NOTE *Virology*

Molecular Characterization of the Equine Herpesvirus 1 Strains RacL11 and Kentucky D

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(Received 2 November 2006/Accepted 29 January 2007)

ABSTRACT. The pathogenicities of RacL11 and Kentucky D strains of equine herpesvirus 1 in the hamster infection model are different from those of Ab4p and the Japanese isolates. Virus genome restriction fragment length polymorphism analysis and sequence comparison of an intergenic region, glycoproteins and tegument genes showed higher conservation but with some strain-specific differences. These results indicate that point nucleotide differences in RacL11 and Kentucky D might be responsible for their pathogenicity in rodent models.

KEY WORDS: EHV-1, pathogenicity.

J. Vet. Med. Sci. 69(5): 573–576, 2007

Equine herpesvirus 1 (EHV-1) causes abortion, respiratory disease, neonatal death and neurological disorders in horses [1, 18]. The EHV-1 genome is a linear double-stranded DNA molecule, and the complete DNA sequence of a British isolate, Ab4p, has been determined [22]. The Ab4p genome is 150,223 bp in length and has been shown to contain 80 open reading frames (ORFs), four of which are duplicated such that the genome contains at least 76 genes. Ab4p has also been reported to be virulent in experimental infection of foals [7]. Recently, another EHV-1 genome sequence has been reported for the V592 strain [12]. The V592 genomic sequence was determined to be 149,430 bp in length. A total of 31 of 76 ORFs had amino acid variations between the two strains [12]. V592 was reported to be of low virulence compared with the highly virulent Ab4 (a parent strain of Ab4p) and Army 183 isolates [20]. However, these strains have not always been used for molecular virulence and pathogenicity analyses of EHV-1. Other strains including RacL11 and Kentucky D have been used for this type of research [3–5, 11, 13–15, 17, 21, 23, 24], although the entire genome sequences of RacL11 and Kentucky D have yet to be reported.

Studies using RacL11 and Kentucky D strains have suggested that there are several virulence- and pathogenicity-related genes for EHV-1 as determined by infections in small rodents as model organisms; these genes include IR6, glycoprotein (g) B, gD, gM, gp2, and [3, 6, 10, 11, 13–15, 17, 21, 23, 24]. Hepatitis was used in hamsters for evaluation of the pathogenicity and virulence of RacL11 and Kentucky D. We previously reported that Ab4p and Japanese field isolates caused encephalitis but not hepatitis, in experimentally inoculated hamsters [16]. Thus, there is a discrepancy in the pathogenicity of EHV-1 in hamsters. Kentucky D was passaged over 300 times through hamsters and in tissue culture [21]. The RacL11 clone of a Rac isolate was virulent beginning at passage 12 in porcine embryonic kidney cells [8, 14]. Both strains were isolated from aborted horse fetuses using newborn hamsters [4, 8].

Determinants of pathogenicity and virulence of EHV-1 include glycoproteins B, D, E, and M as described above. Recently, ORF51 (UL11) of EHV-1 has also been indicated to be involved in cell-to-cell spread of the virus [19]. ORF51 is a tegument gene of the EHV-1 virion. Furthermore, a tegument gene, ORF10 of the Varicella-zoster virus, has been suggested to be a virulence determinant [2]. Thus, it is possible that teguments can be determinants of the virulence and pathogenicity of EHV-1.

Whether the RacL11 and Kentucky D strains have identical sequences in any of these studied genes and whether they are different from those of Ab4p and other Japanese isolates would provide some clues for analysis of the pathogenicity of EHV-1. In the present study, we attempted to identify differences between RacL11, Kentucky D, and other EHV-1 genomic DNAs by restriction site mapping analysis and sequence comparison of several glycoproteins, including gB (UL27), gD (US6), and gE (US8), and tegument genes, including the ORF11 (UL49), ORF46 (UL16), ORF51 (UL11), and ORF76 (US9) genes.

The EHV-1 strains used in this study were RacL11, Kentucky D, Ab4p, and three Japanese field isolates (89c25, 90c16, and 97c5). Ab4p was kindly provided by Dr. A. J. Davison, Glasgow University, UK. RacL11, Kentucky D, and Ab4p were passaged twice in fetal horse kidney cells after arrival at our laboratory, and the strains were propagated as described previously [9, 16].

