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Application of Long PCR Method to Identification of Variations in Nucleotide Sequences among Varicella-Zoster Virus Isolates

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Restriction fragment length polymorphism (RFLP) analysis of whole viral DNA of varicella-zoster virus (VZV) requires the time-consuming and laborious preparation of a large amount of purified viral DNA. RFLP analysis of small DNA fragments amplified by PCR was developed as an alternative method. However, its use has been limited because of the small number of variations in VZV. To overcome these drawbacks and to identify variations in VZV, we developed an RFLP analysis method combined with the long PCR method which has recently been developed for the amplification of DNA fragments between 5 and 35 kb in length. We amplified three DNA regions ranging from 6.8 to 11.4 kb and demonstrated that RFLP analyses of these regions allowed for the classification of 40 VZV isolates in Japan into 17 groups. One-fourth of the isolates contained a nucleotide difference of C versus T, which abolished the *Sty*I site at position 76530; this alteration was linked to the reported *Pst*I site polymorphism at position 69349 (nucleotide positions are based on those of strain Dumas). Nucleotide sequence variation in the examined regions among VZV isolates in Japan was estimated at roughly less than 0.05%, confirming the previously proposed idea that VZV is genetically stable and not highly diversified. Our method will be useful for studies of the molecular epidemiology of VZV.

Restriction fragment length polymorphism (RFLP) analysis of whole viral DNA has been widely used for molecular epidemiological research on herpesviruses (3, 9, 10, 14, 18, 20, 23). However, the method has two drawbacks. First, it requires a large amount of purified viral DNA. Preparation of viral DNA, especially that from cell-associated herpesviruses such as varicella-zoster virus (VZV), is time-consuming and laborious. In addition, passage of virus-infected cells in vitro to obtain sufficient viral DNA may increase the chance of selecting variant viruses with altered nucleotide sequences, although herpesviruses (in this case, VZV) are in general genetically stable (10, 22, 24). Second, detection of polymorphisms with restriction enzymes that yield large numbers of viral DNA fragments must be done in conjunction with Southern blotting, which results in complicated procedures. Recently, RFLP analysis of small DNA fragments amplified by PCR has been developed as an alternative method for determining the molecular epidemiology of herpesviruses (for example, see references 4 and 7). The method can be performed in less time and with smaller amounts of material. However, its application to VZV has been limited, because the lengths of the DNA fragments amplified by conventional PCR are too short to reveal the limited genetic variations among VZV isolates. Reported RFLP analyses of PCR products of VZV are mainly dependent on variations in the copy numbers of repetitive sequences such as R2 and R5 (12, 21). Thus far, only substitutions in a *Pst*I cleavage site and in a *Bgl*I site were reported as markers for the classification of VZV isolates by the PCR method (1, 13, 16, 21).

More recently, a long PCR method has been developed for the amplification of DNA covering nucleotide sequences as long as 35 kb (2, 5, 6, 11). In this study, we applied the method to RFLP analyses of VZV DNA and demonstrated its ease and utility for the identification of nucleotide sequence variations among VZV isolates.

MATERIALS AND METHODS

VZV isolates. Forty VZV isolates from vesicular fluids of Japanese patients with varicella or herpes zoster obtained from 1976 to 1994 in our laboratory were used (see Table 3). The isolates were propagated in human embryonic lung cells, and their genomic DNAs were extracted from nucleocapsid preparations as described previously (22).

DNA primers. The nucleotide sequence of the VZV Dumas strain (GenBank accession number X04370) (8) was used to design the primers listed in Table 1. The three fragments amplified with these primers cover the regions spanning genes 12 to 16, 38 to 43, and 54 to 60, respectively. These regions contain the coding sequences for thymidylate synthetase (gene 13), glycoprotein V (gpV; gene 14), DNA polymerase accessory protein (gene 16), major capsid protein (gene 40), helicase-primase (gene 55), the *Pst*I site polymorphism (13), and R2 and R5 variable regions (8).

