JOURNAL OF VIROLOGY, Sept. 1992, p. 5646–5650
0022-538X/92/095646-05$02.00/0

Vol. 66, No. 9

Characterization of the Deletion and Rearrangement in the *Bam*HI C Region of the X50-7 Epstein-Barr Virus Genome, a Mutant Viral Strain Which Exhibits Constitutive *Bam*HI W Promoter Activity

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Received 11 February 1992/Accepted 2 June 1992

Epstein-Barr virus infection of peripheral B lymphocytes predominantly results in a latent infection, with a concomitant growth transformation of the infected cells. These cells express six nuclear antigens (EBNAs) and three membrane antigens. Transcription of all the EBNA genes is driven by one of two promoters, Cp or Wp, located near the left end of the viral genome, and the activities of these promoters are mutually exclusive. We have previously shown that Wp is exclusively used during the initial stages of B-cell immortalization, followed by a switch to Cp usage. However, several cell lines which appear to have failed to switch from Wp to Cp usage and which exhibit constitutive Wp activity have been identified. In two cases, we have shown that this failure to switch is the result of a deletion of approximately 3.5 kb, spanning Cp. In this paper, we characterize the deletion in one of these cell lines, X50-7, and demonstrate not only that the viral genome in this cell line has sustained a deletion in the region of Cp, but also that there has been a rearrangement into the *Bam*HI C region of viral sequences from the *Bam*HI W and Y fragments.

Epstein-Barr virus (EBV) is a human herpesvirus which infects human B cells and induces transformation in vitro. During latent infection, six viral nuclear antigens (EBNAs) and three membrane-associated proteins are expressed. Six of these antigens are transcribed from a major transcriptional unit that is driven by one of two promoters, Wp or Cp (17, 20). These two promoters are located near the left end of the viral genome and are expressed in a mutually exclusive fashion (19) (see Fig. 1). We have previously shown that Wp is exclusively employed during the initial stages of infection of peripheral B lymphocytes and then a switch to Cp usage occurs. Switching from Wp to Cp usage appears to involve activation of an EBNA 2-dependent enhancer located upstream of Cp (18). Indeed, infection of cells with a nonimmortalizing strain of the virus (clone 13, derived from a subclone of the P3HR-1 cell line), which lacks the EBNA 2 gene, results in Wp activity and failure to switch to Cp usage (18).

Several established EBV-infected cell lines that drive transcription of the EBNA genes from Wp rather than Cp have been identified. We have shown that in the X50-7 and IB4 lymphoblastoid cell lines, constitutive Wp activity is the result of a deletion in the endogenous viral genomes in the *Bam*HI C region spanning Cp. Furthermore, transfection of a Cp-using cell line, JY, with a reporter construct with a 2.8-kb deletion in *Bam*HI C comprising Cp resulted in the utilization of Wp (20). However, it should be noted that the level of Wp activity observed with this construct was significantly lower than the level of Cp activity observed with reporter constructs lacking the deletion. The latter result suggested that activation of Wp in these cell lines might involve more than deletion of Cp.

Probes. The probes used for hybridization to Southern blots were prepared as follows (Fig. 1). The oriP probe was a 1.82-kb *Eco*RI-*Hpa*I fragment, and probe C/ERI was the 3.15-kb *Eco*RI fragment of *Bam*HI C. Probe Y was a 0.35-kb *Hpa*I-*Hin*dIII fragment from the unique region of the viral *Bam*HI Y fragment. Probe X50-2A was an *Eco*RI-*Xho*I fragment from the X50-7/4A4 clone, and the terminal repeat probe was an *Eco*RI (nucleotides 23013 to 26182) fragment from the plasmid p554 (4). Probes were excised from their respective vectors and were labeled by random priming with a degenerate hexamer mix (Boehringer Mannheim).

oriP is present in the deleted *Bam*HI C fragment of the X50-7 EBV genome. To determine whether the latency-associated origin of replication oriP is present in the deleted *Bam*HI C fragment, dC, of the X50-7 genome, *Bam*HI-digested DNA prepared from the X50-7 and B95.8 cell lines was hybridized with an oriP probe (*Eco*RI-*Hpa*I fragment of *Bam*HI C) (Fig. 1). As shown in Fig. 2, the expected 9.2-kb fragment was observed with DNA from the B95.8 cell line, whereas DNA prepared from the X50-7 cell line revealed a single fragment of about 5.7 kb (*Bam*HI dC). This result corresponds to the size of the deleted *Bam*HI C fragment previously observed in the X50-7 cell line and provides direct evidence that oriP is contained within the deleted *Bam*HI C fragment of X50-7.

