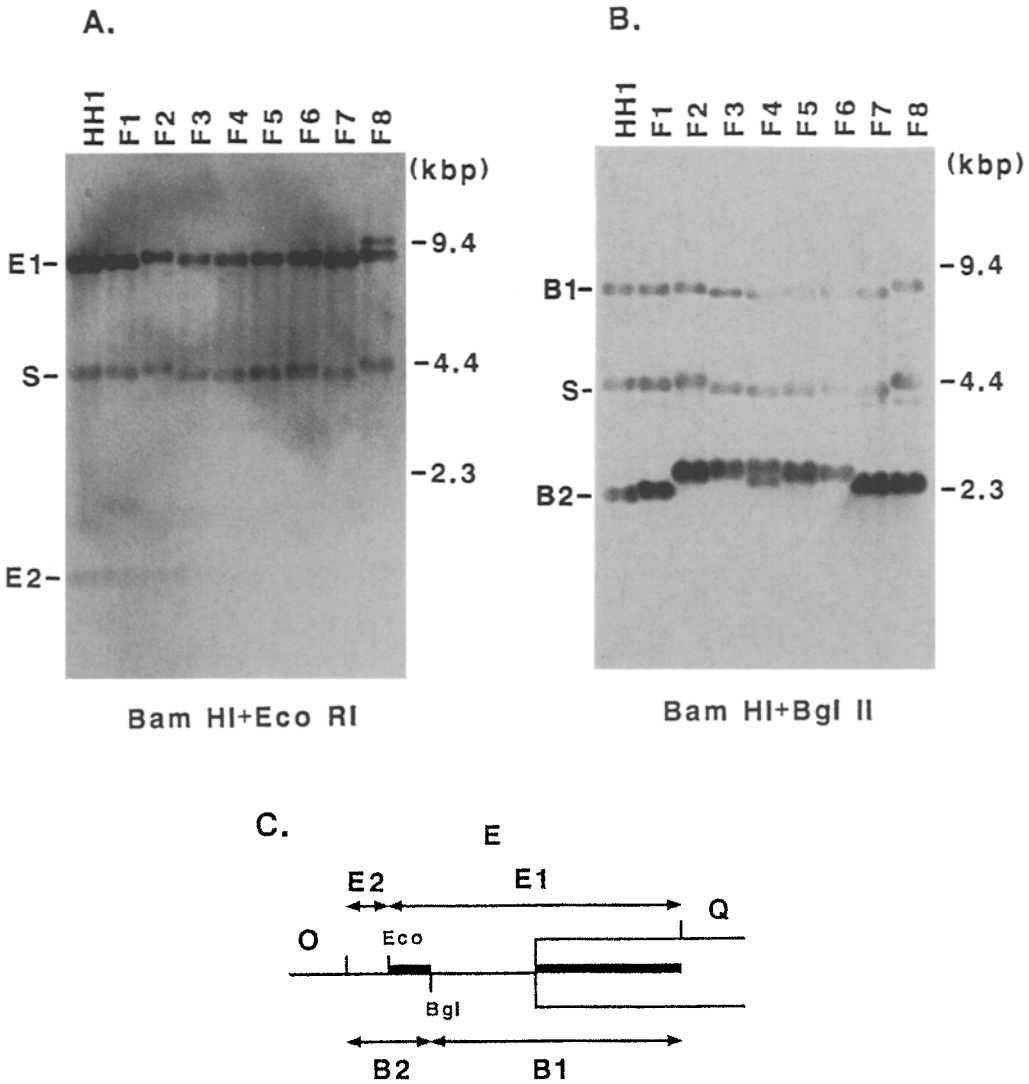

Fig. 6. Hybridization patterns of double digests of the nine EHV-1 DNAs with Bam HI and Eco RI (**A**) or Bgl II (**B**) probed with molecularly cloned *Bam* HI E fragment and restriction map of *Bam* HI E fragment (**C**). E 1 and E 2 fragments were generated from *Bam* HI E fragment by Eco RI digestion. B 1 and B 2 fragments were generated from *Bam* HI E fragment by Bgl II digestion. *Bam* HI S fragment was not cleaved by Eco RI and Bgl II. Variable fragments observed in **A** and **B** were edited and are indicated by the thick line in (**C**). *Eco* Eco RI, *Bgl* Bgl II

between B 1 and S fragments (Fig. 6 B, C). The variation of B 2 fragment was due to variation of right half of the fragment, because E 2 fragment, left half of the B 2 fragment, did not show variability (Fig. 6 C). The positions of these variable fragments are summarized in Fig. 7.

