Discrimination of Herpes Simplex Virus Type 2 Strains by Nucleotide Sequence Variations[▽]

Hisatoshi Kaneko,¹ Takashi Kawana,² Ken Ishioka,¹ Eiko Fukushima,¹ and Tatsuo Suzutani^{1*}

Department of Microbiology, Fukushima Medical University School of Medicine, Fukushima 960-1295,¹ and Department of Obstetrics and Gynecology, University Hospital, Mizonouchi, Teikyo University School of Medicine, Kawasaki 213-8507,² Japan

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We determined the polymorphous 400-bp regions in UL53, US1, and US4 for the discrimination of herpes simplex virus type 2 (HSV-2) strains. Thirty-six HSV-2 clinical strains could be differentiated into 35 groups using these three regions and into 36 groups by additional analysis of three noncoding regions previously reported as polymorphous.

Herpes simplex virus type 2 (HSV-2) often causes genital herpes and, occasionally, meningitis, neonatal infections, and acute retinal necrosis. The study of the relationships between these diseases and virus strains, and the analysis of transmission between individuals, requires accurate and reproducible typing and phylogenetic analyses of clinical strains (4, 9). To achieve this, molecular technologies, in particular restriction fragment length polymorphism (RFLP) analysis, have been widely employed (12, 13, 14). However, the RFLP assay is relatively troublesome, and comparison of results obtained in different laboratories is difficult.

DNA sequencing is much easier to use and is more sensitive

than the RFLP assay in the detection of minor variations between strains. Using this technique, nucleotide sequences are subjected to comparative analysis, and evolutionary relationships between strains are validated. Moreover, the advantage of sequencing is that the results are easily stored and shared electronically; therefore, they can be utilized in laboratories worldwide.

Among human alphaherpesviruses, some genes in both HSV-1 and varicella-zoster virus have already been identified as possessing many nucleotide polymorphisms (1, 2, 6, 8). Recently, polymorphic regions in the noncoding region in HSV-2 were also reported (7), but none have been identified in the

FIG. 1. Nucleotide diversity of each HSV-2 gene. The diversities of 19 UL and 4 US genes were analyzed using sequence data registered in GenBank.

* Corresponding author. Mailing address: Department of Microbiology, Fukushima Medical University School of Medicine, 1 Hikari-gaoka, Fukushima 960-1295, Japan. Phone: 81-24-547-1158. Fax: 81-24-548-5072. E-mail: suzutani@fmu.ac.jp.

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TABLE 1. Positions of and variations in polymorphic sites in 400-bp regions from the UL53, US1, and US4 genes and three noncoding regions, NC1, NC3, and NC4, from 36 fsHSV-2 clinical strains and strain HG52