Cloning the deleted *Bam*HI C fragment from X50-7 EBV genome. To clone the relevant region of *Bam*HI dC from the X50-7 cell line, a genomic library was generated by *Eco*RI and *Xho*I digestion of genomic DNA, followed by cloning into the lambda vector UniZAP-XR (Stratagene). Digestion of X50-7 DNA with these enzymes generates a fragment of ca. 1.5 kb, which hybridizes to an oriP probe (data not shown). Approximately, 10^6 recombinant phages were screened with a ^{32}P-labeled oriP probe. A single positive clone (X50-7/4A4) was isolated and subcloned into the Bluescript vector (Stratagene) by in vivo excision.

When the X50-7/4A4 clone was digested with *Eco*RI and *Xho*I, an apparent doublet of approximately 3.0 kb was observed in addition to the expected 1.5-kb insert (Fig. 3). This digestion was expected to generate a single vector fragment of 3.0 kb, and thus the origin of the other putative

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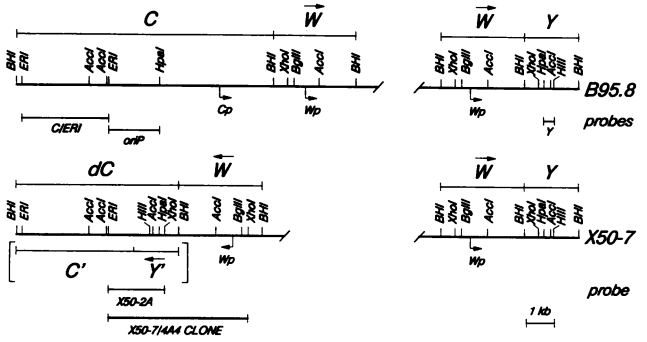

FIG. 1. Restriction enzyme maps of the EBV *Bam*HI C, W, and Y regions in the B95.8 and X50-7 strains. Relative orientations of various fragments are indicated by arrows. The EBNA promoters Wp and Cp and the direction of transcription are also indicated. dC, deleted *Bam*HI C fragment in the X50-7 EBV genome which consists of rearranged *Bam*HI C (C') and *Bam*HI Y (Y') fragments. Restriction endonucleases: BHI, *Bam*HI; ERI, *Eco*RI; HIII, *Hin*dIII. The labeled probes used for hybridization to Southern blots were prepared as described in the text.

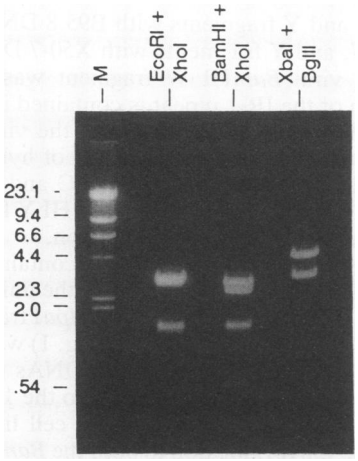

FIG. 3. Ethidium bromide-stained agarose gel of restriction endonuclease-digested clone X50-7/4A4. M, *Hin*dIII-digested bacteriophage lambda DNA markers. Sizes (in kilobases) are on the left.

3.0-kb fragment was unclear. Hybridization of *Eco*RI- and *Xho*I-digested X50-7/4A4 DNA with a *Bam*HI C probe (lacking vector sequences) revealed hybridization to both the 1.5- and the 3.0-kb fragments (data not shown). This result suggested two possibilities: (i) a partial digestion product had been cloned, or (ii) we had recovered a clone containing two distinct inserts. Since the EBV *Bam*HI C probe hybridized to both the 3.0- and the 1.5-kb fragments, this suggested that a partial digestion product may have been cloned. Indeed, a partial digestion product containing a complete *Bam*HI W repeat would be expected to yield a 3.0-kb fragment upon *Eco*RI-*Xho*I digestion, which would hybridize to a *Bam*HI C probe (a portion of the IR-1 repeat is contained in the viral *Bam*HI C fragment).

