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Restriction Fragment Length Polymorphism of Polymerase Chain Reaction Products from Vaccine and Wild-Type Varicella-Zoster Virus Isolates

PHILIP LARUSSA,[1] OCTAVIAN LUNGU,[2] IAIN HARDY,[1] ANNE GERSHON,[1] SHARON P. STEINBERG,[1] AND SAUL SILVERSTEIN[2]*

Departments of Pediatrics[1] and Microbiology,[2] College of Physicians and Surgeons, Columbia University, 701 West 168th Street, New York, New York 10032

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The nucleotide changes that result in two restriction endonuclease polymorphisms that differentiate wild-type varicella-zoster virus (VZV) from the vaccine strain were determined. Oligonucleotide primers that flank these sites were used to amplify the intervening sequences with the polymerase chain reaction to identify VZV DNA in clinical isolates. Restriction enzyme digestion of the amplification products distinguished vaccine and wild-type genomes from one another. This study confirms the feasibility of amplifying VZV sequences so that they may be detected in clinical specimens and provides a molecular epidemiological approach to strain identification of VZV in vesicular lesions.

Varicella-zoster virus (VZV) is the etiologic agent of both varicella (chicken pox) and zoster (shingles). A live attenuated varicella vaccine was developed by serial passage of a Japanese wild-type isolate (Oka) (19). In clinical trials this vaccine was shown to be safe and efficacious in healthy children (21), children with leukemia in remission (8), and healthy adults (9). The vaccine strain latently infects ganglia and on occasion reactivates to cause zoster (7).

In studies of varicella vaccine, it is essential to be able to distinguish between wild-type and vaccine strains of VZV. This has been accomplished by propagation of virus isolates in tissue culture and restriction endonuclease digestion of extracted purified viral DNA (6). Restriction endonuclease cleavage of wild-type VZV DNA with *Bgl*I yields a slowly migrating 28,988-bp A fragment (1, 16). Restriction site polymorphisms in this region of vaccine-type VZV DNA result in the replacement of the A fragment with three smaller fragments, B′, C′, and R′ (1). These fragments are likely to arise as a result of single base changes at two sites within the corresponding sequence of vaccine-type VZV. One of these changes, which results in creation of the B′ and C′ fragments, was mapped to a 250-bp region within the *Hin*dIII-*Bam*HI fragment in *Bam*HI fragment D (1).

The polymerase chain reaction (PCR) was used to amplify DNA in VZV-infected cells and clinical specimens (vesicle fluid). The amplified products were characterized on the basis of the presence of specific restriction fragment length polymorphisms (RFLPs), and their sequences were determined to reveal the molecular basis for the RFLPs present in wild-type and vaccine strains of VZV.

MATERIALS AND METHODS

Specimens. VZV was propagated from clinical samples in human embryonic lung fibroblasts until there was ≥90% cytopathic effect (22). The infected cells were then collected and stored in phosphate-buffered saline (5×10^6 cells per ml) at −70°C. Cell free-virus was prepared from infected cells by sonication (30 s at 20 kHz and 2.2 to 2.6 A) and brief centrifugation. The supernatant was stored at −70°C. Vesicle fluid was collected from patients with chicken pox or zoster by gently unroofing vesicles with a sterile 50-μl capillary tube, and the fluid was stored at −70°C.

DNA probe. A cloned copy of the VZV *Hin*dIII F fragment in pBR322 from the Ellen strain of VZV was obtained from Richard Hyman (11). The plasmid DNA was digested with *Hin*dIII, and the 8,616-bp *Hin*dIII F fragment was separated from plasmid sequences by electrophoresis in a 0.8% agarose gel. The viral insert was extracted from the gel (20) and labeled with ^{32}P to high specific activity by random priming (4).

Preparation of VZV library and VZV clones. Genomic DNAs from wild-type, Bgl^+ wild-type (Bgl^+ WT; see below), and Oka (vaccine) VZV strains were digested to completion with *Bam*HI, and the resulting fragments were ligated into the *Bam*HI site of alkaline phosphatase-treated plasmid pIBI31. The ligation mixtures were transformed into competent *Escherichia coli* DH5α, and the resulting colonies were screened for the presence of clones that contained the *Bam*HI D fragment by using the ^{32}P-labeled probe described above and the method of Grunstein and Hogness (10) (Fig. 1). A 2,055-bp *Hin*dIII-*Bam*HI fragment (positions 94,280 to 96,334) that was subcloned from the isolated *Bam*HI D fragment derived from Oka DNA by using standard procedures (15) was shown to contain a novel *Bgl*I site. This *Hin*dIII-*Bam*HI subclone was digested with *Xba*I and *Sph*I, and the *Bgl*I site was localized within the 732-bp *Xba*I-*Sph*I fragment. This same fragment was subsequently subcloned from the *Bam*HI D fragments of wild-type, Bgl^+ WT, and Oka DNAs into M13, and the DNA sequences were determined as described below.