Gene or region	Position ^a	Variation(s) (no. of strains)
UL53	113584	C (36), T (1)
	113593	A (36), G (1)
	113611	C (33), T (4)
	113713	C (33), G (4)
	113720	A (35), G (2)
	113765	T (31), C (6)
	113805	C (36), G (1)
	113834	A (33), G (4)
	113847	A (25), G (12)
	113864	G (36), A (1)
	113922	G (36), T (1)
	113926	T (36), C (1)
	113944	T (35), G (2)
	113974–113975	TA (25), CG (11), TA (1)
US1	133785	C (35), T (2)
	133807	A (30), G (7)
	133817	T (36), G (1)
	133882	A (33), C (4)
	133892–133900	TCCTCGACC (32), CCCTCGACC (3), ACC (1), deletion (1)
	133910	G (36), A (1)
	133932	G (36), C (1)
	133955	C (32), G (4), T (1)
	134041–134043	CGA (33), TGA (3), deletion (1)
	134049–134054	ATGATG (29), ATGATGATG (3), ATG (2), deletion (2), ATCATGATG (1)
	134082	C (34), A (3)
	134089	T (33), C (4)
	134102	C (36), T (1)
	134117	C (31), G (6)
	134124	C (36), A(1)
US4	138749	A (31), G (6)
	138755–138757	TCG (36), deletion (1)
	138768	G (36), A (1)
	138783	C (36), G (1)
	138807	T (29), C (8)
	138809	C (36), T (1)
	138890	G (35), T (2)
	138911	C (36), T (1)
	138932	C (36), T (1)
	138966	C (35), G (2)
	139011	C (35), T (2)
	139031	C (36), T (1)
	139060	A (34), G (3)
	139092	G (35), C (2)
	139100	C (36), T (1)
	139107	C (36), T (1)
	139145	T (32), C (5)
NC1	40609	G (36), A (1)
	40681	A (35), G (2)
	40684–40687	5A (27), 4A(10)
	40699	A (36), C (1)
	40708	A (36), G (1)
	40735–40737	3A (36), 2A (1)
	40811	T (35), C (2)
	40865	C (36), T (1)
	40866–40871	6C (29), 5C (7), 7C (1)
NC3	48802	C (29), T (8)
	48803–48811	8C (10), 9C (9), 7C (7), 6C (4), 10C (4), 4C (1), 5C (1), 11C (1)
	48812	T (36), C (1)
	48918	C (34), A (3)
	48956	T (34), C (3)
NC4	48957–48963	7C (19), 6C (13), 5C (4), 8C (1)
	84796–84803	4GT (12), 5GT (10), 6GT (9), 8GT (4), 3GT (1), 7GT (1)
	84808	G (33), A (4)
	84809–84812	4G (14), 7G (10), 5G (8), 4G (4), 9G (3), 3G (2)
	84813	C (36), G (1)
	84861	C (27), T (10)

^a Positions of polymorphic sites on HSV-2 strains. The numbering of positions corresponds to that of the HSV-2 strain HG52 total sequence (4).

TABLE 2. Primers and conditions used in PCR-directed sequencing

Gene	Primer	Size of PCR product (bp)	Position ^a	Sequence	PCR conditions
UL53	HSV2-UL53(F)	1,256	112920–112940	GTCGGGACCAACAACCGCCTA	95°C/3 min, (95°C/30 s, 55°C/60 s, 72°C/90 s) × 40, 72°C/10 min
	HSV2-UL53(R)		114135–114117	CGACGTGCGAGGGTGCGTA	
US1	HSV2-US1(F)	1,481	133649–133667	CGATCCCAACATCCGCGCT	95°C/3 min, (95°C/30 s, 55°C/60 s, 72°C/120 s) × 40, 72°C/10 min
	HSV2-US1(R)		135129–135106	CATTACACGTACGAGCGGTGTGCGC	
US4	HSV2-US4c(F)	747	138694–138714	AGCCTGCTGGTGGGGATTACG	95°C/3 min, (95°C/30 s, 55°C/60 s, 72°C/60 s) × 40, 72°C/10 min
	HSV2-US4c(R)		139440–139417	CGTGGCGGTGGTTCGCGCGACCGA	

^a Position of the primer sequence on the DNA sequence of the HSV-2 genome (4).

open reading frames (ORFs). In this study, we identified 400-bp regions with many nucleotide polymorphisms in the HSV-2 genes, the nucleotide sequences of which can be determined with one sequencing reaction.

We used 36 HSV-2 clinical isolates (referred to here as strains 1 to 36) from 36 epidemiologically unrelated Japanese patients with genital herpes infections for more than 20 years. All strains were isolated in Vero cells and stored after a few passages. Virus DNA was extracted from the infected cells or clinical samples by proteinase K treatment and phenol-chloroform extraction.

First, we checked all HSV-2 nucleotide sequence data registered in the GenBank database, and sequence alignments were constructed using Web-based Clustal W alignment programs. Sequence information from partial regions in the ORFs was also analyzed, but data from regions shorter than 400 bp were excluded from the analyses.