Further characterization of the X50-7/4A4 clone by digestion with *Bam*HI and *Xho*I, or with *Xba*I and *Bgl*II, confirmed the initial suspicion that, in addition to the expected 1.5-kb *Eco*RI-*Xho*I fragment from the *Bam*HI dC region, another insert was present (Fig. 3). Digestion with *Bam*HI and *Xho*I generated a complex profile composed of four fragments (ca. 3.0, 2.5, 1.5, and 0.5 kb), with the 3.0-kb fragment corresponding to the cloning vector. Digestion with *Xba*I and *Bgl*II generated only two fragments, the 3.0-kb vector fragment and a 4.4-kb insert fragment. For the initial characterizations, the 1.5-kb *Eco*RI-*Xho*I fragment was subcloned into the Bluescript vector (clone X50-2A).

***Bam*HI dC in X50-7 contains a rearranged fragment from *Bam*HI Y.** To verify that the subcloned 1.5-kb *Eco*RI-*Xho*I fragment of the X50-7/4A4 clone corresponded to the X50-7 *Bam*HI dC fragment, genomic DNAs prepared from the B95.8 and X50-7 cell lines were digested with *Bam*HI, fractionated on agarose gels, and probed with ^{32}P-labeled clone X50-2A. As shown in Fig. 4A, the X50-2A clone hybridized to fragments corresponding in size to the viral

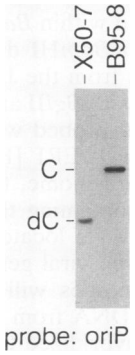

FIG. 2. Genomic Southern blot of *Bam*HI-digested DNA prepared from the B95.8 and X50-7 cell lines. The blot was probed with a fragment of the EBV genome containing oriP (Fig. 1) isolated from the B95.8 strain (1). C, wild-type 9.2-kb *Bam*HI C fragment; dC, deleted form of the fragment in the X50-7 cell line. Genomic DNA was prepared according to a standard protocol (11). Genomic DNA was digested with appropriate restriction endonucleases, fractionated on 0.4 to 0.7% agarose gels, and transferred to nitrocellulose filters (Schleicher & Schuell) by capillary blotting. Hybridization was carried out with ^{32}P-labeled probes as previously described (11).

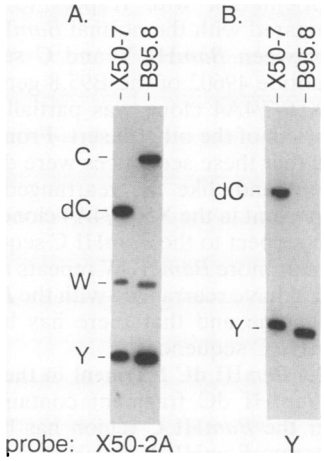

FIG. 4. Genomic Southern blots of *Bam*HI-digested B95.8 and X50-7 DNA. (A) The blot was probed with the X50-2A subclone (Fig. 1). C, wild-type *Bam*HI C fragment; dC, deleted form of the fragment, present in the X50-7 cell line; W, hybridization to the viral 3.0-kb *Bam*HI W fragment; Y, cross-hybridization to the 1.8-kb viral *Bam*HI Y fragment. (B) A blot similar to that in panel A was hybridized with a probe from the unique region of the EBV *Bam*HI Y fragment (which lacks IR-1 sequences; see probe Y in Fig. 1). Y and dC are as defined for panel A.

*Bam*HI C, W, and Y fragments with B95.8 DNA and to the *Bam*HI dC, W, and Y fragments with X50-7 DNA. Hybridization to the viral *Bam*HI W fragment was anticipated, since a portion of the IR-1 repeat is contained in the *Bam*HI C fragment. However, hybridization to the viral *Bam*HI Y fragment was unexpected. The intensity of hybridization of the X50-2A clone to the viral *Bam*HI C and Y fragments suggested that a portion of the viral *Bam*HI Y fragment may have rearranged into the *Bam*HI C region.

