

NOTES

Defective Viral DNA in Epstein-Barr Virus-Associated Oral Hairy Leukoplakia

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Defective Epstein-Barr virus (EBV) has a deleted and rearranged genome (termed het DNA) that disrupts latency and induces standard EBV to replicate in vitro. We used the polymerase chain reaction to detect, in 2 of 10 patient samples, the junction of abnormally juxtaposed EBV DNA fragments *Bam*HI W and Z, a genomic rearrangement responsible for the biologic activity of het DNA. By sequence analysis, the junction in wild-type defective DNA appears to be similar but not identical to the recombination in the DNA of laboratory strain P3HR-1. The presence of this marker for het DNA in the epithelial lesions of two patients suggests a role for defective EBV in a human pathologic process.

Epstein-Barr virus (EBV) is a human herpesvirus that establishes a persistent, replicative infection at oropharyngeal and genital sites (25, 26). The permissiveness of mucosal surfaces for EBV replication contrasts with the stringently latent life cycle of the virus in circulating B lymphocytes. Although differences in EBV activity have been attributed to variables of host cell regulation, an in vitro replicative system has been shown to contain defective EBV whose deleted, rearranged genome has the capacity to induce replication of latent EBV (4, 6, 10, 12, 14, 18, 21, 27). Defective EBV was first identified in a cellular subclone of P3HR-1, a Burkitt's lymphoma-derived cell line (11, 21). The deleted and rearranged genome (het DNA) in defective virus forms self-contained replicons which multiply independently of standard EBV DNA and are capable of cell-to-cell spread (17). The ability to induce replication maps to a 2.7-kilobase-pair WZhet fragment, which is formed by nonhomologous recombination between DNA sequences in *Bam*HI Z and W fragments separated in the standard genome by more than 55 kilobase pairs (Fig. 1) (5, 8). The WZhet recombinant retains the entire open reading frame of standard *Bam*HI-Z, termed BZLF1 (13), encoding a product that *trans* activates the lytic origin of EBV DNA replication (3, 9, 28). The capability of het DNA to activate EBV replication results from up-regulation of BZLF1 by positive regulatory elements newly positioned on either side of the gene (22).

To determine if defective EBV exists in vivo, we used polymerase chain reaction (PCR) amplification to examine DNA from the epithelial lesion oral hairy leukoplakia, which is found predominantly in patients with acquired immunodeficiency syndrome and is known to contain abundant replicative EBV (7; L. Resnick, J. Herbst, and N. Raab-Traub, *J. Am. Acad. Dermatol.*, in press). To test for rearranged EBV DNA, we synthesized oligonucleotide primers framing a 181-base-long sequence spanning the abnormal junction in

the WZhet fragment (Fig. 1). Two ³²P-labeled 30-base oligonucleotides specific for sequences in either the *Bam*HI-Z or -W portion of WZhet DNA were used to detect the PCR product on Southern blots. Oligonucleotides were made with a model 380A DNA synthesizer (Applied Biosystems) using phosphoramidite chemistry. Probes were [γ -³²P]ATP end labeled (ICN Radiochemicals) with cloned T4 polynucleotide kinase (U.S. Biochemicals Corp.) (16). Control DNAs were HR-1 cellular clones 5 (het⁺) and 16 (het⁻) (21) as well as cytomegalovirus, varicella-zoster virus, and human DNA. Test samples consisted of total cellular DNA extracted from the hairy leukoplakia lesions of 10 patients with acquired immunodeficiency syndrome. All samples were positive for EBV DNA by standard Southern blot hybridization. Analysis of the EBV termini identified abundant arrays of terminal fragments representing linear viral DNA of a highly lytic infection (19). In addition to the standard *Bam*HI-Z, a 1.3-kilobase-pair fragment homologous to *Bam*HI-Z was detected at very low levels in the DNA from 1 of the 10 patient samples, suggesting the presence of a rearranged EBV genome (data not shown).