Nucleotide sequences of 28 unique long (UL) genes and 8 unique short (US) genes from more than two strains of HSV-2 were registered in the GenBank database, and the nucleotide diversity of these 36 genes was evaluated. No polymorphism was observed in 13 genes (UL4, UL5, UL22, UL42, UL43, UL45, UL54, UL55, UL56, US2, US3, US5, and US8), which were therefore excluded as candidate genes. Of the remaining 23 genes, we then examined the 8 genes that showed more than 0.5% nucleotide diversity (Fig. 1) and the 400-bp regions with large numbers of polymorphic sites in 6 of the 8 genes, excluding UL28 and UL38. To evaluate the frequency of polymorphic sites in the candidate regions among the clinical isolates, we sequenced the target regions of five HSV-2 clinical strains by a PCR-directed sequencing method, as described previously (5). The homology of the regions from UL1 and UL39 was 100% for all five strains, and just one nucleotide substitution in one strain was identified in the region from UL2. On the other hand, the numbers of polymorphic sites in the 400-bp regions from the UL53, US1, and US4 genes were 8, 3, and 4, respectively. We regarded these 400-bp regions as highly polymorphous regions and carried out sequence analysis of these regions for the other 31 strains (Table 1). The primers and conditions used in PCR for the three regions are summarized in Table 2.

As a result, polymorphisms at 14, 15, and 17 sites among the 36 strains studied were observed in the regions from UL53, US1, and US4, respectively (Table 1). On the basis of the polymorphisms in the three regions, the 36 strains were classified into 35 groups (Table 3). In contrast, six isolates obtained from different recurrent episodes over 20 years for one genital

herpes patient were found to be identical strains (data not shown).

Phylogenetic analyses of the 36 strains and strain HG52 (3) for each of the three regions were carried out using the neighbor-joining method and visualized by MEGA, version 3.1. The shapes of the phylogenetic trees differed from each other; the strains that formed a genetically related cluster in the analysis of one region were dispersed in the phylogenetic trees obtained from the analyses of the other regions. These results indicated that the three target regions are not genetically linked (Fig. 2A, B, and C).

To confirm the accuracy and reliability of our three test regions, we analyzed the sequences of three noncoding regions, noncoding region 1 (NC1), NC3, and NC4, located between the UL19 and UL20, the UL24 and UL25, and the UL37 and UL38 genes, respectively, and reported as target regions for the discrimination of HSV-2 strains (7). The 36 HSV-2 strains were classified into 34 groups by using these three noncoding regions. Moreover, we could differentiate and classify all 36 Japanese strains through additional analysis of the three noncoding regions with more than two different sites between two closely related strains (Table 3).

Furthermore, we analyzed our 36 HSV-2 strains in comparison with 26 American strains registered in GenBank (7) and strain HG52 (3). Phylogenetic analysis of the NC1 (data not shown), NC3 (Fig. 2D), and NC4 (data not shown) sequences found that no specific clusters formed among the 36 Japanese or the 26 American strains. This observation was different from

TABLE 3. Classification of 36 HSV-2 clinical strains with sequence variations in 400-bp regions from the UL53, US1, and US4 genes and three noncoding regions

Region	No. of groups classified	No. of polymorphic sites ^a
UL53	16	0–8
US1	20	0–8
US4	19	0–7
UL53 + US1 + US4	35	0–14
NC1	11	0–5
NC3	21	0–5
NC4	17	0–4
NC1 + NC3 + NC4	34	0–11
Combination of all six regions	36	2–18

^a For every 2 strains among the 36 HSV-2 strains.

FIG. 2. Phylogenetic analyses of 400-bp regions in the UL53 (A), US1 (B), and US4 (C) genes and in NC3 (D). Sequence data from all 36 HSV-2 clinical strains (shown as strains 1 to 36) and strain HG52 (3) were analyzed by the neighbor-joining method. The names of strains with common sequences in the 400-bp regions from UL53 and US4 are shown in red and underlined, respectively. The 26 American strains registered in GenBank (7) were added for the phylogenetic analysis of NC3. These American strains are shown in blue.

those for other human alphaherpesviruses, HSV-1 and varicella-zoster virus, which formed regional genotypes (1, 6, 10, 11). Moreover, our observations described in this report represent common characteristics among HSV-2 strains and provide a method that is applicable worldwide.

In conclusion, the identification and discrimination of HSV-2 strains by three ORF regions were both accurate and

reliable. These results suggest that our method might also have sufficient sensitivity for application to etiological studies worldwide.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been registered in the DDBJ database under accession no. AB290460 to AB290514.

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