To determine whether *Bam*HI dC contains sequences from the *Bam*HI Y region, a probe from the unique region of the viral *Bam*HI Y fragment (a *Hin*dIII-*Hpa*I fragment which lacks any IR-1 sequences; probe Y in Fig. 1) was hybridized to *Bam*HI digests of B95.8 and X50-7 DNAs (Fig. 4B). As expected, the Y probe hybridized only to the 1.8-kb *Bam*HI Y fragment with DNA from the B95.8 cell line. However with X50-7 DNA, hybridization to both the *Bam*HI dC and Y fragments was observed. Thus, a portion of the *Bam*HI Y fragment appears to have rearranged with viral *Bam*HI C sequences in the X50-7 genome. Notably, the latter results provide strong supporting evidence that the X50-2A subclone was derived from the X50-7 *Bam*HI dC fragment.

Sequence analysis of the X50-7/4A4 clone. The complete sequence of the X50-2A subclone, along with partial sequence analysis of the other insert present in the X50-7/4A4 parent clone, is shown in Fig. 5. Sequencing from the *Eco*RI site in the X50-2A subclone (left-hand end with respect to the maps in Fig. 1), the first 727 bp correspond to *Bam*HI C sequences (referred to as C′) and contain 20 copies of the 30-bp direct repeats found in oriP (10). The last base pair from this region corresponds to nucleotide 8031 in the wild-type B95.8 EBV sequence (1). There are 10 bp after this nucleotide, which are not present in the B95.8 genome and which match closely the last 10 bp of the 30-bp repeat consensus (10) (Fig. 5B). Thus, while the last 30-bp repeat in the B95.8 genome is incomplete, it is complete in the X50-7 *Bam*HI dC fragment.

Immediately downstream of the 30-bp repeats is 1,319 bp (799 bp to the *Xho*I site) from the viral *Bam*HI Y region (Y′), which has recombined with *Bam*HI C and is positioned in the opposite orientation with respect to the *Bam*HI C sequences (compared with the normal *Bam*HI Y fragment). The junction between *Bam*HI Y and C sequences corresponds to nucleotide 49602 of the B95.8 genome (1).

The parent X50-7/4A4 clone was partially sequenced to determine the origin of the other insert. From this analysis it was determined that these sequences were derived from the *Bam*HI W repeat and, like the rearranged *Bam*HI Y sequences, were present in the X50-7/4A4 clone in the opposite orientation with respect to the *Bam*HI C sequences. Thus, it appears that one or more *Bam*HI W repeats along with 1,319 bp from *Bam*HI Y have rearranged with the *Bam*HI C region in the X50-7 genome and that there has been a 5,184-bp deletion of *Bam*HI C sequences.

Location of the *Bam*HI dC fragment in the X50-7 genome. Although the *Bam*HI dC fragment contains oriP, it was unclear whether the *Bam*HI C region has been rearranged into the downstream *Bam*HI W and Y region or vice versa. To determine whether *Bam*HI dC is linked to sequences normally upstream of oriP, Southern hybridization analyses with appropriate probes and restriction endonucleases were carried out (Fig. 6). As expected, when the B95.8 DNA was digested with *Bam*HI and probed with C/ERI (a 3.15-kb fragment from *Bam*HI C [Fig. 1]), a 9.2-kb *Bam*HI fragment (Fig. 6A) was revealed. With X50-7 DNA, *Bam*HI dC also hybridized to this probe, indicating that the 3.0-kb fragment