PCR amplification of 1 μ g of total cellular DNA extracted from biopsies of hairy leukoplakia was performed with *Thermus aquaticus* (*Taq*) DNA polymerase in a reaction mixture of 100 μ l as described in detail elsewhere (15). Samples were boiled for 10 min; heating cycles of 2 min at 45°C, 2 min at 63°C, and 1 min at 90°C were performed 30 times on a DNA Thermal Cycler (Perkin Elmer Cetus Corp.). PCR product (18 μ l per well) was electrophoresed on a 3% NuSieve-1% SeaKem agarose gel (FMC Corp.) and transferred to a Nytran membrane (Schleicher & Schuell, Inc.). Blots were prehybridized at 51°C for 3 h in 6 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.2% sodium dodecyl sulfate-5 \times Denhardt solution with 0.5 mg of denatured calf thymus DNA per ml. Hybridization was performed overnight at 51°C in hybridization fluid containing ³²P-labeled probes (10⁶ cpm/ml).

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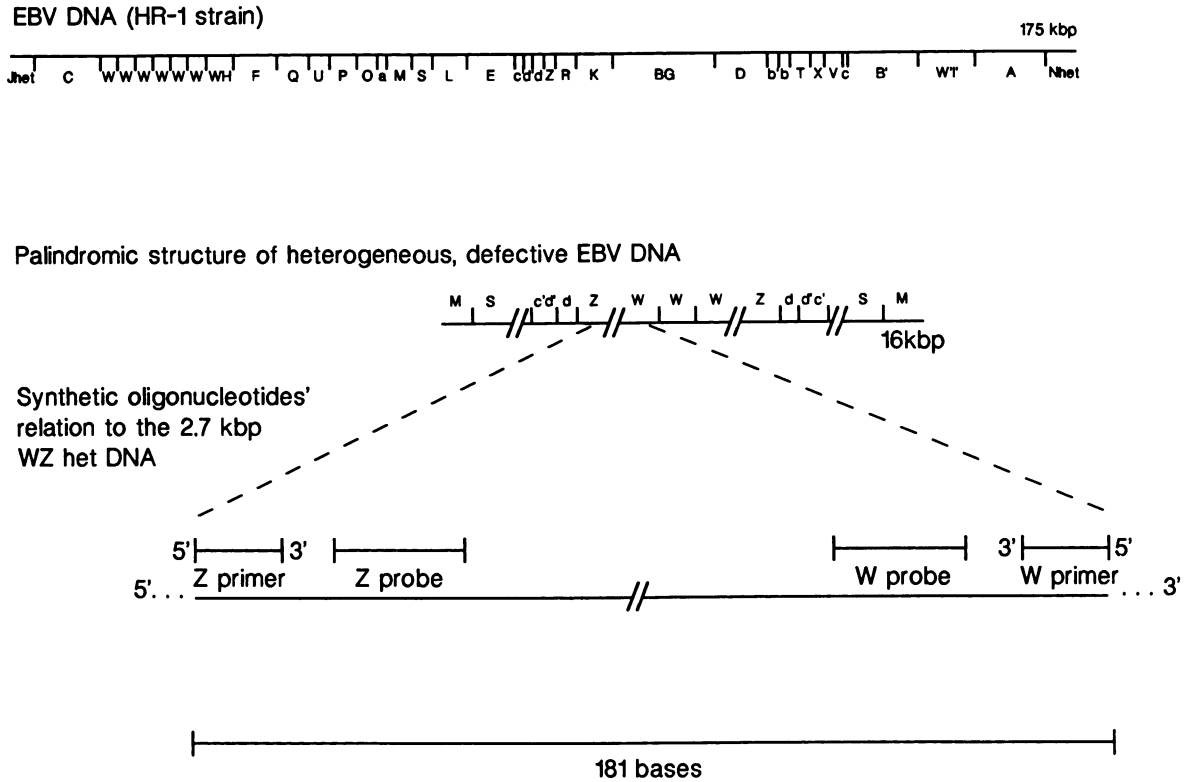


