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

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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-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 involves 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 BamHI C region spanning Cp. Furthermore, transfection of a Cp-using cell line, JY, with a reporter construct with a 2.8-kb deletion in BamHI 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 EcoRI-HpaI fragment, and probe C/ERI was the 3.15-kb EcoRI fragment of BamHI C. Probe Y was a 0.35-kb HpaI-HinIII fragment from the unique region of the viral BamHI Y fragment. Probe X50-2A was an EcoRI-XhoI fragment from the X50-7/4A4 clone, and the terminal repeat probe was an EcoRI (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 BamHI C fragment of the X50-7 EBV genome. To determine whether the latency-associated origin of replication oriP is present in the deleted BamHI C fragment, dC, of the X50-7 genome, BamHI-digested DNA prepared from the X50-7 and B95.8 cell lines was hybridized with an oriP probe (EcoRI-HpaI fragment of BamHI 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 (BamHI dC). This result corresponds to the size of the deleted BamHI C fragment previously observed in the X50-7 cell line and provides direct evidence that oriP is contained within the deleted BamHI C fragment of X50-7.

Cloning the deleted BamHI C fragment from X50-7 EBV genome. To clone the relevant region of BamHI dC from the X50-7 cell line, a genomic library was generated by EcoRI and XhoI 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, 106 recombinant phages were screened with a 32P-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 EcoRI and XhoI, 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 BamHI 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 BamHI C fragment in the X50-7 EBV genome which consists of rearranged BamHI C (C') and BamHI Y (Y') fragments. Restriction endonucleases: BHI, BamHI; ERI, EcoRI; HII, HindIII. The labeled probes used for hybridization to Southern blots were prepared as described in the text.

3.0-kb fragment was unclear. Hybridization of EcoRI- and XhoI-digested X50-7/4A4 DNA with a BamHI 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 BamHI 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 BamHI W repeat would be expected to yield a 3.0-kb fragment upon EcoRI-XhoI digestion, which would hybridize to a BamHI C probe (a portion of the IR-1 repeat is contained in the viral BamHI C fragment).

Further characterization of the X50-7/4A4 clone by digestion with BamHI and XhoI, or with XbaI and BglII, confirmed the initial suspicion that, in addition to the expected 1.5-kb EcoRI-XhoI fragment from the BamHI C region, another insert was present (Fig. 3). Digestion with BamHI and XhoI 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 XbaI and BglII 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 EcoRI-XhoI fragment was subcloned into the Bluescript vector (clone X50-2A).

BamHI dC in X50-7 contains a rearranged fragment from BamHI Y. To verify that the subcloned 1.5-kb EcoRI-XhoI fragment of the X50-7/4A4 clone corresponded to the X50-7 BamHI dC fragment, genomic DNAs prepared from the B9.5 and X50-7 cell lines were digested with BamHI, fractionated on agarose gels, and probed with 32P-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 BamHI-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 BamHI 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 32P-labeled probes as previously described (11).


FIG. 4. Genomic Southern blots of BamHI-digested B95.8 and X50-7 DNA. (A) The blot was probed with the X50-2A subclone (Fig. 1). C, wild-type BamHI C fragment; dC, deleted form of the fragment, present in the X50-7 cell line; W, hybridization to the viral 3.0-kb BamHI W fragment; Y, cross-hybridization to the 1.8-kb viral BamHI Y fragment. (B) A blot similar to that in panel A was hybridized with a probe from the unique region of the EBV BamHI Y fragment (which lacks IR-1 sequences; see probe Y in Fig. 1). Y and dC are as defined for panel A.
BamHI C, W, and Y fragments with B95.8 DNA and to the BamHI dC, W, and Y fragments with X50-7 DNA. Hybridization to the viral BamHI W fragment was anticipated, since a portion of the IR-1 repeat is contained in the BamHI C fragment. However, hybridization to the viral BamHI Y fragment was unexpected. The intensity of hybridization of the X50-2A clone to the viral BamHI C and Y fragments suggested that a portion of the viral BamHI Y fragment may have rearranged into the BamHI C region.

To determine whether BamHI dC contains sequences from the BamHI Y region, a probe from the unique region of the viral BamHI Y fragment (a HindIII-HpaI fragment which lacks any IR-1 sequences; probe Y in Fig. 1) was hybridized to BamHI digests of B95.8 and X50-7 DNAs (Fig. 4B). As expected, the Y probe hybridized only to the 1.8-kb BamHI Y fragment with DNA from the B95.8 cell line. However, with X50-7 DNA, hybridization to both the BamHI dC and Y fragments was observed. Thus, a portion of the BamHI Y fragment appears to have rearranged with viral BamHI 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 BamHI 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 EcoRI site in the X50-2A subclone (left-hand end with respect to the maps in Fig. 1), the first 727 bp correspond to BamHI 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 BamHI dC fragment.