PCR conditions. Forty microliters of a reaction mixture containing 2.5 mM $MgCl_2$, 50 to 200 ng of template viral DNA, 0.2 μM (each) primer, 400 μM (each) deoxynucleoside triphosphate (dNTP), and 2 U of LA *Taq* DNA polymerase (LA PCR kit, version 2; Takara Shuzo Biomedicals, Co. Ltd., Shiga, Japan) were subjected to PCR. PCR conditions were 1 min at 94°C for 1 cycle, 25 s at 98°C and 15 min at 68°C for 30 cycles, and 10 min at 72°C for 1 cycle (Quick Thermo Personal QTP-1; Nippon Genetics, Co. Ltd., Tokyo, Japan). Reactions were done under hot-start conditions by using AmpliWax PCR Gem 50 beads (Perkin-Elmer, Branchburg, N.J.). One microliter of the amplified product was separated by 0.8% agarose gel electrophoresis and was visualized by ethidium bromide staining.

RFLP analyses. The PCR products were dissolved in 30 μl of 10 mM Tris-HCl (pH 8.0)–0.1 mM EDTA after ethanol precipitation. One to three microliters of the solution was used for digestion with the restriction enzymes listed in Table 1. Digestion with these enzymes yielded adequate numbers of fragments for comparison of cleavage patterns.

Nucleotide sequence analysis. DNA fragments of 163 bp containing the *Sty*I cleavage site variation described later were amplified from PCR products of the gene 38 to 43 region with primers 5′-ATCGGATGCGGCTATTCGCA-3′ (positions 76434 to 76453) and 5′-CGTCTTGAATCCGCGGCATT-3′ (positions 76636 to 76617) and were purified after separation by agarose gel electrophoresis. Their nucleotide sequences were determined directly by the dideoxy-chain

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TABLE 1. Primers for long PCR and restriction enzymes used for RFLP analyses

Region	Sequence of primers	Position	Size of amplified product (kbp)	Enzyme(s)		
				6 bp	5 bp	4 bp
Genes 12 to 16	5′-TTATGGTTCTTGGACGTTGTGGACGCCAGGGTA-3′	16970–17002	6.8	*Bam*HI, *Ban*III, *Eco*RI, *Sty*I	*Hin*fI	*Alu*I, *Cfo*I, *Hae*III, *Msp*I, *Rsa*I
	5′-TGGATTTGAGGTCGCGTACAGACGATGCTTTGGAC-3′	23793–23759				
Genes 38 to 43	5′-AGAGCGCCTAAATATGCTATATAACGCCTCCCAGC-3′	68726–68760	11.4	*Bam*HI, *Bgl*I, *Dra*I, *Kpn*I, *Pst*I, *Sty*I	*Hin*fI	*Cfo*I, *Hae*III, *Mbo*I, *Rsa*I, *Taq*I
	5′-ATAGGTCCGATTAACGATGCAGGTAGTGCTGCCTG-3′	80157–80123				
Genes 54 to 60	5′-ATTCACATAACCCTCGAGCATGCCGTTGATACTG-3′	94596–94629	7.7	*Bgl*I, *Dra*I, *Mlu*I, *Pst*I	*Hin*fI, *Mva*I	*Alu*I, *Hae*III, *Msp*I, *Rsa*I
	5′-AAGCACGCTGCCAAGTACGCACTAAGAATGCTTAG-3′	102280–102246				

