Epstein-Barr Virus DNA

X. Direct Repeat Within the Internal Direct Repeat of Epstein-Barr Virus DNA

ANDREW CHEUNG AND ELLIOTT KIEFF*

Section of Infectious Disease, Department of Medicine, Microbiology, Virology and Immunology, Kovler Viral Oncology Laboratories, University of Chicago, Chicago, Illinois 60637

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The 3,360-base-pair internal direct repeat (IR) in Epstein-Barr virus DNA separates the short and long unique DNA domains. IR has a single *Bam*HI site. The juncture between the short unique domain and IR has been mapped by restriction endonucleases and is less than 2,600 nucleotides before the *Bam*HI site in IR. The junction between IR and the long unique domain has been sequenced and is approximately 650 nucleotides after the *Bam*HI site in IR. Thus, relative to the start of IR at the juncture with the short unique domain, the last repeat is at least 90 base pairs short of being complete. There is homology between the 250-nucleotide fragments to the left and the right of the unique *Bam*HI site in IR. A 35-base-pair sequence of the left fragment is directly repeated within the right fragment, once fully and once partially. The implications of these findings are discussed.

Complete restriction endonuclease maps have been derived for at least seven isolates of Epstein-Barr virus (EBV) (2-4, 7-9, 11, 20) and for two isolates of the related herpesvirus papio (12, 13, 16). The common features of this subgroup of herpesviruses include a variable number of direct tandem repeats of a 500- to 600-base-pair sequence (TR) at both ends of the linear virion DNA molecule; a short unique DNA sequence of 15×10^3 base pairs (US), a variable number of tandem direct repeats of a 3,360-base-pair sequence (IR), and a long largely unique DNA sequence of 150×10^3 base pairs (UL). The tandem direct repeats of IR separate US and UL (3, 4, 7, 8, 10, 22).

Aside from its organizational role in EBV DNA, IR is an important functional element in nonpermissively infected, growth-transformed cells. The nucleotide sequence complexity equivalent of one strand of IR is transcribed in nonpermissively infected, transformed cells (14, 26). Most of the RNA encoded by IR is processed and accumulates in polyribosomes (14, 26). In nonpermissively infected, growth-transformed cells IR encodes cytoplasmic polyadenylated, and presumably messenger, RNAs of 3.0×10^3 and 1.5×10^3 bases (26).

Partial denaturation mapping and buoyant density measurements indicate that IR is richer in guanine plus cytosine than most of EBV DNA (5, 10). IR has a single cleavage site for *Bam*HI and *Bgl*II restriction endonucleases (8, 10, 22). Digestion of EBV DNA with either enzyme gen-

erates fragments which are the junction fragments between IR and UL or US and a much more abundant fragment which is the full repeat unit (3, 10, 22; Fig. 1). It has been assumed that the junction fragments together have one complete repeat unit, although neither the beginning nor the end of the repeat has been mapped relative to restriction endonuclease sites within the repeat. The *Bam*HI and *Bgl*II sites are asymmetrically placed relative to each other and divide IR into small and large components (8). Labeled small component was found to hybridize weakly to the large component and vice versa, suggesting that there might be a direct or inverted repeat within IR (8).

The purpose of the experiments described in this communication is (i) to locate the region of homology within IR, (ii) to sequence the homologous regions to determine the extent of homology and whether the homologous sequences are on the same or opposite strands, and (iii) to map the beginning and end of IR relative to restriction endonuclease sites within IR.

MATERIALS AND METHODS

Viral DNAs. Plasmid pDK14, pDK10, and pDK322, which contain the *Bam*HI-C, -V, and -X fragments of EBV (B95-8) DNA cloned into the *Bam*HI site of pBR322 were grown in X1776 under P1 EK2 conditions (3). *Bam*HI-V is the fragment formed by cleavage of adjacent tandem repeats of IR at the single *Bam*HI site in IR. *Bam*HI-C is the fragment extending from the last *Bam*HI site in US to the

FIG. 1. Summary of organizational features and *Bam*HI map of EBV DNA around the internal reiteration. Letters above the line indicate the *Bam*HI fragments (3). *Bam*HI-C is the rightward end of US and the beginning of IR up to the unique *Bam*HI site in IR (3). *Bam*HI-V is a full repeat unit from the unique *Bam*HI site in one repeat to the unique *Bam*HI site in the second repeat (3). *Bam*HI-X is the end of the last repeat of IR and the beginning of UL (3).

*Bam*HI site in the first repeat of IR. *Bam*HI-X is the fragment extending from the *Bam*HI site in the last repeat of IR to the first *Bam*HI site in UL (Fig. 1).

