The Herpes Simplex Virus Type 1 BgK_L Variant, Unlike the BgO_L Variant, Shows a Higher Association with Orolabial Infection than with Infections at Other Sites, Supporting the Variant-Dispersion-Replacement Hypothesis[▽]

Shigeru Ozawa,^{1,2} Hiroyuki Eda,^{1,3} Yasuyuki Ishii,¹ Fumihiko Ban,¹ Toshiyuki Funabashi,⁴ Seiichiro Hata,⁵ Kozaburo Hayashi,⁶ Hiroki Iga,⁷ Takao Ikushima,⁸ Hiroaki Ishiko,¹ Tomoo Itagaki,⁹ Rinji Kawana,¹⁰ Shunsaku Kobayashi,¹¹ Takeo Ogino,¹² Tsuyoshi Sekizawa,¹³ Yoshikazu Shimomura,¹⁴ Hiroshi Shiota,¹⁵ Ryoichi Mori,¹⁶ Takashi Nakakita,¹⁷ Yoshio Numazaki,¹⁸ Yoshikatsu Ozaki,¹⁹ Shigeru Yamamoto,²⁰ Kamesaburo Yoshino,^{2,21}† and Kazuo Yanagi^{1,22}*

Herpesvirus Laboratory, Department of Virology I, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan¹; Yamanashi Institute of Health, Kofu City, Yamanashi Prefecture, Japan²; Tsukuba University, The Program of Environmental Sciences, Tsukuba City, Ibaraki 305-8572, Japan³; Toranomon Hospital, Minato-ku, Tokyo 105-8470, Japan⁴; Osaka University Medical School, Suita City, Osaka Prefecture 565-0871, Japan⁵; Kobe Institute of Health, Kobe City, Hyogo Prefecture 650-0046, Japan⁶; University of Tokushima School of Dentistry, Tokushima City, Tokushima Prefecture 770-8503, Japan⁷; Shizuoka Institute of Environment and Hygiene, Shizuoka, Japan⁸; Shimane Prefectural Institute of Public Health and Environment Science, Matsue City, Shimane Prefecture 690-0122, Japan⁹; Iwate Medical University School of Medicine, Morioka City, Iwate Prefecture 020-8505, Japan¹⁰; Yamaguchi University School of Medicine, Ube City, Yamaguchi Prefecture 755-8505, Japan¹¹; Hiroshima City Institute of Public Health, Hiroshima City, Hiroshima Prefecture 733-8650, Japan¹²; Tohoku University School of Medicine, Sendai City, Miyagi Prefecture 980-8574, Japan¹³; Osaka University School of Medicine, Suita City, Osaka Prefecture 565-0871, Japan¹⁴; University of Tokushima School of Medicine, Tokushima City, Tokushima Prefecture 770-8503, Japan¹⁵; Kyushu University Faculty of Medicine, Fukuoka City, Fukuoka Prefecture 819-0395, Japan¹⁶; Nagoya City Public Health Research Institute, Nagoya City, Aichi Prefecture 467-8615, Japan¹⁷; Sendai National Hospital, Sendai City, Miyagi Prefecture 983-8520, Japan¹⁸; Shiga University of Medical Science, Otsu City, Shiga Prefecture 520-2192, Japan¹⁹; Kurume University School of Medicine, Kurume City, Fukuoka Prefecture 830-0011, Japan²⁰; Institute of Medical Sciences, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan²¹; and Institute of Basic Medical Sciences, Tsukuba University, Tsukuba City, Ibaraki 305-8572, Japan²²

Received 11 December 2006/Returned for modification 18 February 2007/Accepted 21 April 2007

The identification and geographic distribution of the herpes simplex virus type 1 (HSV-1) BgIII restriction fragment length polymorphism (RFLP) variants named BgK_L and BgO_L in clinical isolates from orolabial and cutaneous sites were described in our previous reports, in which the dispersion and replacement of HSV-1 variants were proposed. The base substitution sites deduced from the BgK_L multiple RFLP variations were mapped to the U_L12 (DNase), R_L2 (α0 transactivator), and latency-associated transcript genes in the present study. The results show that the relative frequencies (RFs) of BgK_L are significantly higher in orolabial and cutaneous HSV-1 infections than in ocular infections. For the BgO_L variant, the opposite was found; i.e., the RF of BgO_L was significantly lower in orolabial and cutaneous infections than in ocular infections. No significant differences in the RFs of non-BgK_L:non-BgO_L isolates were observed. The ratio of the BgK_L RF to the BgO_L RF was much higher for the orolabial and cutaneous infection groups than for the ocular infection group, whereas the BgK_L RF-to-non-BgK_L:non-BgO_L RF ratios for the former groups were slightly higher than those for the latter group. The higher efficiency of orolabial and cutaneous infections caused by BgK_L compared to the efficiency of infections caused by BgO_L allows BgK_L to spread more efficiently in human populations and to displace BgO_L, because the mouth and lips are the most common HSV-1 infection sites in children. The present study supports our HSV-1 dispersion-and-replacement hypothesis and suggests that HSV-1, the latency-reactivation of which allows variants to accumulate in human populations, has evolved under competitive conditions, providing a new perspective on the polymorphism or variation of HSV-1.

* Corresponding author. Present address: AIDS Research Center, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81-3-5285-1111. Fax: 81-3-5285-1150. E-mail: kyanagi@nih.go.jp.

† Deceased.

[▽] Published ahead of print on 2 May 2007.

FIG. 1. Geographic regions and areas from which the HSV-1 clinical isolates were collected.

Herpes simplex virus (HSV) type 1 (HSV-1) primary infection occurs at mucosal or cutaneous surfaces, the portals for virus entry (35, 39). In the course of primary infection, HSV-1 establishes latency in the ganglia and occasionally reactivates thereafter, causing recurrent infections at the same site as the primary infection (35, 39). The lifelong latency-reactivation mode of HSV-1 infection may have led to the accumulation of HSV-1 variants in human populations. HSV-1 is transmitted by close contact between individuals (39). These properties of HSV-1 infection make the association of HSV-1 variants with host populations stable (21, 35). HSV-1 is ubiquitous throughout the world (39) and is highly prevalent in Japan (13, 44). HSV-1 restriction fragment length polymorphism (RFLP) is widely used to differentiate various HSV-1 clinical isolates in epidemiologic analyses (11, 18, 32, 33). Recent phylogenetic analyses of clinical HSV-1 isolates have identified “genetic groups” or “genotypic groups” and recombinant viruses (20).