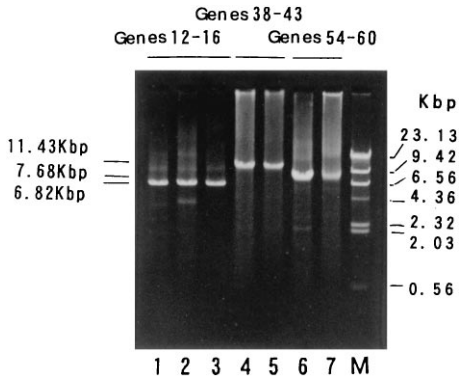

FIG. 1. Long PCR products from DNAs of VZV isolates. The gene 12 to 16 region (lanes 1 to 3), the gene 38 to 43 region (lanes 4 and 5), and the gene 54 to 60 region (lanes 6 and 7) were amplified from different VZV isolates under the optimized conditions described in the Materials and Methods section and were separated in a 0.8% agarose gel. Lane M, bacteriophage λ DNA digested with *Hin*dIII.

termination method with a *Taq* Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.).

RESULTS

Conditions for amplification of long DNA sequences. Conditions for PCR amplification of the 6.8- to 11.4-kb regions of VZV DNA were determined under the conditions suggested by the manufacturer's instruction. First, the concentrations of $MgCl_2$ and dNTP, which are the most critical parameters of long PCR, were determined. In the presence of 0.3 to 0.4 mM dNTPs, reaction mixtures containing 1.0, 1.5, 2.0 or 2.5 mM $MgCl_2$ were compared for their efficiencies in PCR. The reaction mixture containing 2.0 or 2.5 mM $MgCl_2$ was required to yield PCR products, and PCR with the mixture containing 1.0 or 1.5 mM $MgCl_2$ did not yield any product. Second, 0.1 or 0.2 μM DNA primers were sufficient for PCR, and a high concentration of primers (1.0 μM) resulted in the production of additional shorter bands. Third, a 13- to 15-min extension period at 68°C per cycle was sufficient for amplification of the 6.8- to 11.4-kb sequences. Fourth, in addition to VZV DNA prepared by the method described previously (22), that prepared from the supernatant of a sonicated lysate of infected cells after a low-speed centrifugation was competent as the template for amplification. Finally, hot-start reaction conditions effectively reduced the number of background bands. Figure 1 shows the amplification products obtained from two to three isolates by long PCR under the optimized conditions.

VZV DNA was prepared from approximately 10^5 virus-infected cells. The amount of DNA extracted was sufficient for more than 20 long PCRs, and each reaction yielded sufficient product to allow RFLP analysis with more than 20 different restriction enzymes. Whole VZV DNA prepared from about 10^6 infected cells was required for RFLP analysis with 10 different enzymes by the conventional methodology (data not shown). Therefore, the long PCR method required less than 1% of the amount of whole viral DNA required by conventional RFLP analysis.

RFLP analysis of the gene 12 to 16 region. RFLPs of the gene 12 to 16 region among 40 isolates were analyzed by digestion of the long PCR products with *Bam*HI, *Ban*III, *Eco*RI, *Sty*I, *Hin*fI, *Alu*I, *Cfo*I, *Hae*III, *Msp*I, or *Rsa*I. Representative results of the RFLP analyses are shown in Fig. 2. In comparison with strain Dumas, the *Ban*III site at position 18877 and the *Hin*fI site at position 18874 were both absent