PCR. Two sets of primer pairs 20 bp in length were selected (Fig. 1): Nla (GGAACCCCTGCACCATTAAA)/Fok (TCCCTTCATGCCCGTTACAT) and Pst A (TTGAA CAATCACGAACCGTT)/B (CGGGTGAACCGTATTCTG AG). They were synthesized, purified, and stored at −20°C at a concentration of 20 μM in TE buffer (1 mM Tris, 0.1 mM EDTA). Homology between the primers and other regions of the VZV genome and of the genomes of other sequenced herpesviruses (Epstein-Barr virus, herpes simplex virus type

* Corresponding author.

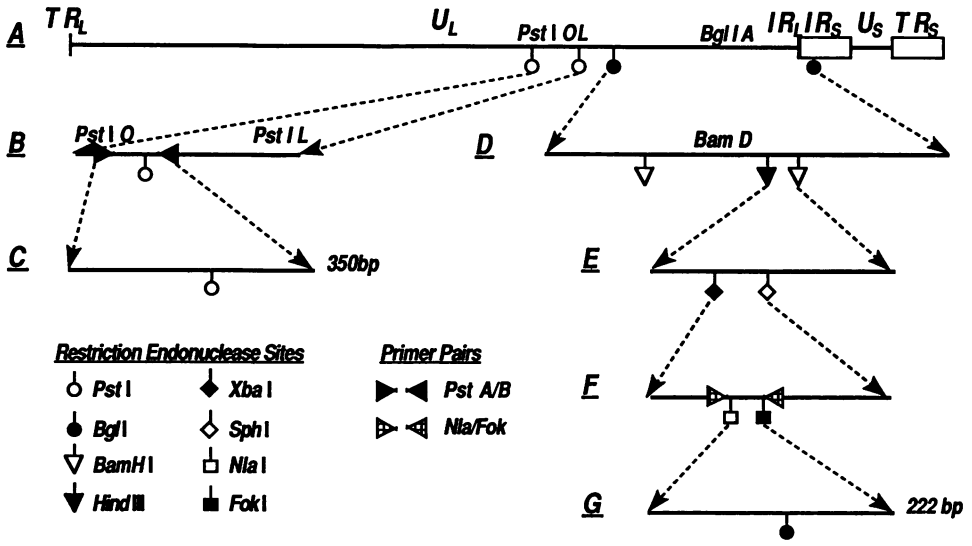

FIG. 1. Strategy for the identification of the *Bgl*I and *Pst*I RFLPs in VZV DNA. (A) Schematic representation of the VZV genome (2). U_L and U_S are the 105-kbp unique long and 5.3-kbp unique short regions, respectively. TR_S and IR_S (7.3-kbp terminal and inverted repeat regions, respectively) flank U_S. TR_L and IR_L (88-bp terminal and inverted repeat regions, respectively) flank U_L. (B) The 7.1-kbp *Pst*I OL fusion fragment present in Oka DNA. (C) The 350-bp amplification product of the Pst A/B primer pair. Wild-type DNA has the *Pst*I site, located in gene 38, depicted within this amplification product, whereas Oka DNA does not. (D) The 29-kbp *Bgl*I A fragment in wild-type DNA. (E) A 2.1-kbp *Hin*dIII-*Bam*HI clone derived from the 8.6-kbp *Bam*HI D fragment. (F) The 732-bp Xba/Sph fragment derived from fragment E. (G) Amplification product of the Nla/Fok primer pair. The *Bgl*I polymorphism is located in gene 54.

1, and cytomegalovirus) was excluded (Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin; GenBank data base). Optimal concentrations of reaction mixture components (Perkin-Elmer Cetus Corp., Norwalk, Conn.) were determined (1.5 mM $MgCl_2$, 200 μM [each] deoxynucleotide triphosphate, 1 μM [each] primer, 2.5 U of recombinant DNA polymerase). The reaction mixture was overlaid with 100 μl of mineral oil. Samples to be amplified were denatured in a boiling water bath for 10 min, centrifuged to remove cellular debris, and stored on ice until used. The sample (10 μl) was added to the reaction mixture (final volume, 100 μl) below the level of the mineral oil.