Total DNA was extracted from infected fetal horse kidney cells by the phenol-chloroform method as described previously [16]. Long and accurate PCR (LA-PCR) was

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performed for the EHV-1 genome as described previously [16]. The LA-PCR products were digested with eight restriction enzymes (SacI, SspI, NaeI, ApaI, EcoRI, HincII, BglII, and StuI). The numbers of restrictions sites examined were 55 for SacI, 25 for SspI, 81 for NaeI, 56 for ApaI, 16 for EcoRI, 149 for HincII, 17 for BglII and 30 for StuI. The digests were electrophoresed on an agarose gel.

The gB, gD, and gE genes were directly sequenced. To sequence the tegument genes, PCR products were cloned with pGEM-T Easy Vector. ORF11, ORF46, ORF51, ORF76, and the intergenic regions of ORF62 and ORF63 were amplified as overlapping PCR fragments. At least 3 clones derived from each amplified PCR product were used for sequencing. The primer sequences for PCR amplification and sequencing are available upon request. Sequence analysis was performed by Dragon Genomics (Mie, Japan). GenetyxMac/ATSQ and GenetyxMac were used for sequence assembly and analysis. The sequences were deposited in the DNA Data Bank of Japan (DDBJ) under accession number BR000259 for ORF46 of the RacL11 and Kentucky D strains (DDBJ applied one accession number representing both strain sequences because the two sequences are identical). The accession numbers for the gB, gD, and gE gene sequences of RacL11 and Kentucky D are AB279606 to AB279611. The EHV-1 genome sequences of Ab4p (NC_001491) and V592 (AY464052) were also used for comparison.

The DNA fingerprints of RacL11 and Kentucky D using BamHI have been reported to vary [1, 7, 13], and Kentucky D is known to be an EHV-1 P electropherotype [1]. We confirmed the lack of the BamHI site of RacL11 and the electropherotype of Kentucky D (data not shown). Then, we compared about 600 restriction sites in the RacL11, Kentucky D, and Ab4p genome. However, we did not find significant differences, although the LA-PCR of fragment 12 was longer in RacL11 than in Kentucky D and Ab4p. The LA-PCR of fragment 12 corresponds to nt 104,320 to 111,287 in the genome sequence of Ab4p and includes a part of ORF62, the intergenic region between ORFs 62–63, and a portion of ORF63. The increased length of fragment 12 of RacL11 was found to be due to the presence of 9 copies of 5'-GCTAGCGCTAACGCTAGG-3' and one copy of 5'-GCTAGCGCTAACGCTAGTG-3', while there were 4 copies in Kentucky D, 3 copies in Ab4p, and 5 copies in V592 (Fig. 1).

We determined the nucleotide sequences of three glycoprotein genes (gB, gD, and gE) of the RacL11 and Kentucky D strains. Although the gB nucleotide sequences and deduced amino acid sequences of Ab4p and V592 were identical, those of the four strains studied (corresponding nt. Sequence 61432 to 64374 in Ab4p) were different (Table 1). The gD nucleotide sequences of the three strains (corresponding nt. sequence 131432 to 132790 in Ab4p) and their deduced amino acid sequences were identical for the N-terminal 428 amino acids but they varied in their C-terminal sequences (Fig. 2). The predicted gD amino acid sequences of RacL11 and Kentucky D were 11 amino acids and 17 amino acids shorter than those of the Ab4p and V592 strains, respectively. Amino acid sequence variations between the strains were also observed from position 429 to

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Ab4p        108927 TTTCACAATTCAATAGAATCATAAATTTAACTTTGGC GCTAGCGCTAACGCTAGG GCTAG
V592        108858 TTTCACAATTCAATAGAATCATAAATTTAACTTTGGC GCTAGCGCTAACGCTAGG GCTAG
Kentucky D     401 TTTCACAATTCAATAGAATCATAAATTTAACTTTGGC GCTAGCGCTAACGCTAGG GCTAG
RacL11         401 TTTCACAATTCAATAGAATCATAAATTTAACTTTGGC GCTAGCGCTAACGCTAGG GCTAG

Ab4p        108987 CGCTAACGCTAGG GCTAGCGCTAACGCTAGG ------------------- ----------
V592        108918 CGCTAACGCTAGG GCTAGCGCTAACGCTAGG GCTAGCGCTAACGCTAG-G GCTAGCAGCT
Kentucky D     461 CGCTAACGCTAGG GCTAGCGCTAACGCTAGG GCTAGCGCTAACGCTAG-G ----------
RacL11         461 CGCTAACGCTAGG GCTAGCGCTAACGCTAGG GCTAGCGCTAACGCTAGTG GCTAGCAGCT