FIG. 1. Organization of the central palindrome of heterogeneous EBV DNA present in HR-1 cellular clone 5 and relation of synthetic oligonucleotides to the 181-base sequence targeted for PCR amplification (restriction endonuclease maps adapted from Rabson et al. [20] and Jenson et al. [12]). Symbol: //, junction of DNA fragments that are not contiguous in the standard HR-1 genome but are abnormally juxtaposed in heterogeneous DNA. Primer sequences used to amplify the WZhet junction were 5' AATAGACAGCCCAGTTGAAA 3' (Z specific) and 5' GTCCAGCGGTTTACGTAAG 3' (W specific). Oligonucleotide probes used for detection of the PCR amplification products were 5' CATGCAGCAGACATTCATCATTTAGAAATG 3' (Z probe) and 5' AGTGGTCCCCCTCCCTAGAACTGACAATTG 3' (W probe). kbp, Kilobase pairs.

After DNA amplification, 2 of 10 patient samples (including the one with an anomalous *Bam*HI-Z signal on standard Southern blot) had a PCR product that hybridized to both the *Bam*HI-Z- and *Bam*HI-W-specific oligonucleotide probes, indicating a genomic rearrangement (Fig. 2, lanes 2 and 9). No product could be detected in the remaining eight patient samples or in the negative controls containing irrelevant and het⁻ DNA. Amplification of het⁺ HR-1 cellular clone 5 yielded two bands 181 and 120 bases in length.

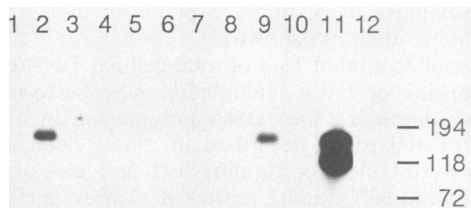


FIG. 2. Rearranged (WZhet) EBV DNA from oral hairy leukoplakia lesions of patients with acquired immunodeficiency syndrome. Southern blot analysis of the PCR products from patient lesions (lanes 1 through 10), positive control HR-1 cellular clone 5 (het⁺) (lane 11), and negative control HR-1 cellular clone 16 (het⁻) (lane 12) demonstrated WZhet DNA in patient samples 2 and 9 when both the Z-specific (above) and W-specific (not shown) probes were used for hybridization. The doublet band in lane 11 represents abnormal product migration due to secondary structure (see text). The molecular weight marker (*Hae*III digestion of ϕ X174 replicative-form DNA) is in base pairs.

To determine the basis of this size variability, we cloned PCR products from the het⁺ control and clinical sample 9 (Fig. 2, lanes 11 and 9, respectively). Primers, phosphorylated at their 5' ends, were used in the PCRs from which DNA for cloning was obtained. Phosphorylated PCR products were blunt end ligated into dephosphorylated *Pvu*II-restricted pATX, and the ligation products were used to transform competent MC1061 cells (16, 23). Positive clones were identified by Benton-Davis screening with the Z-specific probe (2). Recombinant DNA was sequenced by the dideoxy-chain termination method with Sequenase (U.S. Biochemicals) and oligonucleotide primers complementary to pATX vector sites (24). On sequence analysis, the cloned PCR product from the clinical sample was 160 bases in length (Fig. 3). Moreover, the point of recombination between *Bam*HI-W and *Bam*HI-Z in the patient het DNA was shifted in relation to the prototype het DNA (13), so that an additional 45 bases of *Bam*HI-W were included. Recombination occurred at a single adenosine residue shared by both fragments. This alteration led to the preservation within the wild-type recombinant of the eucaryotic promoter sequence TATAAA contained in *Bam*HI-W but interrupted by recombination of *Bam*HI W and Z fragments in laboratory defective virus (13).