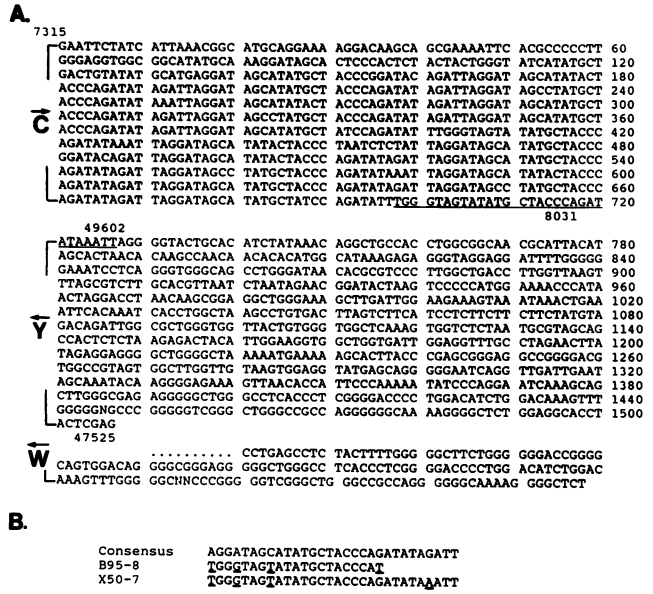

FIG. 5. (A) Complete sequence of the X50-2A subclone and partial sequence of the 3′ end of the parent X50-7/4A4 clone. The last 30-bp direct repeat (10) in oriP is underlined and is juxtaposed with sequences from the viral *Bam*HI Y fragment. The numbers over the sequence correspond to the nucleotide numbers in the sequence of the B95.8 viral strain (1). The origins of the sequences in the X50-7 *Bam*HI dC fragment are indicated, and the arrows indicate the orientations with respect to sequences present in the B95.8 genome. A partial sequence of the fragment adjacent to the rearranged *Bam*HI Y sequences in the X50-7/4A4 clone is shown, with the dots indicating the region not sequenced. Overlapping exonuclease III deletion clones were generated with an Erase-a-base kit (Promega). Double-stranded DNA was sequenced by the chain termination method of Sanger et al. (14). Both strands were sequenced by employing either deletion clones or specific oligonucleotides when a deletion clone was not available for a particular region. (B) Comparison of the last tandem direct repeat in the X50-7 *Bam*HI dC fragment with that present in the B95.8 genome and the consensus sequence (10). Nucleotides which differ from the consensus sequence are underlined.

(*Eco*RI J) immediately upstream of oriP in the wild-type viral genome is also contained within *Bam*HI dC.

To determine whether *Bam*HI dC is linked to the fused terminal repeats, DNAs from the JY, X50-7, and D98-HR1 cell lines were digested with *Bgl*II and *Hpa*I, fractionated on a 0.4% agarose gel, and probed with fragments unique to either *Bam*HI C (probe C/ERI [Fig. 1]) or the terminal repeats. In the wild-type genome, the *Bgl*II and *Hpa*I sites lie outside the region containing the terminal repeats and oriP. Thus, if *Bam*HI dC is located in the same place as *Bam*HI C in the wild-type viral genome, then the terminal repeat and *Bam*HI C probes will hybridize to the same fragment. Indeed, with DNA from all three cell lines, both probes C/ERI and TR hybridized to fragments of the same size, although the size of the fragment varied between cell lines. This variation is predominantly due to the presence of a variable number of terminal repeats. In addition, the presence of a single fragment which hybridized to the terminal repeat probe indicates that the viral genomes present in these cell lines have fused terminal repeats and thus most likely exist as covalently closed circles.

An inverted *Bam*HI W fragment is contiguous with the *Bam*HI dC fragment. A question raised by the data above is

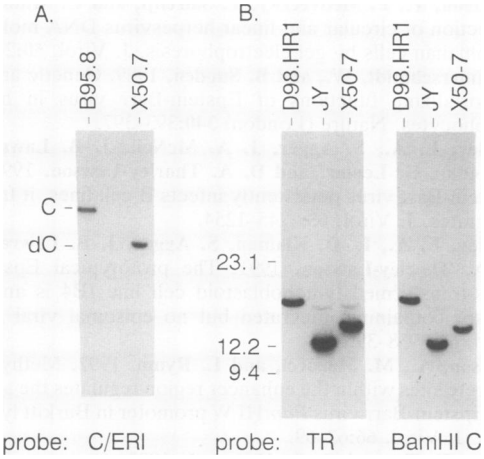

FIG. 6. Genomic Southern blots of the B95.8 and X50-7 DNAs. (A) DNA was digested with *Bam*HI and probed with a 3.0-kb *Eco*RI fragment (C/ERI [Fig. 1]) from the viral *Bam*HI C region. C, wild-type *Bam*HI C fragment; dC, deleted form of the fragment in the X50-7 genome. (B) Genomic DNAs from cell lines D98-HR1 (D98.HR1), JY, and X50-7 were digested with *Bgl*II and *Hpa*I and probed with terminal repeats (TR) and a 3.0-kb *Eco*RI fragment from *Bam*HI C (probe C/ERI [Fig. 1]). The migration of *Hin*dIII-digested bacteriophage lambda DNA fragments is indicated. Sizes (in kilobases) are on the left.