The HSV-1 variant BgK_L comprised 27.1% of clinical isolates from patients with orolabial and cutaneous infections in Japan (21, 22). Almost all BgK_L variant isolates possess the three different SalI RFLP variations designated SaCFJ_M, SaE_L, and SaGH_M, as well as the KpnI RFLP variation KpM_S (8, 21). Thus, the BglIII K_L variation is a surrogate RFLP marker for the five highly associated mutations (21). The geographic distribution of BgK_L forms a gradient, suggesting that it dispersed from Shikoku Island to the surrounding regions of Japan (21). Furthermore, the contrasting geographic distribution of BgK_L and BgO_L variants suggests that the former may have displaced the latter (8).

In the present study, we sought genes that may have been affected by the base substitutions deduced from the BgK_L and BgO_L RFLP differences by computer-aided analyses. We took this approach because the very large size of the HSV-1 genome, which contains at least 77 genes (GenBank) (31), makes experimental analyses challenging. To investigate whether any altered pathogenic properties of BgK_L compared to those of

BgO_L and the rest of the isolates, referred to as non-BgK_L: non-BgO_L (8), were observed in different lesion sites, we further analyzed the RFLPs of clinical isolates from patients with ocular or genital HSV-1 infections. We then compared the frequency of each variant, referred to here as the variant relative frequency (RF), in the different lesion site groups of HSV-1 isolates. The significance of these results in terms of the dispersion and differentiation of HSV-1 is discussed.

MATERIALS AND METHODS

Viruses and cells. HSV-1 clinical isolates were obtained from 104 patients with HSV-1 infections of the eyes (herpes simplex keratitis and/or conjunctivitis) and from 40 patients with genital infections (genital HSV). The clinical specimens, the cell inoculation procedures, and the identification and typing of the isolates have been described previously (21). All clinical isolates were obtained before 1986, as were the HSV-1 isolates, which were mostly from the patients with orolabial HSV or skin infections described in our previous report. The geographic distribution of the 104 HSV-1 isolates from the eyes (keratitis) in the different regions are represented by letters on the map in Fig. 1, as follows (1): 82 from regions A (Shikoku Island), B (the Chugoku region), and C (the Kinki region); 11 from region D (Kyushu Island); and 11 from regions E (the Chubu region) and F (the Tohoku region) (Fig. 1). The distribution of the 40 HSV-1 isolates from genital infections was 8 from regions A, B, and C; 19 from region D; and 13 from regions E and F (Fig. 1). In this report, Shikoku Island comprises the Tokushima and Kagawa Prefectures; the Chugoku region comprises the Hiroshima, Shimane, and Yamaguchi Prefectures, the Kinki region comprises the Osaka and Shiga Prefectures, the Kyushu region comprises the Fukuoka and Miyazaki Prefectures; the Chubu region comprises the Yamanashi, Shizuoka, and Aichi Prefectures; and the Tohoku region comprises the Fukushima, Miyagi, Akita, and Iwate Prefectures (Fig. 1). The geographic distribution of the isolates from patients with HSV-1 infections of the mouth/lips (orolabial) or skin/eyelids (cutaneous) was described previously (21), and the numbers of isolates from these sites are indicated separately in Table 1. The standard laboratory HSV-1 strain F was kindly provided by B. Roizman (University of Chicago). The laboratory strain HF was described previously (43–45). HSV-1 from virus stocks that had been prepared shortly after isolation were propagated on Vero cells (36, 41, 42, 45) in Eagle’s minimal essential medium supplemented with 5% calf serum in a humidified incubator containing 5% CO₂.

Viral DNAs and restriction endonuclease cleavage analyses. DNA extraction and analyses of the restriction endonuclease cleavage patterns or the restriction enzyme fragment length polymorphisms of HSV-1 clinical isolates were per-

TABLE 1. RFs of BgK_L, BgO_L, and non-BgO_L:non-BgK_L among HSV-1 clinical isolates from different anatomical lesion sites and geographic areas with different BgK_L and BgO_L RFs

Geographic area(s) ^a	HSV-1 clinical isolate	No. (RF) of BgK _L , BgO _L , and non-BgO _L :non-BgK _L isolates ^b					No. (%) of isolates		Total no. (%) of isolates
		Mouth/lips	Skin/eyelids		Eyes	Genitals	Mouth/lips/skin/eyelids	Eyes/genitals	
			Skin	Eyelids					
A-B-(c) (high BgK _L RF)	BgK _L	55 (47.8)	21 (60.0)	8 (40.0)	24 (29.6)	0 (0.0)	84 (49.4)	14 (34.1)	108 (42.8)
	BgO _L	0 (0.0)	0 (0.0)	0 (0.0)	6 (7.4)	0 (0.0)	0 (0.0)	4 (9.8)	6 (2.4)
	Non-BgO _L :non-BgK _L	60 (52.2)	14 (40.0)	12 (60.0)	51 (63.0)	1 (100)	86 (50.6)	23 (56.1)	138 (54.8)
	Subtotal	115 (100)	35 (100)	20 (100)	81 (100)	1 (100)	170 (100)	41 (100)	252 (100)
A-B-C (high BgO _L RF)	BgK _L	58 (46.0)	22 (46.8)	8 (40.0)	24 (29.3)	0 (0.0)	88 (45.6)	32 (29.1)	112 (39.6)
	BgO _L	1 (0.8)	0	0	6 (7.3)	0 (0.0)	1 (0.5)	6 (5.5)	7 (2.5)
	Non-BgO _L :non-BgK _L	67 (53.2)	25 (53.2)	12 (60.0)	52 (63.4)	8 (100)	104 (53.9)	72 (65.4)	164 (57.9)
	Subtotal	126 (100)	47 (100)	20 (100)	82 (100)	8 (100)	193 (100)	110 (100)	283 (100)
D (low BgK _L RF)	BgK _L	14 (30.4)	17 (33.3)	1 (25)	1 (9.1)	3 (15.8)	32 (31.7)	5 (14.7)	36 (27.5)
	BgO _L	3 (6.5)	3 (5.9)	0	1 (9.1)	0 (0.0)	6 (5.9)	1 (2.9)	7 (5.3)
	Non-BgO _L :non-BgK _L	29	31 (60.8)	3 (75)	9 (81.8)	16 (84.2)	63 (62.4)	28 (82.4)	88 (67.2)
	Subtotal	46	51	4	11	19	101	34	131
E-F (lowest BgK _L RF)	BgK _L	17 (10.7)	4 (8.7)	0 (0.0)	0 (0.0)	0 (0.0)	21 (10.2)	0 (0.0)	21 (9.1)
	BgO _L	11 (6.9)	3 (6.5)	0 (0.0)	2 (18.2)	1 (7.7)	14 (6.8)	3 (12.0)	17 (7.4)
	Non-BgO _L :non-BgK _L	131 (82.4)	39 (84.8)	1 (100)	9 (81.8)	12 (92.3)	171 (83.0)	22 (88.0)	192 (83.5)
	Subtotal	159	46	1	11	13	206	25	230

^a Geographical locations are shown in Fig. 1. A-B-(c), Shikoku-Chugoku-Osaka area (21); A-B-C, Shikoku-Chugoku-Kinki area (8); D, Kyushu area (8, 21); E-F, Chubu-Tohoku area (8, 21).