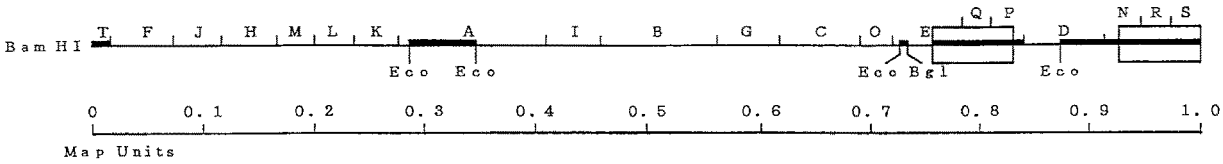

Fig. 7. The Bam HI restriction map (prototype arrangement only) of the EHV-1 HH 1 strain. *Bam* HI fragments exhibiting variation among nine Japanese EHV-1 isolates are indicated by the thick line. *Eco* Eco RI, *Bgl* Bgl II

Discussion

In this report, we cloned all *Bam* HI DNA fragments of EHV-1 HH 1 strain and constructed precise physical maps of the virus genomic DNA to study heterogeneities of EHV-1 isolates in Japan. The restriction endonuclease analysis and the physical maps of the HH 1 strain were essentially similar to those of the HVS-25 strain obtained by Whalley et al. [34, 35] but not the mouse cell adapted L-M strain by Henry et al. [21]. We confirmed the revised Bam HI map of the HVS-25 strain [35] but not the original one [34]. In Bgl II restriction profile, the migration distance of the M fragment of HH 1 is slightly longer than that of HVS-25, which had almost the same electrophoretic mobility of K and L fragments. We found new fragments in the Eco RI and Bgl II maps. To notice these differences between strains might be very important especially in mapping genes.

Electrophoretic profiles of the Japanese field isolates of EHV-1 showed some differences among the viruses, as a consequence of addition or deletion of DNA sequences, not to loss or gain of restriction endonuclease cleavage sites resulting in the generation of new fragments. Such variations were commonly observed among EHV-1 isolates in other countries [5, 13, 30]. The results of our extensive hybridization studies showed that heterogeneity was observed in both terminals of U_L (*Bam* HI T and E fragments), the center of U_L (*Bam* HI A fragment) and all fragments located in S region (Fig. 7). The variability of *Bam* HI A fragment has not been reported yet because of its high molecular weight. These variations were compared between isolates before and after the vaccine use, but there was no considerable difference between them. However, it may be important to survey the emergence of other genotype viruses as in the United States [5, 6] to control effectively EHV-1 infection.

Recently, the mapping of several EHV-1 genes was carried out [2, 3, 7, 17, 24, 35, 36]. Telford et al. [32] reported the complete DNA sequence of a pathogenic British isolate Ab-4 of EHV-1 and compared it with that of the previously sequenced herpes simplex virus type-1 (HSV-1) and VZV. Based on these reports, we analyzed the variable fragments observed in our experiments in detail. On A 1 subfragment of *Bam* HI A fragment, there might be seven genes, genes 24 (partial), 25, 26, 27, 28, 29 and 30 (partial) based on the nomenclature by Telford et al. [32]. The gene 24, which is a counterpart of

HSV-1 gene UL 36 encoding tegument protein, contains repeat sequences [32]. Whittaker et al. [37] reported that molecular weight variation of gp 2 was observed among EHV-1 strains, particularly in the vaccine strain "Rinomune". The gp 2 is one of major EHV-1 glycoproteins [1] and might be corresponding to the gene 28 which is a counterpart of HSV-1 gene UL 32 [9, 32]. Therefore, there is a possibility that genetic alterations of these regions may influence the antigenicity and pathogenicity. We are now studying molecular aspects of the gene 24 and gp 2 gene from field isolates and attenuated strains.

On D 1 subfragment of *Bam* HI D fragment, there might be four genes, genes 69 (partial), 70, 71 and 72 (partial) [32]. It was noticed that gene arrangements in U_S region by Telford et al. [32] were on the genome of inverted isomer but not on prototype isomer [7, 17]. The gene 71, which is a counterpart of HSV-1 gene US 5 encoding a putative membrane glycoprotein contains two sets of tandem reiterations [32]. The heterogeneity in D 1 fragment might be explained mainly by variable frequencies of the repeats and might influence the antigenicity and pathogenicity. We plan to examine molecular aspects of this gene.

Chowdhury et al. [14] reported that some EHV-1 isolates from ruminants showed a DNA restriction pattern more divergent from the reference EHV-1 strain and have slightly different properties in cross-neutralization. The heterogeneity at both genomic U_L ends, IR and TR regions might be explained by variable frequencies of tandem repeats [12, 19, 32, 38]. Although the heterogeneities in these regions were observed in our EHV-1 field isolates, they were particularly frequent when EHV-1 was passaged in non-equine cells [4, 29]. A direct relationship between the alteration of these regions and antigenicity or pathogenicity remains to be demonstrated.

In general, the EHV-1 genome is stable during virus replication in equine cells [1]. The reason why the DNAs of field isolates have some different electrophoretic restriction profiles is obscure. It may be helpful to consider the latency of EHV-1 infection and pathological relationship with other equine herpesviral infections in order to resolve the question [11, 16].

Acknowledgements

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