Separation and size determination of restriction endonuclease fragments. Restriction endonucleases were purchased from Bethesda Research Laboratories (Bethesda, Md.) and used under conditions specified by the manufacturer. Restriction digests of cloned EBV DNAs were electrophoresed at 40 V and 4°C in 1% agarose gels in a buffer consisting of 40 mM Tris-hydrochloride (pH 7.8), 1 mM EDTA, and 5 mM potassium acetate. The size of fragments was determined relative to intact and restriction endonuclease fragments of ϕ X174 and pBR322 DNAs (25).

Preparation for blot hybridization. After electrophoresis, the DNA fragments in the gel were denatured in situ with 1 M KOH, neutralized with 1 M Tris-hydrochloride (pH 7)–1 M HCl, and rinsed with 6× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate). The DNA was transferred onto nitrocellulose filters (Millipore Corp.) as described by Southern (24). The filters were then rinsed with 2× SSC (pH 7), blotted, dried, and baked for 2 h at 80°C in a vacuum oven.

Filter hybridization and autoradiography. All steps in filter hybridization were carried out at 37°C. Baked nitrocellulose filters were presoaked in hybridization buffer which consists of 50% formamide, 5× SSC, 0.5% sodium dodecyl sulfate, 20 mM Tris-hydrochloride (pH 7.4), and 0.08% each of Ficoll, bovine serum albumin, and polyvinylpyrrolidone for 30 to 60 min (6, 27). The filters were then allowed to hybridize with alkaline-denatured, nick-translated (21) radioactive probe in hybridization buffer for 24 h. After hybridization the filters were washed twice in hybridization buffer for 1 h and then in 2× SSC (pH 7) for 1 h. The filters were then air dried and exposed to X-ray film.

DNA sequence analysis. DNA fragments were treated with bacterial alkaline phosphatase (Bethesda Research Laboratories), labeled at their 5' end with polynucleotide kinase (Bethesda Research Laboratories) and [γ -³²P]dATP (Amersham Corp.), cut with restriction enzyme, separated on agarose gel, and identified by autoradiography. The DNA fragments were eluted and purified by DEAE chromatography (18). In other experiments, end-labeled DNA strands were separated on acrylamide gels. The chemical degradation method was used for nucleotide sequence determination (17). The sequences were analyzed for regions of homology and reading frames on an Amdahl computer by using previously derived programs (15, 19).

RESULTS

Restriction endonuclease map of IR. *Bgl*II, *Pvu*II, *Xho*I, *Sst*I, or *Sst*II cut the *Bam*HI-V (IR) insert only once, whereas *Hin*fI, *Hha*I, *Alu*I, and *Mbo*II each cut *Bam*HI-V at several sites. The location of the single-cut enzyme sites was determined from the size of the fragments generated by cleavage of pDK 14 with these enzymes individually and in combination with *Bam*HI. The location of multicut restriction endonuclease sites was also determined in part by sequential cleavage and determination of the size of fragments. The map was completed by determining the size of partial digestion products. For these experiments *Bam*HI-V was labeled at the 5' ends. The labeled DNA was then cut with *Bgl*II to separate the 760- and 2,600-base-pair fragments, each labeled only at the 5' *Bam*HI end. The sizes of the products of partial digestion of the 760- and 2,600-base-pair fragments with a second enzyme were used to determine the distance from the labeled *Bam*HI end to the internal restriction endonuclease sites for the second enzyme (Fig. 2). The restriction endonuclease maps are summarized in Fig. 3. Although only part of IR has been sequenced, for conciseness fragments of IR are subsequently denoted by their map coordinates in base pairs, as shown in Fig. 3.

Map of homology within IR. The labeled fragment between nucleotides 0 and 760 (0–760 fragment) of IR (*Bam*HI-V) hybridizes much less to the 760–3,360 fragment of *Bam*HI-V than to the 0–760 fragment, indicating that there is only partial homology between the two fragments (Fig. 4a). The part of the 0–760 fragment homologous to the 760–3,360 fragment is the 0–245 component (Fig. 4b lane 3, 4d lane 2, and 4e lane 2). The part of the 760–3,360 fragment homologous to the 0–760 fragment is the 3,120–3,360 component (Fig. 4b lane 2, 4c lane 2, and 4f lane 2). Thus, the region of homology is within 250 nucleotides on each side of the *Bam*HI site in IR.