Thirty cycles of amplification were performed (denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 2 min) in a DNA thermocycler (Perkin-Elmer Cetus). Ten microliters of the amplified product was analyzed by gel electrophoresis in a 1.8% agarose gel containing ethidium bromide (0.5 μg/ml), electrophoresed at 45 mA, and visualized with shortwave UV illumination of the gel.

Restriction enzyme digestion. The remainder of the amplification product was divided and subjected to restriction enzyme digestion with 16 U of *Bgl*I or 20 U of *Pst*I (New England BioLabs, Beverly, Mass.) at 37°C overnight. The digests were analyzed after ethanol precipitation and resuspension in 10 μl of TE buffer by electrophoresis in a 4% agarose gel (three parts NuSieve GTG, 1 part Seaplaque GTG agarose [both from FMC BioProducts, Rockland, Maine]).

Sequence analysis. The DNA from the *Xba*I-*Sph*I subclones of each strain of VZV were sequenced by the dideoxy-chain termination technique (18) with a ^{T7}Sequencing kit (Pharmacia Inc., Piscataway, N.J.). Sequences were compiled and analyzed by using the DNASIS software package (Pharmacia LKB, Bromma, Sweden) and an MS-DOS microcomputer.

RESULTS

Localization of restriction endonuclease polymorphisms in Oka and wild-type VZV DNAs. Computer analysis of the DNA sequence of the 28,988-bp *Bgl*I A fragment from wild-type VZV reveals three nucleotide strings, which, upon mutation of a single base, would generate *Bgl*I recognition sites within the 250-bp fragment to which the *Bgl*I site has been mapped (1). A search of the VZV sequence (2) indicated that these strings are located at positions 95,014, 95,242, and 95,251. A 732-bp *Xba*I-*Sph*I fragment containing the *Bgl*I restriction site was subcloned into M13 (Fig. 1). Sequence analysis identified a T-to-C transition at position 95,242 that creates the novel *Bgl*I site, resulting in the B′ and C′ fragments. Two 20-bp oligonucleotides (Nla/Fok) flanking this novel *Bgl*I site were synthesized and used as primers to amplify the intervening 222 bp in wild-type and Oka DNA. The amplification product of wild-type DNA lacks a *Bgl*I site and is therefore not cleaved after incubation with *Bgl*I, whereas the amplification product of vaccine-type DNA is cleaved with *Bgl*I, resulting in two fragments of 137 and 85 bp (Fig. 1).

However, *Bgl*I cleavage of DNA from some VZV wild-type strains results in the generation of B′, C′, and R′ fragments that comigrate with those of the vaccine strain (1, 6). Analysis of the amplified products from two of these Bgl^+ WT strains containing additional *Bgl*I cleavage sites revealed that their location and sequences are identical to that found in Oka (data not shown). RFLP analysis was therefore extended to other areas of the VZV genome to aid in the differentiation between wild-type, Bgl^+ WT, and Oka DNAs. A second primer pair (Pst A/B) was chosen to amplify a 350-bp fragment that asymmetrically brackets a *Pst*I site present in all wild-type isolates that we have analyzed, including the Bgl^+ WT strain, but which is absent from Oka (Fig. 1).

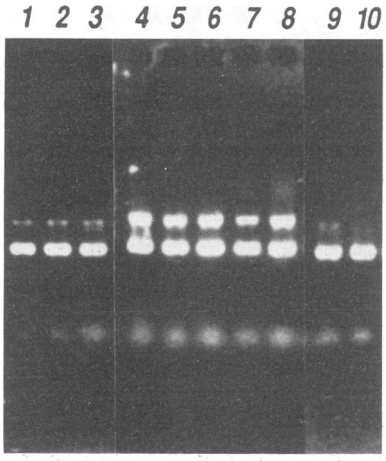

FIG. 2. Simultaneous amplification with both primer pairs of VZV DNA from various virus strains. Amplification products of purified VZV DNA (1 pg) from WT, Bgl$^+$ WT, and Oka strains (lanes 1 through 3, respectively), cell-free virus from WT, Bgl$^+$ WT, and Oka strains (lanes 4 through 6, respectively), infected cells from WT and Bgl$^+$ WT strains (lanes 7 and 8, respectively) and VZV vaccine (Oka), and vesicle fluid from a patient with chicken pox (lanes 9 and 10).

Analysis of DNA from clinical isolates. Simultaneous amplification of VZV sequences with both primer pairs was performed on samples of purified VZV DNA, cell-free virus, infected cells, vaccine, and initially from one vesicle fluid specimen from a patient with chicken pox. Representative examples are shown in Fig. 2. Control samples that did not amplify included purified herpes simplex virus type 1 DNA (1 and 100 pg), cytomegalovirus Towne strain DNA (100 pg), and cellular DNA (100 pg) from cells infected with Epstein-Barr virus, herpes simplex virus type 1 or 2, or cytomegalovirus (data not shown).