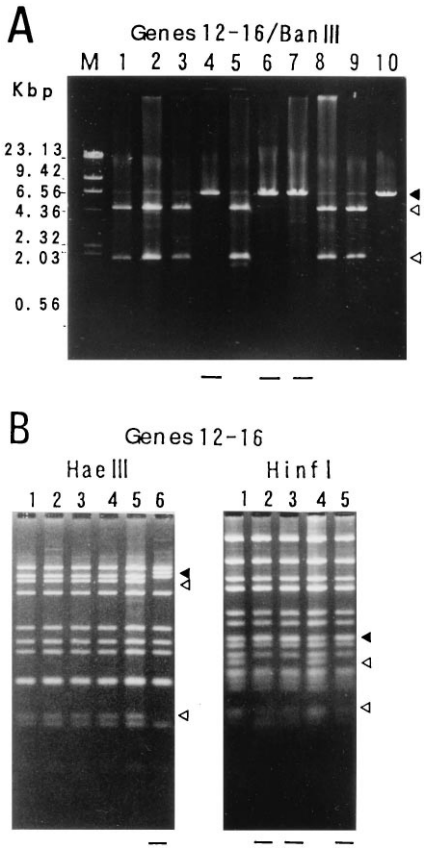

FIG. 2. RFLP analyses of the gene 12 to 16 regions of various VZV isolates. The enzymes used were *Ban*III (A) and *Hae*III and *Hin*fI (B). Samples were run on 0.8% (A) and 4% (B) agarose gels. The isolates in lanes 1 to 10 are independent and different among the three photographs. The restriction enzyme site which separates the band indicated with a closed arrowhead into the two bands indicated with two open arrowheads was absent from the isolates marked with minus signs at the bottom of each panel. In panel B, the *Hin*fI digests indicated with a closed arrowhead contain a single band (257 bp) in lanes 1 to 5 and an additional band (259 bp) in lanes 2, 3, and 5. The digests indicated with the lower open arrowhead contain a single band (77 bp) in lanes 2, 3, and 5 and two bands similar in length (76 and 77 bp) in lanes 1 and 4. Lane M, bacteriophage λ DNA digested with *Hin*dIII.

from 4 of the 40 isolates examined (Table 2; Fig. 2). Because the recognition sequences of the two enzymes partially overlap, it is likely that the four isolates contained a single nucleotide alteration of either a T at position 18877 or a C at position 18878. An additional *Sty*I, *Rsa*I, or *Msp*I site and the absence of an *Rsa*I or *Hae*III site were detected as minor variations (Table 2; Fig. 2). Although the *Cfo*I site at position 19118 described in the Dumas strain was not observed in any of the isolates, the digestion patterns obtained with *Bam*HI, *Eco*RI, *Alu*I, or *Cfo*I were identical among the 40 isolates.

RFLP analysis of the gene 38 to 43 region. In the gene 38 to 43 region, a base substitution from A to G at nucleotide 69349 in a *Pst*I site was previously identified as a marker for grouping VZV isolates (13). Lack of the *Pst*I site was observed in 11 of the 40 isolates. Figure 3A shows the *Pst*I digestion patterns for some of the isolates that contained this alteration (lanes 5, 7, 10, and 11). The absence of the *Sty*I site at position 76530 was observed in the same isolates that lacked the *Pst*I site at position 69349 (Fig. 3B, lanes 5, 7, 10, and 11). DNA fragments of 163 bp containing the *Sty*I site alteration were amplified from 9 of the 11 mutants, and the nucleotide sequences of these fragments were determined. The nucleotide sequences of the nine mutants were identical and they contained a one-base substitution, C to T, at position 76530 in the *Sty*I site compared with that of the Dumas strain. RFLPs of the gene 38 to 43 region among the isolates were observed in digests with *Cfo*I and *Hin*fI, but not in those with *Bam*HI, *Bgl*I, *Dra*I, *Kpn*I, *Hae*III, *Mbo*I, *Rsa*I, and *Taq*I (Table 2).

RFLP analysis of the gene 54 to 60 region. It was reported that although the Oka vaccine strain had one *Bgl*I site in the gene 54 to 60 region, most of isolates in the United States lacked the *Bgl*I site (1, 16). All of the 40 Japanese isolates examined contained the *Bgl*I site identical to that in the Oka vaccine strain (data not shown).

*Pst*I digestion of this region yielded two fragments. The shorter digest was heterogeneous in length (Fig. 4). The *Hin*fI, *Mlu*I, and *Mva*I fragments that overlap the short *Pst*I fragment also varied in length (data not shown). Taking account of the differences of fragment length, the variations seem to be derived from variations of the copy number of the R5 repeat sequence. *Dra*I, *Mlu*I, *Hin*fI, *Mva*I, *Alu*I, *Hae*III, and *Msp*I digests did not reveal any polymorphisms except for those caused by R5 copy number variation.