^b RFs (in parentheses) are percentages of the indicated HSV-1 variants among the HSV-1 clinical isolates, and the numbers of isolates indicate the numbers of HSV-1 clinical isolates obtained in the indicated anatomical lesion sites and geographic areas.

formed as described previously (21). Computer-aided searches for HSV-1 genes possibly affected in the BgK_L and BgO_L variants were performed by using the databases and tools of the National Center for Biotechnology Information (NCBI; HSV-1 strain 17, NCBI accession no. NC_001806, GenBank accession no. X14112) and REBSites (REBASE) (29).

Statistical methods. Fisher's exact test and Yates' correction test (21) were carried out by using Stat Flex software (version 5.0; Japan), as indicated, to compare the RFs of BgK_L, BgO_L, and non-BgK_L:non-BgO_L between the different lesion site groups of the HSV-1 clinical isolates in each of the previously defined geographic areas (8, 21), with the high, low, and lower RFs of BgK_L or BgO_L being categorized. The level of significance was a *P* value of <0.05 (two tailed).

RESULTS

Computer-aided search for HSV-1 genes possibly affected in the BgK_L and BgO_L variants. The loss of the BglII cleavage site between the BglII K (genomic residues 14588 to 25147, 10,560 bp, strain 17) and Q/#13 (genomic residues 25148 to 25378, 231 bp, strain 17) fragments (8, 21) indicates a mutation at or adjacent to residues 25147 to 25148 in the coding region of the U_L12 gene (genomic residues 25007 to 26887, strain 17, DNase/alkaline nuclease) (26, 31), as summarized in Table 2. The loss of the SalI cleavage sites between the SalI C and J fragments and between the SalI F and J fragments, resulting in SaCFJ_M (8, 21), indicates a mutation in the repeated sequence "b" and its inverted sequence "b'" (30) (at or adjacent to genomic residues 5462 to 5463 and 12904 to 120905, respectively, in strain 17 DNA) (Table 2). This mutation may affect the gene for R_L2 (residues 2261 to 5489 and residues 120882 to 124110, strain 17, α0 or ICP0 protein) and latency-associated

transcript (LAT) genes (residues 118802 to 127143) (NCBI gene identification 2828259) (9, 31, 34, 46).

The SaE_L variant with the unusually large SalI E fragment (8, 21) implies a mutation(s) in or at the terminus/termini of its genomic DNA coordinate at residues 107380 to 14517 (7,138 bp) that overlaps or contains the genes for U_L50, U_L54, U_L51, U_L52, and U_L53 (NCBI accession no. NC_001806, GenBank accession no. X14112) (Table 2). Similarly, the SaGH_M variations indicating the loss of both SalI G and H fragments may cause mutations in the 10 genes shown in Table 2 (NCBI accession no. NC_001806, GenBank accession no. X14112).

The loss of the BglII cleavage site between the BglII #13(Q) (residues 25148 to 25378) and O (residues 25379 to 30671) fragments in BgO_L (8, 21) causes a mutation at genomic bases 25378 to 25379, which are also in the coding region of the U_L12 gene, the same gene that is affected by the loss of the BglII cleavage site between the BglII K and Q/#13 fragments (Table 2).

The RFs of BgK_L are higher in orolabial and cutaneous HSV-1 isolates than in ocular isolates in geographic areas with high RFs of BgK_L. We examined the BglII RFLP patterns of clinical isolates from ocular (39, 40) or genital (39) HSV-1 infections compared with those of isolates from orolabial or cutaneous infections. In area A-B-(c) [where (c) represents Osaka] (Fig. 1), where BgK_L is highly prevalent (21), the differences in the BgK_L RFs between the orolabial, cutaneous (skin), and ocular groups were statistically significant (*P* < 0.005 by chi-square test by use of a three [lesion sites]-by-two [BgK_L and non-BgK_L] contingency table) (Table 1). The RFs

TABLE 2. HSV-1 genes possibly affected by RFLP variations in BgK_L and BgO_L variants

RFLP variation and characteristics	Genomic residues at or around which the base substitution has occurred	Gene(s) containing the genomic residues	Open reading frame (CDS ^a) or transcript of gene	Gene function
BgK _L Loss of the Bgl II cleavage site between the Bgl II K and Q/#13 fragments	25147–25148	U _L 12	CDS 25007–26887	DNase
SaCFJ _M SaCJ _M Loss of the SalI cleavage sites between the SalI C and J fragments	5462–5463 (in the terminal b sequence)	R _L 2	CDS 2261–2317, 3083–3749, and 3886–5489 (joined)	α0 (also called ICP 0, IE110) (a transcriptional regulator, proteasome-dependent degradation of several cellular proteins, reactivation from latency)
SaCJ _M		LATs	Transcripts	Down-regulation of viral gene expression and prevention of apoptosis
SaFJ _M Loss of the SalI cleavage sites between the SalI fragments F and J	120904–120905 (in the internal “b” sequence)	RL2	CDS 120882–122485, 122622–123288, and 124054–124110 (joined)	
SaFJ _M		LATs	Transcripts (118802–127143)	
SaE _L The unusually large SalI E fragment	In 107380–14517 (7138 bp)	U _L 50	107010–108125	Deoxyuridine triphosphatase
		U _L 54	113596–115282	α27 (also called ICP 27) (an immediate-early transcriptional regulator)
		U _L 51 U _L 52 U _L 53	108276–109011 109048–112224 112179–113195	Virion glycoprotein K
SaGH _M SaG _M Lack of the SalI G fragments	In 136309–143028 (6720)	U _S 3	135189–137531	Tegument protein (protein phosphorylation, apoptosis, nuclear egress)
		U _S 4	136702–137531	Glycoprotein G (cell-to-cell spread)
		U _S 5	137618–138452	Glycoprotein J (function unknown)
		U _S 6	138333–142048	Glycoprotein D (cell attachment)
		U _S 7	139785–140957	Glycoprotein I (cell-to-cell spread)
		U _S 8	141243–142895	Glycoprotein E (cell-to-cell spread)
		U _S 8A	142744–143223	Nucleolus-associated (function unknown)
SaH _M Lack of the SalI H fragments	In 54827–58701	U _L 28	53058–58320 (CDS 55802–58159)	DNA packaging
		U _L 27 U _L 29	53058–56080 (CDS 53080–55794) 58463–62053 (CDS 58463–62053)	Glycoprotein B Single-stranded DNA binding protein
KpM _S Lack of the KpnI M fragment	In 44391–49349 (4959 bp)	U _L 22	43824–46581	Glycoprotein H (cell entry, cell-to-cell spread)
		U _L 23	46672–47802	Thymidine kinase (nucleotide metabolism)
		U _L 24	47737–48546	Nuclear protein (function unknown)
		U _L 25	48813–50555	DNA packaging tegument protein (DNA encapsidation)
BgO _L Loss of the Bgl II cleavage site between the Bgl II Q/13 and O fragments	25378–25379	UL12	CDS 25007–26887	DNase