Twenty-four additional vesicle fluid samples (15 from patients with varicella and 9 from patients with zoster) were analyzed. VZV DNA was detected in 23 of the 24 samples examined (Table 1). Vesicle fluid specimens from patients with herpes simplex virus gingivostomatitis did not amplify (data not shown). Virus culture and countercurrentimmunoelectrophoresis (CIE) (5) for VZV had previously been performed on some of these vesicle fluids (Table 1). Nine of 10 vesicle fluids that were both VZV culture and CIE positive were also PCR positive. VZV DNA was detected by PCR amplification in three instances when vesicle fluids were culture negative but CIE positive.

The products of simultaneous PCR amplification were digested with *Bgl*I and *Pst*I and analyzed by electrophoresis in a 4% agarose gel. A schematic representation of the digestion patterns of wild-type, Bgl$^+$ WT, and Oka DNAs is shown in Fig. 3A. The 222-bp amplification product from wild-type isolates is not cleaved by *Bgl*I, whereas its 350-bp amplification product is cleaved by *Pst*I. In contrast, the 222- and 350-bp amplification products of Bgl$^+$ WT isolates are cleaved by *Bgl*I and *Pst*I, respectively. The 222-bp Oka amplification product is cleaved by *Bgl*I, whereas its 350-bp amplification product is not cleaved by *Pst*I. Representative examples of clinical specimens with these restriction endonuclease cleavage patterns are shown in Fig. 3B. When the amplification products of vesicle fluids were digested and electrophoresed, none exhibited the Oka pattern, 6 exhibited the Bgl$^+$ WT pattern (4 from a single patient on successive days of chicken pox in 1978), and 17 had the wild-type pattern. It is possible that a recombinant virus with the

TABLE 1. Correlation of DNA amplification with virus culture and CIE[a]

Specimen no.	Day	Diagnosis	Culture	CIE	Amplifies with:	
					Nla/Fok	Pst A/B
1	1	V	ND	ND	+	+
2	2	V	ND	ND	+	+
3	2	V	+	+	+	+
4	2	V	+	+	+	+
5	3	V	+	ND	+	+
6	3	V	+	+	+	+
7	5	V	ND	ND	+	+
8	5	V	+	+	−	−
9	5	V	+	+	+	+
10	6	V	+	ND	+	+
11	7	V	ND	ND	+	+
12	9	V	−	+	+	+
13	?	V	ND	ND	+	+
14	?	V	ND	+	+	−
15	?	V	+	ND	+	+
16	1	Z	−	+	+	+
17	1	Z	+	+	+	+
18	2	Z	+	+	+	+
19	4	Z	+	ND	+	−
20	5	Z	+	+	+	+
21	9	Z	−	+	+	+
22	?	Z	ND	ND	+	+
23	?	Z	ND	+	+	+
24	?	Z	+	ND	+	−

[a] V and Z, clinical diagnosis of varicella and zoster, respectively; Day, day the sample was obtained from the lesion; ND, not done. The number of positive samples were 20, 3, and 0, respectively, with both primer pairs, only Nla/Fok, and only Pst A/B. Only one sample was negative with both primer pairs; this sample was CIE and culture positive at the time it was procured but was found to be contaminated at the time of amplification (12 years later).