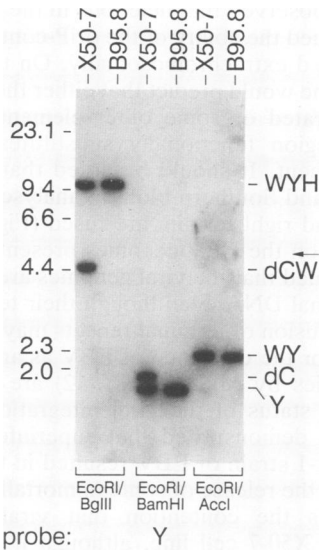

FIG. 7. Genomic Southern blots of the B95.8 and X50-7 DNAs. DNA was digested with *Eco*RI and *Bgl*II, *Eco*RI and *Bam*HI, or *Eco*RI and *Acc*I, and the blots were probed with a fragment from the unique region of *Bam*HI Y (probe Y [Fig. 1]). WYH, a fragment derived from the *Bam*HI W, Y, and H regions of the EBV genome; WY, a fragment from *Bam*HI W and Y; dCW and dC, rearranged fragments present in the X50-7 genome. The sizes (in kilobases) of *Hin*dIII-digested bacteriophage lambda DNA fragments are given.

whether the presence of *Bam*HI W in the X50-7/4A4 clone occurred as a result of cloning a partial *Xho*I digestion product (failure to cleave at the *Xho*I site present in the Y′ sequences of the *Bam*HI dC fragment). To address this question, Southern blot analyses of the B95.8 and X50-7 DNAs digested with appropriate restriction endonucleases were probed with a labeled fragment from the *Bam*HI Y unique region (this fragment hybridizes to the *Bam*HI dC fragment from X50-7 but does not hybridize to the *Bam*HI C fragment of B95.8; see probe Y in Fig. 1) (Fig. 7). The choice of restriction enzymes was based on the deduced X50-7 map illustrated in Fig. 1. As an internal control for probe specificity, the DNAs were digested with *Eco*RI and *Bam*HI. This resulted in detection of a 1.8-kb fragment which was present in both cell lines and which corresponds to the wild-type *Bam*HI Y fragment. In addition, an *Eco*RI-*Bam*HI fragment of ca. 2.0 kb, generated from *Bam*HI dC, was detected with DNA from the X50-7 cell line. However, hybridization of the probe to fragments from the B95.8 *Bam*HI C region (or from the *Bam*HI W region) was not observed.

When the B95.8 and X50-7 DNAs were digested with *Eco*RI and *Acc*I, a single fragment of ca. 2.3 kb hybridized to the probe in both cases. This fragment corresponds to an *Acc*I fragment containing the *Bam*HI W and Y sequences (Fig. 1). Since no DNA fragments of other sizes from X50-7 hybridized to the probe, this is evidence that the *Bam*HI W fragment adjacent to *Bam*HI dC is in the same orientation as the *Bam*HI Y sequences. Finally, *Eco*RI and *Bgl*II digestion of B95.8 DNA revealed a single large fragment that hybridized to the probe. This fragment was also detected with X50-7 DNA, and its size corresponds to the size of the expected fragment containing sequences from the *Bam*HI W, Y, and H regions of the viral genome. With X50-7 DNA, in addition to the large fragment, a smaller fragment, which is composed of sequences from the *Bam*HI dC and W regions of the X50-7 genome, was also detected. The size of this smaller fragment (4.6 kb) corresponds to the size which was predicted on the basis of an inverted orientation of the *Bam*HI W fragment adjacent to *BamHI* dC. If the adjacent *Bam*HI W fragment had the normal orientation, the predicted size of the fragment would be ca. 2.3 kb.