Sequence of homologous fragments within IR. The 0–245 *Bam*HI-*Pst*I fragment and 3,120–3,360 *Hin*fI-*Bam*HI fragment (Fig. 3)

FIG. 2. Mapping of multicut restriction endonuclease sites by analysis of partial digest products (23). *Bam*HI-V (5' end labeled) was digested with *Bgl*II (a) to give 760-base-pair (lane 1) and 2,600-base-pair (lane 2) fragments. Both fragments were subjected to partial digestion by enzymes indicated at the top of b, c, d, and e. The size of the partial digestion products (given in nucleotides in the figure) was determined from their electrophoretic mobility as described in the text.

FIG. 3. Restriction enzyme map of the internal reiteration. Restriction endonuclease sites of one- or two-cut enzymes were derived from double digestion. The sites of multiple-cut enzymes were derived from partial digestion of 5' end-labeled DNA fragments. The scale at the bottom is in percentage of overall length and in nucleotide pairs (based on size estimates).

were sequenced by the chemical degradation method (17). The nucleotide sequences are shown in Fig. 5 as a continuous sequence beginning at the *Hinf*I site and extending through the *Bam*HI site to the *Pst*I site. An identical 35-nucleotide sequence, CCAGCCAGCCGGA-GGGACCCCGCAGCCCGGGCG, occurs as a direct repeat 202 to 168 nucleotides before and 91 to 125 nucleotides after the *Bam*HI site. Also, an identical 17-nucleotide subsequence of the 35, GCCGGAGGGACCCCGGC, occurs as a direct repeat 212 to 228 nucleotides after the *Bam*HI site. These results are indicated on the summary

map (Fig. 1). Several other shorter direct and inverted repeats are also present.

Junction between IR and US or UL. The internal reiteration begins at the right end of *Bam*HI-C (3, 7, 8, 10). *Eco*RI, which makes two cuts at the left end of *Bam*HI-C, separates US components of *Bam*HI-C from the right end of *Bam*HI-C which contains the beginning of IR (Fig 6A). The distance from the beginning of the first copy of IR to the *Bam*HI site in IR (which separates *Bam*HI-C from *Bam*HI-V [Fig. 1]) was determined by cleaving *Bam*HI-C with restriction endonucleases which cut IR before the

*Bam*HI site. Enzymes which cut *Bam*HI-C between the beginning of IR and the first *Bam*HI site should yield the same size fragment from *Bam*HI-C as from *Bam*HI-V. *Pvu*II and *Sst*I, which cut IR at 840 and 2,500 nucleotides before the *Bam*HI site, generate IR homologous fragments of 840 and 2,500 nucleotides from *Bam*HI-C (Fig. 6A). *Bgl*II, which cuts IR at 2,600 nucleotides before the *Bam*HI site, does not cut *Bam*HI-C (recombinant or virion DNA). Thus, the beginning of IR in *Bam*HI-C is less than 2,600 and probably more than 2,500 nucleotides before the *Bam*HI site in IR.

The internal reiteration ends in *Bam*HI-X (Fig. 1). *Hind*III makes a single cut in *Bam*HI-X and separates UL components of *Bam*HI-X from the left end of *Bam*HI-X, which contains the end of IR (Fig. 6B). *Pst*I, which cuts IR at 245 nucleotides after the *Bam*HI site, and *Xho*I, which cuts IR at 550 nucleotides after the *Bam*HI site, both generate homologous frag-

ments of identical size from *Bam*HI-X (Fig. 6B). However, *Bam*HI-X (recombinant or virion DNA) lacks the *Bgl*II site at 760 nucleotides. Labeled IR hybridizes almost half as much to the part of *Bam*HI-X to the right of the *Xho*I site at 550 nucleotides as it does to the 0-550 fragment which is in common to *Bam*HI-V and -X (Fig. 6B). These data suggest that IR extends for 100 to 200 nucleotides past the *Xho*I site into *Bam*HI-X and stops before the *Bgl*II site at 760 nucleotides in IR.

Sequence at the end of the last repeat of IR. To define more precisely the end of the last repeat of IR in *Bam*HI-X, the nucleotide sequences of the regions to the right of the *Xho*I cut sites in *Bam*HI-V and -X were sequenced by chemical degradation. The nucleotide sequences of *Bam*HI-V and -X (Fig. 7) are identical for 117 nucleotides past the *Xho*I site. Thereafter, the nucleotide sequences diverge. Thus, US begins 118 nucleotides after the *Xho*I site in *Bam*HI-X.