Sequences of all het⁺ clones agreed with the published sequence of the targeted 181-base segment (Fig. 3, top sequence). When the PCR product was subjected to electrophoresis on a denaturing polyacrylamide gel, the doublet

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1470      1480      1490      1500      1510      1520      1530
AA TAGACAGCCC AGTTGAAATA TGCATGGCAT GCAGCAGACA TTCATCATT AGAAATGTAT
AA TAGACAGCCC AGTTGAAATA TGCATGGCAT GCAGCAGACA .....

      1540      1550      1560      1570      1580      1590
CCAAGATTTC ATTAAGTTCC GGGGTCAGGC GCGACTCCAG ATTCAAAATAC CAGGGGCAGT
.ctcctggcg ctcctgatgcg accagaaata gctgcaggac cactttATAC CAGGGGCAGT

      1600      1610      1620      1630      1640      1650
GGTCCCCCTC CCTAGAACTG ACAATGCGCT GCTGTCTGGC TTACGTAAAC GCGCTGGAC
GGTCCCCCTC CCTAGAACTG ACAATGgCT GCTGTCTGGC TTACGTAAAC GCGCTGGAC

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FIG. 3. Altered WZhet junction of defective EBV DNA in hairy leukoplakia lesions. The top line is the sequence from 15 clones of PCR product obtained with the HR-1 clone 5 (het⁺) control and is identical to the predicted 181-base published sequence (13). The bottom sequence is a 160-base variant from clinical sample 9. Numerical designations are sequence coordinates of the 2.7-kilobase-pair WZhet DNA per Jenson et al. (13). Stippled areas, *Bam*HI-Z; unstippled areas, *Bam*HI-W; boxed residues, bases shared by both fragments that form the recombination site; lowercase letters, additional 45 bases of standard *Bam*HI-W; underlining, putative promoter sequence TATAAA; boldface type, region of PCR primers. Base 1618 in the patient sample differs from that in het DNA but concurs with the *Bam*HI-W sequence of EBV prototypes B95-8 and HR-1 (12).

seen on the agarose gel (Fig. 2) resolved into a single 181-base band, indicating that secondary structure was the cause of this aberrant migration pattern (data not shown).

Ready detection of the W-Z rearrangement by PCR but not by standard blot hybridization techniques suggests that a minimal number of defective virions may be generated. Although defective EBV replicates preferentially to standard virus in tissue culture (17), this advantage may not hold for heterogeneous cell populations *in vivo*. However, a low copy number does not lessen the potential biologic significance of the defective virus. In contrast to defective interfering particles that inhibit replication by appropriating the viral replicative machinery, the het DNA of defective EBV appears to enhance replication by *trans* activation of the lytic origin of DNA replication, a mechanism that conceivably would operate if only a single copy of the defective genome were present.

The apparent absence of het DNA in eight clinical samples could be interpreted as evidence that the defective DNA is not causal but rather the occasional by-product of active virus replication. However, in experimental systems with other viruses, in which the introduction of defective virus clearly altered the disease course, isolation of defective interfering particles has proven difficult (1). In our study, the variability in the W-Z junction of wild-type het DNA may further complicate detection of this molecular alteration by PCR when the point of recombination occurs outside the region demarcated by a particular primer set (Fig. 3). Alternatively, single base substitutions in the regions of het DNA recognized by the PCR primers may interfere with primer annealing.

The presence of rearranged genomic EBV DNA in a lesion noted for its abundant replicative virus may represent a novel strategy by which persistent viruses move, by way of recombinational events, from a latent to a replicative stage in their life cycle. Because the ability of viruses to generate defective particles *in vitro* often depends on the cell type infected (1), the detection of defective EBV molecules in hairy leukoplakia lesions suggests that epithelial cells may be critical for EBV recombinational events. The enhanced susceptibility of the epithelium to this mode of viral pathogenesis may underlie the divergent biology of EBV at lymphoid and epithelial sites.

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