RFLP analysis of the Oka vaccine strain. In addition to the 40 isolates, the three regions of the Oka vaccine strain were amplified by long PCR. The PCR products were digested with all restriction enzymes listed in Table 1 except *Mlu*I. The *Pst*I site at position 69349 and the *Sty*I site at 76530 were absent from the Oka vaccine strain, and the R5 copy number was two. The other restriction sites examined were the same as those in the Dumas strain. As a result, the Oka vaccine strain was classified into group 8-b (Table 2).

Epidemiological and RFLP analyses of 40 VZV isolates. Clinical data for patients from whom 40 VZV strains were isolated are presented in Table 3. Isolates 202, 224, and 225 were obtained from patients who were hospitalized in the same ward during the same period. Because the isolates from the three patients were classified into the same RFLP group, it is likely that the patients were infected with the same virus in the hospital, as reported previously (22). The other isolates were found to have no epidemiological relationship to one another. Isolates 75, 124, and 367 were obtained from neonates within 19 days after birth. Two of three neonates (infected with isolates 124 and 367) were clinically suspected of being infected in utero. No individual who received the Oka varicella vaccine was included in our study except the individual infected with isolate 429. Isolate 429 was grouped into group 1-c, which is different from that of the vaccine strain. Seven isolates (isolates 10, 202, 224, 225, 302, 378, and 435) were grouped into group 8-b which is the same as that of the Oka vaccine strain. However, these isolates had not originated from the Oka strain, because they were isolated before 1987, when the Oka vaccine became commercially available in Japan, and none of the seven patients had received test varicella vaccines. No correlation was found among the year of virus isolation, primary or secondary infection, the clinical severity of the infection, and RFLP groups. Because all except one of the isolates used in this study were obtained from patients who lived in or around Tokyo, there is no geographical bias to the RFLP grouping.

DISCUSSION

Progress in molecular epidemiology of VZV infections has been hampered by two factors: the difficulty in obtaining a large quantity of viral DNA because of the highly cell-associated nature of the virus and the high degree of conservation from isolate to isolate. In this study, we established the condi-

TABLE 2. RFLPs found in 40 VZV isolates

Variation group	RFLP[a]											No. of isolates
	Genes 12 to 16					Genes 38 to 43				Genes 54 to 60		
	*Ban*III/*Hin*fI	*Hae*III	*Msp*I	*Rsa*I	*Sty*I	*Pst*I	*Sty*I	*Cfo*I	*Hin*fI	*Rsa*I	R5[b]	
1-a	+	+	+	+	+	+	+	+	+	+	1	1
1-b	+	+	+	+	+	+	+	+	+	+	2	15
1-c	+	+	+	+	+	+	+	+	+	+	3	3
2-b	+	−	+	+	+	+	+	+	+	−	2	1
3-b	+	+	++	+	+	+	+	+	+	+	2	1
4-b	+	+	+	−	+	+	+	+	+	+	2	1
5-b	+	+	+	++	+	+	+	+	+	+	2	1
6-b	+	+	+	+	+	+	+	+	++	+	2	1
7-b	+	+	+	+	+	+	+	+	−	+	2	1
8-a	+	+	+	+	+	−	−	+	+	+	1	1
8-b	+	+	+	+	+	−	−	+	+	+	2	7
9-b	+	+	+	+	+	−	−	−	+	+	2	1
9-c	+	+	+	+	+	−	−	−	+	+	3	1
10-b	+	+	+	+	+	−	−	+	+	++	2	1
11-a	−	+	+	+	+	+	+	+	+	+	1	1
11-b	−	+	+	+	+	+	+	+	+	+	2	2
12-b	−	+	+	+	++	+	+	+	+	+	2	1

[a] +, restriction site identical to that of the Dumas strain; ++, gain of an additional restriction site; −, loss of a restriction site.
[b] Copy number of R5 on the basis of length variations of *Pst*I, *Hin*fI, *Mlu*I, and *Mva*I digests.