FIG. 4. Mapping of the homologous regions in IR. The coordinates of the DNA fragments are indicated in base pairs between *Bam*HI sites in the orientation shown in Fig. 1 and 3. The coordinates are those shown in Fig. 3. Blots of *Bam*HI-V digested with *Bgl*II (a) or *Pst*I, *Xho*I, *Bgl*II, and *Pvu*II (b) were hybridized with labeled *Bam*HI-V (lane 1), with labeled (0-760) probe (lane 2), or with labeled (760-3,360) probe (lane 3). Homologous regions were further defined by hybridization of labeled (0-760) probe to a blot of an *Mbo*II digest of the part of *Bam*HI-V from coordinates 2,520 to 3,360 (c), by hybridization of labeled (2,520-3,360) probe (d) or of labeled (0-245) probe (e) to blots of *Pst*I- and *Pvu*II-digested *Bam*HI-V, and by hybridization of labeled (0-245) probe to blots of a *Hinf*I digest of the 2,520-3,360 fragment (f).

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          30                                60                                90
GAG TCC AGA GGT CAG GGG CAC CTC AGG GTG CCC CCG GGT CCC AGG CCA GCC GGA GGG ACC CCG GCA GCC CCG GCG GCC CCA GAG GCC GGT
GLU SER ARG GLY GLN GLY HIS LEU ARG VAL PRO PRO GLY PRO ARG PRO ALA GLY GLY THR PRO ALA ALA ARG ALA ALA PRO GLU ALA GLY

          120                                150                                180
TCC TCG CCC CTT CCC CGG GCT TCA GAG CCC AGG ATG TCC CCC AGA AGG GAC CCT AGG CGT CCC CTC TCC TCC CCT CCA GGC CCG AGC CTC
SER SER PRO LEU PRO ARG ALA SER GLU PRO ARG MET SER PRO ARG ARG ASP PRO ARG ARG PRO LEU SER SER PRO PRO GLY PRO SER LEU

          210                                240                                270
TCC CTC GCG GAG AGG CCT CTT TGG GCC CTC AAG TCC AGC CCC ACC GAG ACC CGA GTG GCC CCG ATC OCC CCA CCG GCC CTT CTC TCT GTC
SER LEU ALA GLU ARG PRO LEU TRP ALA LEU LYS SER SER PRO THR GLU THR ARG VAL ALA ARG ILE PRO PRO PRO ALA LEU LEU SER VAL

          300                                330                                360
CCC CTG CTC CTC TCC AAC CTT CGC TCC ACC CTA GAC CCC AGC TTC TGG CCT CCC CCG GTC CAG CAG GCC ACC CCG AGG GAC CCC GGC ACC
PRO LEU LEU LEU SER ASN LEU ARG SER THR LEU ASP PRO SER PHE TRP PRO PRO ARG VAL HIS GLN ALA SER ARG ARG ASP PRO GLY SER

          390                                420                                450
CCG GGC GAG TCG CCT TCC CTC TCC CAAT GGC CTC TCC TTC CCG CCT CCC ACC CGA GCC CCC TCA GCT TGC CTC CCC ACC GGG TCC ATC AGG
PRO GLY GLU SER PRO SER LEU SER HIS GLY LEU SER PHE PRO PRO THR ARG ALA PRO SER ALA CYS LEU PRO THR GLY SER ILE ARG

          480
CCG GGC GGA GGG ACC CCG GGC GCC CGG TGT CAG TCC CCC CTG CAG
PRO ALA GLY GLY THR PRO ALA ALA ARG CYS GLN SER PRO LEU GLN

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FIG. 5. Nucleotide sequence of the homologous DNAs around the *Bam*HI site in IR. The 242 nucleotides in the *Hin*FI-*Bam*HI fragment to the left of the *Bam*HI site were sequenced by determining the 200 nucleotides from each end. The 253-nucleotide *Bam*HI-*Pst*I fragment to the right of the *Bam*HI site was also sequenced by determining the 200 nucleotides from each end and 120 to 150 nucleotides from the *Hin*FI site at 367 to 371 nucleotides in the figure. The continuous nucleotide sequence is shown from the *Hin*FI site through the *Bam*HI site (indicated by an arrow between nucleotides 242 and 243) to the *Pst*I site. Regions of identity are indicated by boxes; regions of similarity are indicated by underlining, with asterisks indicating unmatched bases. The amino acid sequence is shown in the only reading frame which does not have a termination codon.

DISCUSSION

The data which are summarized in Fig. 1 confirm that there is homology within the internal reiteration in EBV DNA and indicate that the homology is due to the direct repeat of a 35-nucleotide sequence to the left and the right of the unique *Bam*HI site in IR. A direct repeat of the 17-nucleotide subsequence of the 35 begins 87 nucleotides after the second 35-nucleotide sequence. This 17-nucleotide subsequence is part of a larger sequence duplication, since there is extensive homology to the 35-nucleotide sequence on both sides of the 17-nucleotide repeat. Thus, there are three direct repeats of a highly conserved sequence in this region of IR. These repeats are likely to have important *cis* or *trans* function.