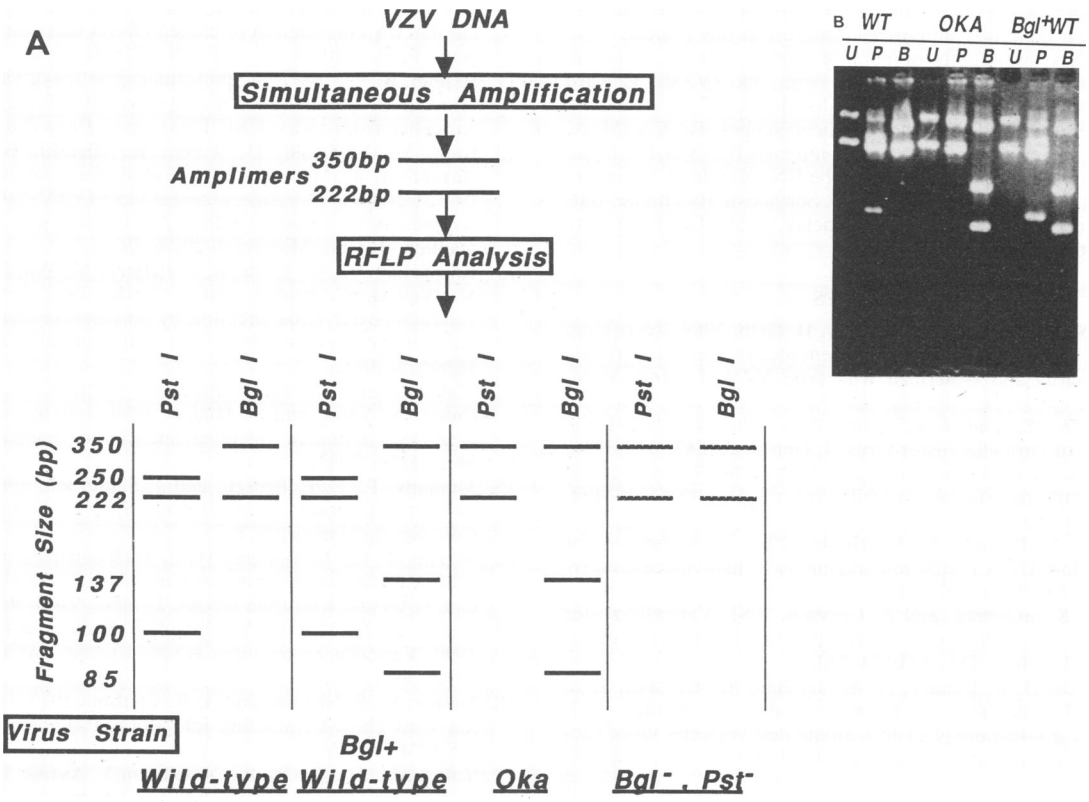

FIG. 3. RFLP analysis of VZV DNA amplified by PCR. (A) Schematic representation of the predicted digestion patterns. The virus strain designated Bgl^-, Pst^- is a theoretical recombinant that we have not detected in these analyses. (B) Representative examples of amplification products from wild-type (lanes 1 through 3), Oka (lanes 4 through 6), and Bgl^+ WT (lanes 7 through 9) strains that were undigested (U) or digested with *Pst*I (P) or *Bgl*I (B).

wild-type 222-bp amplification product and a 350-bp Oka product might arise and that these PCR products would not be cleaved by either enzyme. This pattern was not identified in any of the clinical isolates analyzed in this study.

DISCUSSION

Previous studies (12–14, 17) have demonstrated the utility of the PCR as a diagnostic technique for the detection of VZV DNA in clinical specimens. The present investigation confirms the validity of this approach and extends its utility to differentiation between strains of VZV without having to propagate the virus. This approach is particularly useful for the analysis of patients with minimal skin lesions or who present for diagnosis in a late stage of illness after their skin lesions have dried and infectious VZV is no longer present.

Amplification with the two primer pairs, Nla/Fok and Pst A/B, demonstrated the presence of VZV DNA in all but one of the clinical specimens from patients with VZV infection. Amplification was not successful in this instance because the sample was contaminated with bacteria (culture and CIE had been performed 12 years before the present study). Three clinical samples were successfully amplified with Nla/Fok but not with Pst A/B. If the amplification product of the Nla/Fok primer pair exhibits the wild-type phenotype, the strain type can still be identified. However, if the Nla/Fok amplification product contains a *Bgl*I restriction site and there is no Pst A/B product to analyze, it would not be possible to differentiate between Bgl^+ WT and vaccine strains. We are therefore examining other RFLPs present in all wild-type strains to address this problem. Although it appears that CIE is as sensitive as the PCR, analysis was biased in favor of CIE because only vesicular lesions were sampled in the original CIE analysis (5). The PCR would be expected to be more sensitive than CIE because vesicular fluid is not required for successful amplification of VZV (13).

The PCR in conjunction with restriction enzyme digestion of the amplified product successfully differentiated VZV strains, identifying the isolates as either wild-type, Bgl^+ WT, or vaccine strains. The specificity of this approach was demonstrated by our inability to amplify DNA sequences from any of the closely related herpesviruses, by generation of PCR amplification products of the predicted size, and by conservation of previously defined restriction endonuclease sites within the amplified sequences. This approach obviates the need to confirm the origin of the amplification products by Southern blot hybridization.

The single base pair substitution responsible for the novel *Bgl*I polymorphism was present in approximately 20% of the samples analyzed in this study. Although creation of this novel *Bgl*I restriction site by in vitro recombination between vaccine and wild-type virus has been demonstrated (3), the identification of this RFLP in clinical isolates from as early as 1978, before the introduction of VZV vaccine into the United States, indicates that this RFLP arose as the result of a single-base-pair mutation.

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