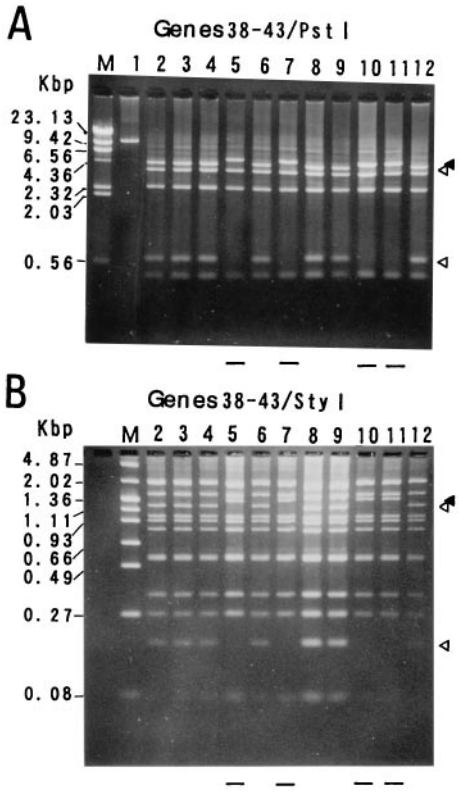

FIG. 3. RFLP analyses of the gene 38 to 43 region digested with *Pst*I (A) and *Sty*I (B). The isolates in lanes 2 through 12 are identical between the two panels. The digests were analyzed in 1.4% (A) and 4.0% (B) agarose gels. The undigested amplified product is in lane 1 of panel A. Bacteriophage λ DNA digested with *Hin*dIII and pHY300PLK DNA digested with *Hin*dIII and *Hae*III (Takara Shuzo, Co., Ltd.) were used as size standards in lane M of panels A and B, respectively. Symbols are as described in the legend to Fig. 2.

tions for PCR amplification of VZV genomic segments longer than 6 kb and demonstrated the ease and utility of using the long PCR products for discriminating VZV isolates. The long PCR method enabled us to shorten the time and reduce the amount of labor required to prepare the materials needed for RFLP analysis. The three regions amplified in this study cover about 20% of the VZV genome. In addition to the three regions, we also amplified a different 15-kb sequence under the same conditions established in the study. However, RFLP analyses of this region of some isolates failed to distinguish them from each other (data not shown). Considering the capacity of the long PCR method, fewer than 10 sets of primers may be sufficient to span the entire VZV genome. It is necessary to analyze the remaining regions, which may give more information for classifying VZV isolates.

During preliminary experiments, some difficulty was encountered in amplification of the region containing the R4 repeat sequence (data not shown). It is possible that R4 was unstable

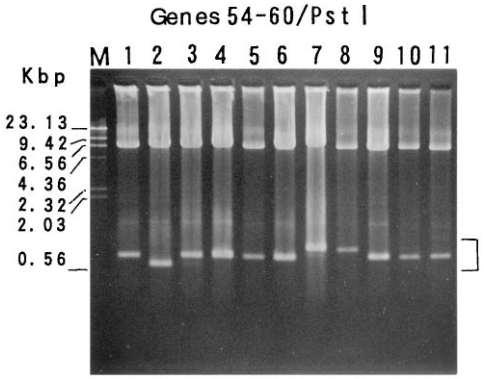

FIG. 4. Analysis of R5 copy number. *Pst*I digests of DNA fragments amplified from the gene 54 to 60 region were separated in a 1.4% agarose gel. Bands marked with a bracket contain R5 and differ in length by increments of the R5 length (112 bp). The copy numbers of R5 of the isolates are one (lane 2), two (lanes 1, 3 to 6, and 9 to 11), and three (lanes 7 and 8). Lane M, bacteriophage λ DNA digested with *Hin*dIII.