The fragments which have been sequenced on both sides of the *Bam*HI site in IR are 242 and 253 nucleotides (Fig. 5). These sequences are continuous in IR and are 495 of the total 3,360 nucleotides of IR. Through the continuous 495-nucleotide sequence, there is no polypeptide chain termination codon in the reading frame beginning GAG. There is one termination codon (TAG) in the second reading frame and one in the third. Translation from the opposite strand is blocked by at least two terminator codons in each reading frame. The amino acid sequence which could make maximum use of the coding capacity of this sequence by translating it in frame from the first nucleotide is shown below the nucleotide sequence in Fig. 5.

The *Bam*HI site in IR is less than 2,600 nucleotides after the beginning of IR in *Bam*HI-C. The nucleotide sequence which begins IR has

not been determined. Relative to the start, which is less than 2,600 nucleotides before the *Bam*HI site, the last repeat of IR would be expected to terminate more than 760 nucleotides after the last *Bam*HI site so as to terminate in a complete repeat. However, the last repeat terminates 117 nucleotides after the *Xho*I site and before the *Bgl*II site at 760. The *Xho*I site is approximately 550 nucleotides after the *Bam*HI site. Thus, the last repeat stops approximately 670 nucleotides after the last *Bam*HI site or at least 90 nucleotides short of completing the last copy of IR (relative to the start of IR at the US-IR junction).

Presumably, the repetitions of IR are structurally or functionally useful to EBV. The number of repeats of IR varies within populations of molecules produced by a single isolate of EBV (8). The average number of repeats also varies among EBV isolates (11). The B95-8 isolate which has a deletion of approximately 15×10^3 nucleotides in the UL tends to have 10 repeats of IR, whereas other isolates of EBV and of herpesvirus papio tend to have six repeats of IR (11-13). This suggests that the number of repeats is partially dependent on overall DNA size. Increases in overall size have also been reported with passage and may be due to an average increase in the number of repeats of IR (1). One consequence of the finding in this study of a deletion in the last (or first) repeat of IR is that it raises the possibility that multiple repeats could be generated from a parental molecule which consists of less than two complete repeats.

Although deletions as a result of homologous recombination between the repeats within IR

FIG. 6. Mapping of the juncture of IR and US (BamHI-C; panel A) or UL (BamHI-X, panel B). BamHI-C (panel A) or BamHI-X (panel B) was digested with restriction endonucleases which cut within IR or within the adjacent US or UL to determine the extent of IR in BamHI-C and BamHI-X. Blots of restriction endonuclease fragments of BamHI-C, -X, or -V were hybridized to labeled BamHI-V to indicate the fragments which contain sequences of IR. The recombinant DNAs were digested with EcoRI or HindIII or with a combination of BamHI and SstI, PvuII, XhoI, or PstI. The numbers indicate the coordinates of the fragment within IR in base pairs relative to the map shown in Fig. 3 or the size of the fragment in base pairs. Fragments consisting of both pBR322 and EBV DNA are indicated by "P" and the letter of the EBV DNA fragment.

FIG. 7. Nucleotide sequences of the DNAs to the right of the XhoI sites in BamHI-V and -X. The DNAs were 5' end labeled at the XhoI site, and the nucleotide sequences were determined by chemical degradation (17).

might be expected, the deletion of part of IR outside of these regions and at the juncture of IR with UL or US is a surprising finding which could be of significance. IR and the adjacent part of UL encode at least one and possibly two abundant cytoplasmic polyadenylated RNA(s) in nonpermissively infected, growth-transformed cells (14, 26). Translation of an mRNA from IR would be blocked once in all three reading frames between the *Xho*I site and the switch into UL (Fig. 7). In the reading frame beginning CGA (Fig. 7) a second terminator sequence TAG occurs 162 nucleotides after the *Xho*I site in IR (Fig. 7). This terminator is deleted by the switch into UL at 118 nucleotides after the *Xho*I site in the last copy of IR at the IR-UL juncture in *Bam*HI-X (Fig. 7). If the terminator codon TAG, which is five nucleotides to the right of the *Xho*I site, were eliminated by RNA processing, a consequence of the deletion of the last part of IR in *Bam*HI-X could be that a message extending from IR into UL would be translated by using codons from both IR and UL.

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ADDENDUM IN PROOF

The *Sst*I site in *Bam*HI-C (Fig. 6) is within the US component. Thus, the extent of the deletion of sequences in the incomplete copy of IR, relative to the beginning of IR, is larger than anticipated from the data reported here.

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