^a CDS, coding sequence (NCBI, HSV-1 strain 17, NCBI accession no. NC_001806, GenBank accession no. X14112).

TABLE 3. BgK_L-to-BgO_L frequency ratios for orolabial, cutaneous, and ocular lesion groups of HSV-1 clinical isolates

Geographic area(s) ^a	Ratio for lesion sites from which HSV-1 clinical isolates were obtained ^c			
	Mouth/lips	Skin/eyelids	Mouth/lips/skin/eyelids	Eyes
A-B-(c) (high BgK _L RF)	>55 (55:0)	>29 (29:0)	>84 (84:0)	4.0 (24:6)
A-B-C (high BgO _L RF)	58 (58:1)	>30 (30:0)	88 (88:1)	4.0 (24:6)
D (low BgK _L RF)	4.7 (14:3)	6.0 (18:3)	5.3 (32:6)	— ^b
E-F (lowest BgK _L RF)	1.6 (17:11)	1.3 (4:3)	1.5 (21:14)	—

^a A-B-(c), Shikoku-Chugoku-Osaka area (21); A-B-C, Shikoku-Chugoku-Kinki area (8); D, Kyushu area (8, 21); E-F, Chubu-Tohoku area (8, 21).

^b —, not shown because the sample numbers are too low.

^c Values in parentheses represent numbers of BgK_L isolates:numbers of BgO_L isolates.

of BgK_L in the skin ($P < 0.005$), orolabial ($P = 0.016$), and orolabial/cutaneous ($P < 0.005$) groups were significantly higher than those in the ocular group. There was also a significant difference in the RFs of BgK_L between orolabial, cutaneous (skin/eyelids), and ocular infections ($P = 0.010$, chi square, three-by-two contingency table). Finally, the RFs of BgK_L in the cutaneous (skin/eyelids) ($P = 0.011$) and the orolabial/cutaneous (skin/eyelids) ($P < 0.005$) groups were significantly higher than those in the ocular group.

In region D (Fig. 1), where the RF of BgK_L is low (21), the RFs of BgK_L in orolabial and cutaneous (skin) infections were higher than those in ocular and genital infections (Table 1). However, the number of samples from ocular infections was small and the differences in the RFs of BgK_L between the orolabial, cutaneous (skin), ocular, and genital groups did not achieve statistical significance (Fig. 1). In regions E and F, where the RF of BgK_L is lower (21), the RFs of BgK_L in orolabial and cutaneous (skin) infections were higher than those in ocular and genital infections (Table 1). Again, however, the ocular and genital sample numbers; were small and the differences between the RFs in the orolabial, cutaneous (skin), ocular, and genital groups did not reach significance ($P = 0.39$ by chi-square test by the use of a four [lesion sites]-by-two contingency table).

These results indicate that the RFs of BgK_L in orolabial and cutaneous infections are higher than those in ocular infection in geographic areas where the RF of BgK_L is high.

The RF of BgO_L is lower in the orolabial and cutaneous groups than in the ocular group in geographic areas with high RFs of BgK_L. In regions A, B, and C, where the RF of BgO_L is low (8), there were significant differences in the RFs of BgO_L in the orolabial, cutaneous (skin/eyelids), ocular, and genital groups ($P = 0.010$ by chi-square test by use of a four [lesion sites]-by-two [BgO_L and non-BgO] contingency table) (Table 1). The RF of BgO_L in the ocular group was significantly higher than those in the orolabial ($P = 0.016$), cutaneous ($P = 0.026$), and combined orolabial/cutaneous (skin/eyelids) ($P < 0.003$) groups. In addition, the RF of BgO_L in the ocular group was significantly higher than that in the genital group ($P < 0.003$).

Similar statistical analyses were performed for geographic areas in which the RF of BgO_L is high (8). No statistically significant differences in the RFs of BgO_L were found between the orolabial, cutaneous (skin/eyelids), ocular, and genital groups in region E ($P = 0.88$ by chi-square test by use of the four-by-two contingency table), region F ($P = 0.48$ by chi-

square test by use of the four-by-two contingency table), regions E and F ($P = 0.57$), or region D ($P = 0.68$) (Table 1).

These results indicate that the RF of BgO_L is lower in the orolabial and cutaneous (skin) groups than in the ocular group in area A-B-C, where the RF of BgO_L is low.

No differences in the RFs of non-BgK_L:non-BgO_L exist between the orolabial, cutaneous, ocular, and genital groups. No significant differences in the RFs of non-BgK_L:non-BgO_L were found between the orolabial, cutaneous (skin/eyelids), and ocular groups in the clinical isolates from area A-B-(c) ($P = 0.15$ by chi-square test by use of the three [lesion sites]-by-two [the non-BgK_L:non-BgO_L isolate group and the BgK_L or BgO_L isolate group] contingency table) or between the mouth/lips, cutaneous (skin/eyelids), and ocular groups in regions A, B, and C ($P = 0.33$ by chi-square test by use of the three-by-two contingency table) (Table 1). There were also no statistically significant differences in the RFs of non-BgK_L:non-BgO_L between the orolabial, cutaneous, ocular, and genital groups in regions E and F ($P = 0.81$ by chi-square test by use of the four-by-two contingency table) or region D ($P = 0.20$ by chi-square test by use of the four-by-two contingency table) (Table 1).