Conclusions. In an effort to determine what sequences lie upstream of Wp in a cell line that constitutively utilizes Wp, the region of the X50-7 *Bam*HI dC fragment which had sustained a deletion was cloned and characterized. Characterization of the X50-7/4A4 clone, and subsequent confirmation by Southern blot analyses, revealed that there had been a significant rearrangement of the *Bam*HI W and Y sequences into the *Bam*HI C region of the X50-7 genome with the deletion of the 5,184-bp *Bam*HI C sequence. In addition, the *Bam*HI W and Y sequences are present in inverted orientations. The X50-7 *Bam*HI dC fragment contains 727 bp from oriP, which is composed of 20 tandem copies of the 30-bp direct repeats (10), but lacks the dyad symmetry elements. Immediately downstream of the 30-bp direct repeats is 1.3 kb of the *Bam*HI Y sequence, which is contiguous with sequences from the *Bam*HI W region of the EBV genome. At least one complete *Bam*HI W repeat is present in an inverted orientation, and we cannot rule out the possibility that two or more copies have been rearranged. It is attractive to speculate that this rearrangement has led to constitutive Wp activity in the X50-7 cell line. To date, however, we have been unable to generate reporter constructs containing inverted *Bam*HI W repeats to test this hypothesis.

Previous studies have identified two essential elements in oriP required for maintenance of the virus in an episomal form during latency. The first element consists of multiple copies of a 30-bp direct repeat, and the second element is a 65-bp sequence with dyad symmetry (10). From sequence analysis of the X50-7 *Bam*HI dC fragment, it appears that the oriP of X50-7 lacks the dyad symmetry element. Chittenden

et al. (2) have observed that deletions in the dyad symmetry element abolished the ability of the oriP-containing plasmids to be maintained extrachromosomally. On the basis of this observation, one would predict that either the X50-7 genome must be integrated or some other element(s) outside the *Bam*HI dC region functionally substitutes for the dyad symmetry element. It should be noted that our restriction endonuclease and Southern blotting analyses demonstrated that the left and right termini are fused (Fig. 6). However, earlier studies on the EBV genomes present in the IB4 cell line have revealed that the viral genomes are integrated into the chromosomal DNA even though their termini are fused (6). Thus, the fusion of terminal repeats may not be taken as the evidence for the existence of EBV as an episome.

Earlier studies by Miller et al. (12) are important with respect to the status of the viral integration in the X50-7 genome. They demonstrated that superinfection of X50-7 with the P3HR-1 strain of EBV resulted in the induction of lytic cycle and the release of some immortalizing virus. This result supports the contention that viral episomes are present in the X50-7 cell line, although it is possible that the immortalizing virus recovered was the result of recombination between integrated X50-7 genomes and episomal P3HR-1 genomes. Our preliminary experiments with Gardella gel electrophoresis (3) have indicated the existence of EBV in X50-7 as an episome (data not shown). We cannot rule out the possibility of the presence of polymorphic forms of the virus. Although none of our restriction analyses of X50-7 indicates that the virus in these cells is polymorphic, further studies involving different experimental approaches, such as in situ hybridization analysis, will be required to resolve this question.

A number of deletions in the *Bam*HI C and W regions of EBV have been reported. Two Burkitt's lymphoma cell lines, Daudi and P3HR-1, have sustained deletions whose upstream junction maps within the *Bam*HI W repeats and extends through *Bam*HI Y and into the adjacent *Bam*HI H fragment (8, 9). A deletion of approximately 0.3 kb in the *Bam*HI C region was reported for another Burkitt's Lymphoma cell line, Raji (16). The latter is of interest, since Raji does not exhibit either Cp or Wp activity (19). In addition, studies with EBV-converted BL-41 cell lines have revealed deletions in the *Bam*HI C region of the viral genome in three different cell lines (5).

All the EBNA genes appear to be transcribed from either Wp or Cp (17), with the notable exception of the EBNA 1 transcripts present in group 1 Burkitt's lymphoma cell lines (7, 13, 15). As the translated proteins of these transcripts are undoubtedly involved in many of the functions essential for viral transformation of B lymphocytes, one would imagine that there exists a large negative selective pressure against viral genomes with gross rearrangements within this region. Therefore, the frequency of rearrangements in this region may be higher than has been observed. The underlying mechanism involved in the recombination within this region is unclear.

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