Immediately downstream of the 30-bp repeats is 1,319 bp (79 bp from the XhoI site) from the viral BamHI Y region (Y'), which has recombined with BamHI C and is positioned in the opposite orientation with respect to the BamHI C sequences (compared with the normal BamHI Y fragment). The junction between BamHI 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 BamHI W repeat and, like the rearranged BamHI Y sequences, were present in the X50-7/4A4 clone in the opposite orientation with respect to the BamHI C sequences. Thus, it appears that one or more BamHI W repeats along with 1,319 bp from BamHI Y have rearranged with the BamHI C region in the X50-7 genome and that there has been a 5,184-bp deletion of BamHI C sequences.

Location of the BamHI dC fragment in the X50-7 genome. Although the BamHI dC fragment contains oriP, it was unclear whether the BamHI C region has been rearranged into the downstream BamHI W and Y region or vice versa. To determine whether BamHI 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 BamHI and probed with C/ERI (a 3.15-kb fragment from BamHI C [Fig. 1]), a 9.2-kb BamHI fragment (Fig. 6A) was revealed. With X50-7 DNA, BamHI dC also hybridized to this probe, indicating that the 3.0-kb fragment (EcoRI J) immediately upstream of oriP in the wild-type viral genome is also contained within BamHI dC.

To determine whether BamHI dC is linked to the fused terminal repeats, DNAs from the JY, X50-7, and D98-HR1 cell lines were digested with BglII and HpaI, fractionated on a 0.4% agarose gel, and probed with fragments unique to either BamHI C (probe C/ERI [Fig. 1]) or the terminal repeats. In the wild-type genome, the BglII and HpaI sites lie outside the region containing the terminal repeats and oriP. Thus, if BamHI dC is located in the same place as BamHI C in the wild-type viral genome, then the terminal repeat and BamHI C probes will hybridize to the same fragment. Indeed, with DNA from all the 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 BamHI W fragment is contiguous with the BamHI dC fragment. A question raised by the data above is
whether the presence of BamHI W in the X50-7/4A4 clone occurred as a result of cloning a partial XhoI digestion product (failure to cleave at the XhoI site present in the Y' sequences of the BamHI 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 BamHI Y unique region (this fragment hybridizes to the BamHI dC fragment from X50-7 but does not hybridize to the BamHI 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 EcoRI and BamHI. This resulted in detection of a 1.8-kb fragment which was present in both cell lines and which corresponds to the wild-type BamHI Y fragment. In addition, an EcoRI-BamHI fragment of ca. 2.0 kb, generated from BamHI dC, was detected with DNA from the X50-7 cell line. However, hybridization of the probe to fragments from the B95.8 BamHI C region (or from the BamHI W region) was not observed.

When the B95.8 and X50-7 DNAs were digested with EcoRI and AccI, a single fragment of ca. 2.3 kb hybridized to the probe in both cases. This fragment corresponds to an AccI fragment containing the BamHI 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 BamHI W fragment adjacent to BamHI dC is in the same orientation as the BamHI Y sequences. Finally, EcoRI and BglII 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 BamHI 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 BamHI 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 BamHI W fragment adjacent to BamHI dC. If the adjacent BamHI 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 BamHI 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 BamHI W and Y sequences into the BamHI C region of the X50-7 genome with the deletion of the 5,184-bp BamHI C sequence. In addition, the BamHI W and Y sequences are present in inverted orientations. The X50-7 BamHI 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 BamHI Y sequence, which is contiguous with sequences from the BamHI W region of the EBV genome. At least one complete BamHI 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 BamHI 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 BamHI 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 X0-7 genome must be integrated or some other element(s) outside the BamHI 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 X0-7 genome. They demonstrated that superinfection of X0-7 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 X0-7 cell line, although it is possible that the immortalizing virus recovered was the result of recombination between integrated X0-7 genomes and episomal P3HR-1 genomes. Our preliminary experiments with Gardella gel electrophoresis (3) have indicated the existence of EBV in X0-7 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 X0-7 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 BamHI 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 BamHI W repeats and extends through BamHI Y and into the adjacent BamHI H fragment (8, 9). A deletion of approximately 0.3 kb in the BamHI 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 BamHI 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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