These results indicate that there are no significant differences in the RFs of non-BgK_L:non-BgO_L between the orolabial, cutaneous, ocular, and genital groups in any of the geographic areas, independent of the RFs of BgK_L and BgO_L.

The BgK_L RF-to-BgO_L RF ratio in the orolabial/cutaneous groups is much higher than that in the ocular group, whereas the BgK_L RF-to-non-BgK_L:non-BgO_L RF ratio in the orolabial/cutaneous group is only slightly higher than that in the ocular group. The BgK_L RF-to-BgO_L RF and the BgK_L RF-to-non-BgK_L:non-BgO_L RF ratios in the orolabial, cutaneous (skin/eyelids), and ocular infection groups were calculated from the data in Table 1 (see Tables 3 and 4, respectively). The BgK_L RF-to-BgO_L RF ratios in the orolabial infections were at least 14-fold greater than those in ocular infections in regions A, B, and C, which had low RFs of BgO_L, and area A-B-(c), which had high RFs of BgK_L. These regions largely overlap and are denoted "high-BgK_L/low-BgO_L areas" (Table 3). Also, the BgK_L RF-to-BgO_L RF ratios in cutaneous infections were at least sevenfold greater than those in ocular infections in the high-BgK_L/low-BgO_L areas (Table 3). Thus, the BgK_L RF-to-BgO_L RF ratios in orolabial/cutaneous infections were at least 22-fold higher than those in the ocular group in the high-BgK_L/low-BgO_L areas (Table 3). There was no difference in the BgK_L RF-to-BgO_L RF ratios between the orolabial and cutaneous infection groups like that seen in low-BgK_L/high-BgO_L

TABLE 4. BgK_L-to-non-BgK_L:non-BgO_L frequency ratios for orolabial, cutaneous, ocular, and genital lesion groups of HSV-1 clinical isolates

Geographic area(s) ^a	Ratio for lesion sites from which HSV-1 clinical isolates were obtained ^c				
	Mouth/lips	Skin/eyelids	Mouth/lips/skin/eyelids	Eyes	Genitals
A-B-(c) (high BgK _L RF)	0.92 (55:60)	1.1 (29:26)	0.98 (84:86)	0.47 (24:51)	— ^b
D (low BgK _L RF)	0.48 (14:29)	0.53 (18:34)	0.51 (32:63)	0.11 (1:9)	0.19 (3:16)
E-F (lowest BgK _L RF)	0.13 (17:131)	0.10 (4:40)	0.12 (21:171)	0.0 (0:9)	0.0 (0:12)

^a A-B-(c), Shikoku-Chugoku-Osaka area (21); D, Kyushu area (21); E-F, Chubu-Tohoku area (21).^b —, not shown because the sample number of genital infections are too low.^c Values in parentheses represent numbers of BgK_L isolates:numbers of BgO_L isolates.

region D and the lower-BgK_L/high-BgO_L regions E and F (Table 3).

The BgK_L RF-to-non-BgK_L:non-BgO_L RF ratios in the orolabial, cutaneous, and orolabial/cutaneous groups were only twofold higher than those in the ocular infection group in the high-BgK_L-RF area (Table 4). The ratio of the low-BgK_L RF for region D to the non-BgK_L:non-BgO_L RF in the orolabial/cutaneous groups was 4.6-fold higher than that in the ocular infection group and 2.7-fold higher than that in the genital infection group, although the numbers of ocular and genital infections were low (Table 4). In lower-BgK_L-RF area E-F, the BgK_L RF-to-non-BgK_L:non-BgO_L RF ratio in the orolabial/cutaneous group was also higher than those in the ocular and genital groups, but the numbers of ocular and genital infections were low (Table 4). The BgK_L RF-to-non-BgK_L:non-BgO_L RF ratio was the same in the orolabial and cutaneous groups in all geographic areas (Table 4).

To compare these BgK_L RF-to-BgO_L RF and BgK_L RF-to-non-BgK_L:non-BgO_L RF ratios more quantitatively, the ratio of the BgK_L RF-to-BgO_L RF ratio ([A]) to the BgK_L RF-to-non-BgK_L:non-BgO_L RF ratio ([B]) was calculated from the data shown in Tables 3 and 4. For simplicity, this is referred to as the ratio "[A] to [B]." No differences in the [A]-to-[B] ratios between the orolabial and cutaneous groups were found in each of the geographic areas (Table 5). In contrast, the [A]-to-[B] ratios for the orolabial group (>60), cutaneous group (>26), and orolabial/cutaneous group (>86) were much larger than the [A]-to-[B] ratio for the ocular group (8.5) in area A-B-(c), in which the number of samples in the ocular group was large enough for the data to be more meaningful (Table 5).

DISCUSSION

To further address the question of whether BgK_L gradually dispersed and replaced BgO_L in different geographic areas in Japan (8, 21), in the present study we analyzed the RFs of BgK_L and BgO_L in HSV-1 clinical isolates from ocular and genital infections. Previous RFLP studies had suggested that there were either statistically significant differences (6), possibly significant differences (5), or no significant differences (10) between the encephalitic, facial, genital, and ganglionic groups of HSV-1 clinical isolates. However, whether the HSV-1 transmission efficiency varies in the different RFLP variants has not been addressed, to the best of our knowledge.

The main findings in and conclusions from the present study are as follows. First, the BgK_L variation may affect the DNase (alkaline nuclease) genes (U_L12 and U_L12.5) (7) required for

DNA maturation and encapsidation, which interact with ICP8 (24, 26–28, 31). The SaCFJ_M mutation may affect the function of the immediate-early *trans*-activator α0 gene (also called ICP0 [31] or IE110 [23]), a nonspecific *trans*-activator promoting viral infection and viral gene expression (31, 37), as well as apoptosis (34). Additionally, the SaCFJ_M variation may affect LATs (9, 46), which are transcribed from the "b" and "b'" repeat sequences of the L component, which are antisense to mRNAs of ICP0, γ₁34.5, and possibly, ICP4 (4, 31) and some of which contain open reading frames (17, 25, 31). LATs composed of three major components, ≥8.3 kb-, 2.0 kb-, and 1.3- to 1.45-kb molecules, down-regulate viral gene expression in neurons and prevent infected neurons from undergoing apoptosis (1, 14, 15, 25, 31, 38). The SaE_L variation may cause a mutation of (i) the deoxyuridine triphosphatase (U_L50) essential for viral replication in the nervous system; (ii) α27 (also called ICP27) (U_L54), an essential multifunctional immediate-early protein required for the expression of post-α genes, viral inhibition of RNA splicing, and inhibition of transcription of host genes; (iii) U_L51, which is involved in HSV-1 maturation and egress; and (iv) virion glycoprotein K (UL53), which is required for gB-mediated virus-induced cell fusion (NCBI accession no. NC_001806, GenBank accession no. X14112) (Table 2). In addition, the SaGH_M and KpM_S variations suggest