TABLE 3. Clinical data and RFLP variation groups for 40 VZV isolates

Isolate no.	Date of isolation	Age[a]	Sex[b]	Clinical diagnosis[c]	Underlying disease[d]	Geographical location[e]	RFLP group
9	May 1976	13 y	M	Z	Lymphosarcoma	Tokyo	1-b
10	May 1976	10 y	M	V	None	Unknown	8-b
57	Apr. 1984	1 y	M	V	Failure to thrive	Tokyo	1-b
75	Apr. 1984	19 d	F	V	None	Tokyo	10-b
78	Apr. 1984	8 y	F	V	None	Tokyo	11-a
79	Apr. 1984	5 y	F	V	None	Tokyo	1-b
80	Apr. 1984	4 y	M	V	Encephalitis	Tokyo	1-b
124	June 1984	13 d	M	V	None	Hyogo	6-b
132	June 1984	1 m	F	V	None	Tokyo	8-a
135	June 1984	8 y	M	V	None	Tokyo	3-b
136	June 1984	35 y	M	V	None	Tokyo	1-b
160	Sept. 1984	2 m	F	V	None	Tokyo	9-c
197	Nov. 1984	5 m	M	V	None	Tokyo	1-b
199	Nov. 1984	48 y	M	Z	ALL	Tokyo	1-b
201	Nov. 1984	64 y	F	Z	None	Tokyo	5-b
202[f]	Dec. 1984	11 y	M	Z	AML	Saitama	8-b
205	Dec. 1984	49 y	F	Z	SLE	Tokyo	9-b
224[f]	Dec. 1984	3 y	M	V	Malignant hystiocytosis	Tokyo	8-b
225[f]	Dec. 1984	5 y	M	V	Anaphylactoid purpura	Tokyo	8-b
223	Jan. 1985	13 y	M	Z	ALL	Saitama	1-a
227	Jan. 1985	10 y	M	V	Epilepsy	Tokyo	12-b
260	Mar. 1985	1 y	F	V	Fever convulsion	Tokyo	1-b
273	May 1985	43 y	F	Z	Rhabdomyosarcoma	Ibaraki	11-b
302	June 1985	6 y	M	V	Epilepsy	Tokyo	8-b
364	June 1985	1 y	F	V	None	Tokyo	1-b
365	June 1985	10 m	M	V	Fever convulsion	Tokyo	4-b
367	June 1985	12 d	F	V	None	Tokyo	1-b
378	June 1985	20 y	F	V	None	Tokyo	8-b
429[g]	Dec. 1985	1 y	M	V	None	Tokyo	1-c
433	Feb. 1986	8 y	F	Z	Hodgkin's disease	Tokyo	2-b
442	Feb. 1986	5 y	M	Z	ALL	Tokyo	7-b
435	Apr. 1986	5 y	M	V	None	Tokyo	8-b
436	Apr. 1986	4 y	F	V	Nephrotic syndrome	Tokyo	1-b
437	May 1986	5 m	F	V	Epilepsy	Chiba	1-c
438	May 1986	5 y	M	Z	ALL	Tokyo	1-b
439	July 1986	5 y	F	V	Bronchial asthma	Tokyo	1-b
430	Apr. 1987	5 y	M	Z	Hodgkin's disease	Chiba	1-c
431	June 1987	14 y	F	Z	AML	Tokyo	1-b
292	Apr. 1988	13 y	F	Z	Louis-Bar syndrome	Tokyo	11-b
810	June 1994	1 y	F	V	None	Tokyo	1-b