Ab4p                --------- ------------------- ------------------ ---------------
V592                --------- ------------------- ------------------ ---------------
Kentucky D          --------- ------------------- ------------------ ---------------
RacL11         521 AACGCTAGG GCTAGCGCTAACGCTAGG GCTAGCGCTAACGCTAGG GCTAGCGCTAACGCT

Ab4p        109080 --- ------------------- ------------------ GCTAGCAATGAGGCTGGCCAC
V592        108966 AGG ------------------- ------------------ GCTAGCAATGAGGCTGGCCAC
Kentucky D     509 --- ------------------- ------------------ GCTAGCAATGAGGCTGGCCAC
RacL11         581 AGG GCTAGCGCTAACGCTAGG GCTAGCGCTAACGCTAGG GCTAGCAATGAGGCTGGCCAC
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Fig. 1. Intergenic region of ORF62 and ORF63. Dashes (–) indicate deletion of nucleotides. Repeat sequences are indicated in bold. Each repeat unit is separated by a space.

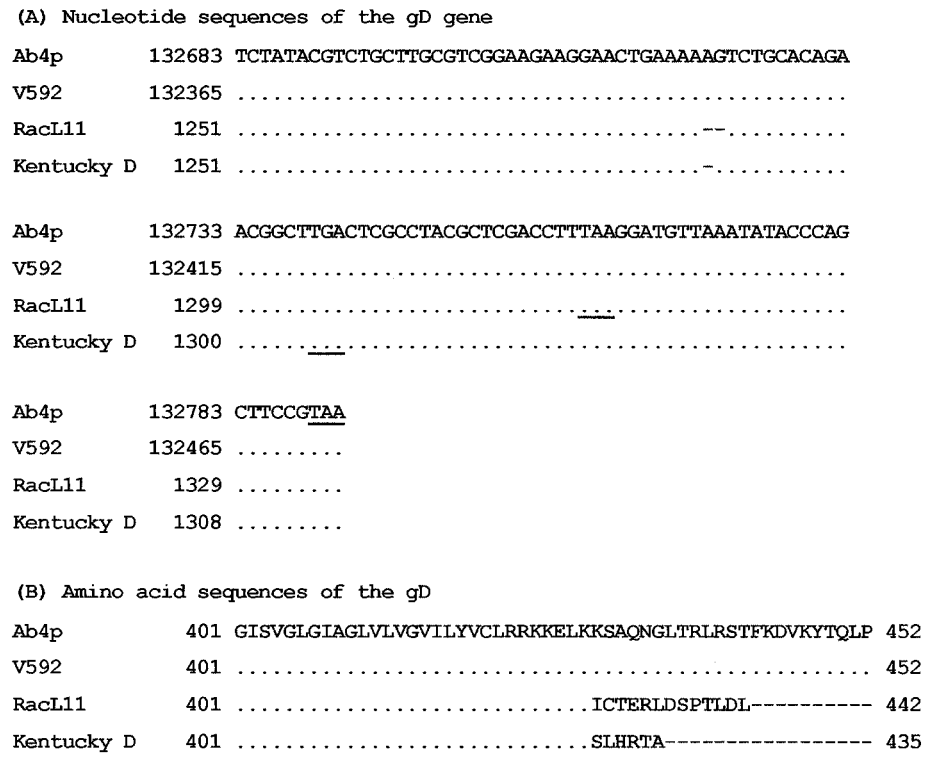

Fig. 2. Comparison of the gD sequences. A portion of multiple alignments of nucleotide sequences (A) and predicted amino acid sequences (B) is shown. The underlined areas in the panel A indicate the stop codons. Lack of bases in the RacL11 and Kentucky D sequences caused the frameshift shown in panel B. Dots (.) indicate an identical base or amino acid residue related to the sequence of Ab4p. Dashes (–) indicate deletion.

the carboxy terminus of gD. However, the gE gene sequences corresponding to nt 34,405 to 136,057 in the Ab4p genome were absolutely identical in RacL11, Kentucky D, Ab4p, and V592.