TABLE 5. Comparison of BgK_L/BgO_L and BgK_L/non-BgK_L:non-BgO_L ratios^c

Geographic area(s) ^a	Lesion site	[A]	[B]	Ratio of [A] to [B]
A-B-(c)(high BgK _L RF)	Orolabial	>55	0.9	>60
	Cutaneous	>21	1.5	14
	Orolabial/cutaneous	>76	1.0	>76
	Ocular	4.0	0.47	8.5
D (low BgK _L RF)	Orolabial	4.7	0.48	9.8
	Cutaneous	5.7	0.55	10
	Orolabial/cutaneous	5.2	0.52	10
	Ocular	— ^b	0.11	—
E-F (lowest BgK _L RF)	Orolabial	1.6	0.13	12
	Cutaneous	1.3	0.10	13
	Orolabial/cutaneous	1.5	0.12	13
	Ocular	—	0.0	—

^a A-B-(c), Shikoku-Chugoku-Osaka area (21); D, Kyushu area (8, 21); E-F, Chubu-Tohoku area (8, 21).^b —, not shown because the sample numbers were too small (less than five).^c [A], BgK_L/BgO_L; [B], BgK_L/non-BgK_L:non-BgO_L ratio.

that some other genes may have been mutated functionally in the BgK_L variant (NCBI accession no. NC_001806, GenBank accession no. X14112) (Table 2). Thus, BgK_L with its widely distributed base substitutions in the genome, may have a biological property(ies) different from that of non-BgK_L viruses, which may exert combined effects on HSV-1 growth and/or transmission. The highly diversified and closely associated set of five mutations that are widely distributed in the HSV-1 genome, namely, BgK_L, SaCFJ_M, SaE_L, SaGH_M, and KpM_S (21), imply that more unidentified base-substitution mutations are also likely to be closely associated with the BgK_L variation, affecting the biological properties of BgK_L.

The finding that BgO_L (8, 21) causes a base substitution mutation in the coding region of the same DNase gene that is affected by BgK_L, i.e., the loss of the BglII cleavage site between the BglII K and Q/#13 fragments, may imply that DNA maturation and encapsidation mediated by the viral DNase affects HSV-1 propagation and the efficiency of HSV-1 infection at different human anatomical sites. Whether these genes or other genes are related to a hypothesized functional mutation(s) that affects the efficiency of infection at different sites remains a major question to be addressed in future studies.

The second main finding presented here is that the BgK_L variant is associated relatively more frequently with orolabial and cutaneous infections than with ocular infections, in contrast to BgO_L, while there were no differences in the RFs of non-BgK_L:non-BgO_L viruses between the orolabial, cutaneous, ocular, and genital groups. The BgK_L RF-to-BgO_L RF ratios in the orolabial and cutaneous groups were much higher than those in the ocular group, whereas the BgK_L RF-to-non-BgK_L:non-BgO_L RF ratios in the orolabial and cutaneous groups were only slightly higher than those in the ocular group. The variation in the RFs of BgK_L between different lesion sites, in contrast to those of non-BgK_L:non-BgO_L, suggests that the former possesses a functional mutation(s) that probably affects the frequency of viral reactivation/recurrence in the host and/or the efficiency of transmission to another individual. Non-BgK_L:non-BgO_L viruses are heterogeneous in terms of their RFLPs and probably in terms of the efficiency of orolabial, cutaneous, and ocular infection. BgO_L, in contrast, is relatively homogeneous compared to non-BgK_L:non-BgO_L viruses (8) and may have slightly decreased infectivity for the mouth/lips/skin.

Third, primary HSV-1 infections usually occur in young children in Japan (21, 44), and the mouth and lips are the most commonly infected sites, with gingivostomatitis being the main manifestation (2, 3, 16, 39). Primary infection in young adults is often detected as pharyngitis (12, 35, 39). Hence, the relatively strong association of BgK_L but not BgO_L with the orolabial lesion site contributes to transmission between individuals and, thus, the dispersion of BgK_L. Similarly, the more frequent association of BgK_L with skin infection compared with that of BgO_L enhances the dispersion of BgK_L. Furthermore, because reinfection with a different HSV-1 strain was indicated by the recent finding of "frequent homologous recombination" or "recombinant viruses" in clinical HSV-1 isolates (20), not only primary infection but also reinfection with BgK_L may also play a significant role in its dispersion.

Fourth, the strong association of BgK_L but not BgO_L with orolabial and cutaneous infections supports the graphic

BgK_L dispersion-replacement hypothesis that has been proposed in our previous article (8, 21), providing at least part of the explanation of the mechanism of this dispersion-replacement.

Fifth, RFLP analyses with an increasing number of restriction endonucleases and more precise sequence analyses of genomic DNAs from HSV-1 clinical isolates will show more HSV-1 RFLP variants, genomic types, and genomic recombinants. This would reflect the accumulation of HSV-1 nonlethal mutations in human populations through the lifelong latency-reactivation mode of infection. The biological properties of HSV-1 variants and genotypes may be more or less varied. Our previous studies focusing on the BgK_L and BgO_L pair of variants (8, 21) led us to propose the HSV-1 dispersion-replacement hypothesis. The data in the present study further suggest that HSV-1 has evolved from randomly generated variants/mutants under competitive or selective pressure. This conclusion provides a new viewpoint from which to consider base substitution rates and evolutionary timescales of HSV-1 (19).

Sixth, how BgK_L and BgO_L appeared and affected viral diversification in Japan is an intriguing but challenging question. HSV-1 variants spread very slowly in and between populations, probably taking generations and centuries, because HSV-1 establishes a lifelong latent infection and is transmitted by close contact between individuals (8, 21). However, BgK_L will spread increasingly faster in the future as social conditions (8, 21) rapidly change.

Finally, the present study is the first, to the best of our knowledge, to suggest that an HSV-1 RFLP variation affects the efficiency of viral transmission between individuals. This suggests that the composition of HSV-1 variants in a human population may change as a result of the displacement of one HSV-1 variant by others. Such gradual alterations in the frequencies of different HSV-1 variants with different pathogenic or biological properties in human populations will have impacts on the evolution of HSV-1. The genetic and biological properties of the HSV-1 variants that may influence primary infection, latency, reactivation, and recurrence will be the subjects of further study in the future.