[a] y, year; m, month; d, day.
[b] M, male; F, female.
[c] Z, zoster; V, varicella.
[d] ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; SLE, systemic lupus erythematosus.
[e] Chiba, Saitama, and Ibaraki are situated around Tokyo, and Hyogo is in the west of Japan, far from Tokyo.
[f] Isolates 202, 224, and 225 were taken from the patients who were hospitalized in the same ward at the same period.
[g] The patient was inoculated with the Oka varicella vaccine 9 days before the onset of varicella.

during passage of the virus, as suggested by the others (10, 21). Similarly, difficulty in cloning R3 and the instability of R3 have been reported (8). Although a difference in R2 copy number among isolates was reported (15, 21), we found no obvious difference in R2 copy number among the isolates that we analyzed. Although the overall G+C content of VZV DNA is 46%, those of R1, R2, R3, R4, and R5 are 71, 63, 86, 82, and 44%, respectively. Because the amplification of repeat sequences with high G+C contents has been reported to be difficult (17), the results regarding the R2 copy number should be confirmed by another method. However, because RFLP analyses combined with long PCR do not necessarily depend on variations in the repeat sequences to distinguish isolates, it could be possible to design regions for amplification that do not contain repeat sequences with high G+C contents, such as R1 to R4.

RFLP analysis of the long PCR products identified 12 variable loci among 40 isolates on the basis of the presence or absence of restriction enzyme cleavage sites. Combination of these loci with the R5 copy number allowed classification of the 40 isolates into 17 groups (Table 2). One-fourth of the isolates contained a nucleotide alteration of C to T at position 76530, and that alteration was linked to the reported *Pst*I site polymorphism at position 69349. The *Sty*I site-less alteration will be a useful marker for molecular epidemiology. We neither observed any difference in in vitro growth property among the groups nor found any relationship between the group(s) and the clinical presentations of the patients from whom the isolates were obtained.

On the basis of our observations, we were able to calculate and approximate the rate of nucleotide sequence variation among Japanese VZV isolates. The calculation was performed as follows: (number of restriction enzyme cleavage sites as identified from electrophoresis patterns) × (number of nucle-

otide residues recognized by the enzyme) for each enzyme. The values for each of the enzymes used were summed. The sum was multiplied by the number of epidemiologically independent isolates, which was 38. The result, 65,000, is the total number of nucleotide residues analyzed. The total number of variations in all of the isolates examined, 28, was divided by the total number of residues analyzed (65,000). The result is the frequency of nucleotide sequence variations. Although this formula is based on a simple assumption that each RFLP was caused by one base alteration and does not account for variations in repeated sequence copy number, we think that the result provides a close approximation of reality. The extent of variations was calculated to be 0.043%; in other words, one base per 2.3 kb of sequence would be expected to differ from isolate to isolate, confirming the genetic stability and limited diversity of VZV isolates (9, 24). The nucleotide sequence diversity of herpes simplex virus type 1 isolates in Japan was estimated to be 0.37% (19).

Our method will be helpful for investigating variations in VZV DNA that are based on geographical distribution, differences in clinical presentations, and consequences of antiviral drug treatment. Application of the method to molecular epidemiology based on geographical distribution and to distinguishing patients with VZV reactivation from those with VZV reinfection is under way. The Oka vaccine is now used not only in Japan but also in several countries, including the United States. Therefore, identification of the genetic markers specific for the vaccine strain should be necessary for quality control and monitoring of vaccination programs. Although some RFLP loci for distinguishing the Oka vaccine and its parent strains from other field isolates were previously proposed on the basis of analyses of whole viral DNA (9, 10, 21), genetic markers unique to the Oka vaccine strain have not yet been conclusively established. In this study, we demonstrated that the Oka vaccine strain is classified in group 8-b (Table 2). To discriminate the vaccine strain from any isolates, it will be essential to examine RFLPs with different restriction enzymes and to analyze the other regions of the VZV genome.

Finally, because field isolates of human herpesviruses 6A, 6B, and 7 and human cytomegalovirus are highly cell associated and are difficult to grow, the long PCR RFLP method will also be useful in studies with these viruses.

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