ORF11 and ORF51, which correspond to nt 12,549 to 13,463 and 92,783 to 93,007 in the Ab4p genome, respectively, were identical among the RacL11, Kentucky D, V592, and Ab4p strains (data not shown). The ORF46 nucleotide sequences of the RacL11 and Kentucky D strains corresponding to nt 86,620 to 87,732 in the Ab4p genome were identical but were different from those of Ab4p on three nucleotides (data not shown). ORF46 of V592 was almost identical to those of RacL11 and Kentucky D, with only one nucleotide difference. Comparison of the predicted amino acid sequences showed that one amino acid was conserved among RacL11, Kentucky D, and V592 at the residual number 140, i.e., serine in the RacL11, Kentucky D, and V592 strains and phenylalanine in Ab4p and Japanese isolates (Table 1). The RacL11 and Kentucky D ORF76 sequences corresponding to 136,782 to 137,411 in the Ab4p genome harbored differences in three nucleotides that cause one amino acid difference between the two strains examined (i.e., position 210 is valine in RacL11 and alanine in Kentucky D). The other four amino acid differences were found among the strains examined (Table 1).

The RacL11 and Kentucky D strains have been reported to cause hepatitis in a hamster model [17, 21]. We attempted to identify differences in genome DNA between the virulent RacL11 and Kentucky D strains and the Ab4p and V592 strains of EHV-1. At first, we compared the restriction site maps of RacL11, Kentucky D, and Ab4p. However, the restriction maps with the 8 restriction enzymes used in the present study did not show any genetic differences that could explain the higher pathogenicity of the RacL11 and Kentucky D strains. The only difference that we found was a difference in the copy number of repeat sequences in the intergenic region of ORF62 and ORF63. Thus, drastic changes, such as deletion, inversion, and insertion of certain size sequences, might not occur in the genome DNAs of virus as a result of passaging in hamsters.

Then, we focused our attention on glycoproteins, including gB, gD, and gE. All of these glycoproteins have been suggested to play some roles in the pathogenicity of EHV-1 [5, 10, 15, 17, 21, 23, 24]. This led us to believe that some specific sequence differences may be related to RacL11 and Kentucky D causing hepatitis. However, we only found highly conserved or identical sequences among all the strains examined. Although our results did not identify a

Table 1. Amino acid differences among the RacL11, Kentucky D, and Ab4p gB, ORF46, and ORF76 sequences

	gB						ORF46								ORF76				
	15	268	734	922	955	976	16	124	140	254	263	271	346	359	29	128	150	210	219
Ab4p	N	A	V	M	K	D	S	F	F	T	P	Q	G	S	E	F	Q	V	P
V592	N	A	V	M	K	D	S	F	S	T	P	Q	G	S	E	S	Q	V	P
RacL11	H	V	A	M	K	D	S	F	S	T	P	Q	G	S	E	S	Q	V	R
Kentucky D	N	A	V	R	Q	N	S	F	S	T	P	R	G	T	E	S	Q	A	R
89c25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	P	F	F	A	P	Q	G	S	E	S	Q	V	R
90c16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	S	S	F	T	T	Q	G	S	G	S	Q	V	R
97c5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	S	F	F	T	P	Q	R	S	E	F	K	V	R

The numbers under the gene names are the positions of the amino acids in the deduced sequences.

gene in RacL11 and Kentucky D that is responsible for causing hepatitis, they did suggest that it is not gE. Even if gB and gD are responsible for causing hepatitis, the mechanism through which the RacL11 and Kentucky D strains cause hepatitis in hamsters is likely different.

The gD of RacL11 and Kentucky D has been suggested to be shorter than that of Ab4p and V592. The predicted amino acid sequence of the gD of RacL11 is identical to that of Kentucky A [5]. We could not confirm size differences among the strains in the present study because we did not possess the required identification tools, such as gD specific antiserum for immunological detection. Ordinary SDS-polyacrylamide gel electrophoresis was not helpful in finding the differences in gD using whole virion preparations. Future work is required to confirm the size differences and virological effects of gD in each strain.

Other researchers have proposed that a tegument protein might be involved in the pathogenicity of herpesvirus [2]. However, most of the tegument gene sequences in the present study were also conserved among the strains examined, although the ORF46 sequences of RacL11 and Kentucky D were the same and were different from those of Ab4p. Interestingly, ORF 46 amino acid sequence of the EHV-1 V592 strain was identical to those of the RacL11 and Kentucky D strains, although we were unable to find any reports on the pathogenicity of the EHV-1 V592 strain in hamsters. Further experiments are required to reveal the involvement of ORF46 in the pathogenicity of EHV-1. Sequencing of the entire genome and comprehensive proteomics of the RacL11 and Kentucky D strains would probably be helpful in resolving this problem.

ACKNOWLEDGMENTS. This study was supported in part by Grants-in-Aid for Scientific Research (12660283 and 14560264 to H. F.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by the Japan Racing Association.

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