ACKNOWLEDGMENTS

We are grateful to T. Higashi, Institute of Basic Medical Sciences (Tsukuba University), K. Ishizuka (The Program of Environmental Sciences, Tsukuba University), and A. Oya and T. Kitamura (National Institute of Health) for support with this study. We thank Y. Abe (Yamaguchi University) for HSV-1 clinical isolates from Yamaguchi and Chugoku, Y. Eizuru and Y. Minamishima (Miyazaki University) for HSV-1 clinical isolates from Miyazaki and Kyushu, M. Futamura (Aichi Prefectural Colony Hospital) for HSV-1 clinical isolates from Aichi and Chubu, S. Furukawa (Kanazawa Medical School) for HSV-1 clinical isolates from Ishikawa and Chubu, T. Kurimura (Tottori University) for HSV-1 clinical isolates from Shimane and Chugoku, T. Morishima for HSV-1 clinical isolates from Aichi and Chubu, S. Sato and J. Shirai (Iwate Medical University School of Medicine) for HSV-1 clinical isolates from Iwate and Tohoku, M. Sato (University of Tokushima School of Dentistry) for HSV-1 clinical isolates from Tokushima and Shikoku, the Fukushima Medical School Hospital for HSV-1 clinical isolates from Fukushima and Tohoku, the Momoyama Hospital for HSV-1 clinical isolates from Osaka and Kinki, and the National Zentui Hospital for HSV-1 clinical isolates from Kagawa and Shikoku. We thank Ryo Kitamura for technical assistance.

Financial support for this research was provided by the Yamanashi Institute of Health and the Ministry of Health, Welfare and Labor.

REFERENCES

- Amelio, A. L., N. V. Giordani, N. J. Kubat, J. E. O'Neil, and D. C. Bloom. 2006. Deacetylation of the herpes simplex virus type 1 latency-associated transcript (LAT) enhancer and a decrease in LAT abundance precede an increase in ICP0 transcriptional permissiveness at early times postexplant. *J. Virol.* **80**:2063–2068.
- Amir, J., L. Harel, Z. Smetana, and I. Varsano. 1999. The natural history of primary herpes simplex type 1 gingivostomatitis in children. *Pediatr. Dermatol.* **16**:259–263.
- Birek, C., and G. Ficarra. 2006. The diagnosis and management of oral herpes simplex infection. *Curr. Infect. Dis. Rep.* **8**:181–188.
- Burton, E. A., C. S. Hong, and J. C. Glorioso. 2003. The stable 2.0-kilobase intron of the herpes simplex virus type 1 latency-associated transcript does not function as an antisense repressor of ICP0 in nonneuronal cells. *J. Virol.* **77**:3516–3530.
- Chaney, S. M., K. G. Warren, J. Kettys, A. Zbitnue, and J. H. Subak-Sharpe. 1983. A comparative analysis of restriction enzyme digests of the DNA of herpes simplex virus isolated from genital and facial lesions. *J. Gen. Virol.* **64**(Pt. 2):357–371.
- Chaney, S. M., K. G. Warren, and J. H. Subak-Sharpe. 1983. Variable restriction endonuclease sites of herpes simplex virus type 1 isolates from encephalitic, facial and genital lesions and ganglia. *J. Gen. Virol.* **64**(Pt. 12):2717–2733.
- Draper, K. G., G. Devi-Rao, R. H. Costa, E. D. Blair, R. L. Thompson, and E. K. Wagner. 1986. Characterization of the genes encoding herpes simplex virus type 1 and type 2 alkaline exonucleases and overlapping proteins. *J. Virol.* **57**:1023–1036.
- Eda, H., S. Ozawa, K. Yoshino, and K. Yanagi. 2007. Contrasting geographic distribution profiles of the herpes simplex virus type 1 BgOL and BgKL variants in Japan suggest dispersion and replacement. *J. Clin. Microbiol.* **45**:771–782.
- Farrell, M. J., A. T. Dobson, and L. T. Feldman. 1991. Herpes simplex virus latency-associated transcript is a stable intron. *Proc. Natl. Acad. Sci. USA* **88**:790–794.
- Hamelin, C., A. Pramatarova, J. Portnoy, and M. Gerson. 1991. Restriction endonuclease patterns of herpes simplex virus DNA: subtyping of HSV-1 and HSV-2 strains from genital and nongenital lesions. *Comp. Immunol. Microbiol. Infect. Dis.* **14**:39–45.
- Hayward, G. S., N. Frenkel, and B. Roizman. 1975. Anatomy of herpes simplex virus DNA: strain differences and heterogeneity in the locations of restriction endonuclease cleavage sites. *Proc. Natl. Acad. Sci. USA* **72**:1768–1772.
- Holbrook, W. P., G. T. Gudmundsson, and K. T. Ragnarsson. 2001. Herpetic gingivostomatitis in otherwise healthy adolescents and young adults. *Acta Odontol. Scand.* **59**:113–115.
- Hondo, R. 1974. A seroepidemiological study of herpes simplex virus. *Jpn. J. Med. Sci. Biol.* **27**:205–213.
- Jones, C., M. Inman, W. Peng, G. Henderson, A. Doster, G. C. Perng, and A. K. Angeletti. 2005. The herpes simplex virus type 1 locus that encodes the latency-associated transcript enhances the frequency of encephalitis in male BALB/c mice. *J. Virol.* **79**:14465–14469.
- Kent, J. R., W. Kang, C. G. Miller, and N. W. Fraser. 2003. Herpes simplex virus latency-associated transcript gene function. *J. Neurovirol.* **9**:285–290.
- Kolokotronis, A., and S. Dumas. 2006. Herpes simplex virus infection, with particular reference to the progression and complications of primary herpetic gingivostomatitis. *Clin. Microbiol. Infect.* **12**:202–211.
- Lock, M., C. Miller, and N. W. Fraser. 2001. Analysis of protein expression from within the region encoding the 2.0-kilobase latency-associated transcript of herpes simplex virus type 1. *J. Virol.* **75**:3413–3426.
- Locker, H., and N. Frenkel. 1979. BamI, KpnI, and SalI restriction enzyme maps of the DNAs of herpes simplex virus strains Justin and F: occurrence of heterogeneities in defined regions of the viral DNA. *J. Virol.* **32**:429–441.
- McGeoch, D. J., S. Cook, A. Dolan, F. E. Jamieson, and E. A. Telford. 1995. Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses. *J. Mol. Biol.* **247**:443–458.
- Norberg, P., T. Bergstrom, E. Reikabdar, M. Lindh, and J. A. Liljeqvist. 2004. Phylogenetic analysis of clinical herpes simplex virus type 1 isolates identified three genetic groups and recombinant viruses. *J. Virol.* **78**:10755–10764.
- Ozawa, S., H. Eda, K. Hayashi, K. Yoshino, and K. Yanagi. 2006. Geographical distribution of the herpes simplex virus type 1 BgKL variant in Japan suggests gradual dispersion of the virus from Shikoku Island to the other islands. *J. Clin. Microbiol.* **44**:2109–2118.
- Ozawa, S., K. Yoshino, H. Eda, and K. Yanagi. 1989. Localization of a variant of herpes simplex virus type 1 in the western Japan. *Kansenshogaku Zasshi* **63**:39–43. (In Japanese.)
- Perry, L. J., F. J. Rixon, R. D. Everett, M. C. Frame, and D. J. McGeoch. 1986. Characterization of the IE110 gene of herpes simplex virus type 1. *J. Gen. Virol.* **67**(Pt. 11):2365–2380.
- Porter, I. M., and N. D. Stow. 2004. Virus particles produced by the herpes simplex virus type 1 alkaline nuclease null mutant ambUL12 contain abnormal genomes. *J. Gen. Virol.* **85**:583–591.
- Randall, G., M. Lagunoff, and B. Roizman. 2000. Herpes simplex virus 1 open reading frames O and P are not necessary for establishment of latent infection in mice. *J. Virol.* **74**:9019–9027.
- Reuven, N. B., S. Antoku, and S. K. Weller. 2004. The UL12.5 gene product of herpes simplex virus type 1 exhibits nuclease and strand exchange activities but does not localize to the nucleus. *J. Virol.* **78**:4599–4608.
- Reuven, N. B., and S. K. Weller. 2005. Herpes simplex virus type 1 single-strand DNA binding protein ICP8 enhances the nuclease activity of the UL12 alkaline nuclease by increasing its processivity. *J. Virol.* **79**:9356–9358.
- Reuven, N. B., S. Willcox, J. D. Griffith, and S. K. Weller. 2004. Catalysis of strand exchange by the HSV-1 UL12 and ICP8 proteins: potent ICP8 recombinase activity is revealed upon resection of dsDNA substrate by nuclease. *J. Mol. Biol.* **342**:57–71.
- Roberts, R. J., T. Vincze, J. Posfai, and D. Macelis. 2005. REBASE—restriction enzymes and DNA methyltransferases. *Nucleic Acids Res.* **33**:D230–D232.
- Roizman, B. 1979. The structure and isomerization of herpes simplex virus genomes. *Cell* **16**:481–494.
- Roizman, B., and D. M. Knipe. 2001. Herpes simplex viruses and their replication, p. 2399–2459. In D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), *Fields virology*, vol. 2, 4th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
- Roizman, B., and M. Tognon. 1983. Restriction endonuclease patterns of herpes simplex virus DNA: application to diagnosis and molecular epidemiology. *Curr. Top. Microbiol. Immunol.* **104**:273–286.
- Roizman, B., and M. Tognon. 1982. Restriction enzyme analysis of herpesvirus DNA: stability of restriction endonuclease patterns. *Lancet* **i**:677.
- Sanfilippo, C. M., and J. A. Blaho. 2006. ICP0 gene expression is a herpes simplex virus type 1 apoptotic trigger. *J. Virol.* **80**:6810–6821.
- Stanberry, L. R., D. M. Jorgensen, and A. J. Nahmias. 1997. Herpes simplex viruses 1 and 2, p. 419–454. In A. S. Evans and R. A. Kaslow (ed.), *Viral infections of humans—epidemiology and control*. Plenum Medical Book Company, New York, NY.
- Tada, A., N. Sekine, M. Toba, and K. Yoshino. 1977. An analysis of factors influencing the isolation rate of herpes simplex virus. *Microbiol. Immunol.* **21**:219–229.
- Van Sant, C., P. Lopez, S. J. Advani, and B. Roizman. 2001. Role of cyclin D3 in the biology of herpes simplex virus 1 ICPO. *J. Virol.* **75**:1888–1898.
- Wang, Q. Y., C. Zhou, K. E. Johnson, R. C. Colgrove, D. M. Coen, and D. M. Knipe. 2005. Herpesviral latency-associated transcript gene promotes assembly of heterochromatin on viral lytic-gene promoters in latent infection. *Proc. Natl. Acad. Sci. USA* **102**:16055–16059.
- Whitley, R. J. 2001. Herpes simplex virus, p. 2461–2509. In D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), *Fields virology*. Lippincott Williams & Wilkins, Philadelphia, PA.
- Whitley, R. J., and B. Roizman. 2002. Herpes simplex viruses, p. 375–401. In D. G. Richman, R. J. Whitley, and F. G. Hayden (ed.), *Clinical virology*, 2nd ed. ASM Press, Washington, DC.
- Yanagi, K. 1981. Irreversible conversion of the physical state of herpes simplex virus preceding inactivation by thermal or antibody treatment. *J. Virol.* **38**:737–748.
- Yasumura, Y., and Y. Kawakita. 1988. 1. Isolation and establishment of Vero cells. a. Studies on SV40 in tissue culture—preliminary step for cancer research in vitro, p. 2–19. In B. Shimizu and T. Terasima (ed.), *Vero cells—origin, properties and biomedical applications*. Soft Science Publications, Tokyo, Japan.
- Yoshino, K., H. Taniguchi, and S. Taniguchi. 1959. Titration of herpes simplex virus in one-day eggs by an improved inoculation method and its application to the serum neutralization test. *J. Immunol.* **83**:246–251.
- Yoshino, K., S. Taniguchi, and R. Furuse. 1962. A serological survey for antibodies against herpes simplex virus with special reference to comparatively heat-labile complement-fixing antibodies. *Jpn. J. Mol. Sci. Biol.* **15**:235–247.
- Yoshino, K., K. Yanagi, and K. Abe. 1982. Efficacy of intradermal administration of herpes simplex virus subunit vaccine. *Microbiol. Immunol.* **26**:753–757.
- Zwaagstra, J. C., H. Ghiasi, S. M. Slanina, A. B. Nesburn, S. C. Wheatley, K. Lillycrop, J. Wood, D. S. Latchman, K. Patel, and S. L. Wechsler. 1990. Activity of herpes simplex virus type 1 latency-associated transcript (LAT) promoter in neuron-derived cells: evidence for neuron specificity and for a large LAT transcript. *J. Virol.* **64**:5019–5028.