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Molecular cloning of the complete Epstein-Barr virus genome as a set of overlapping restriction endonuclease fragments

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ABSTRACT

A complete collection of fragments of Epstein-Barr virus DNA, obtained by cleavage with restriction endonuclease Eco RI, has been cloned. Fourteen different internal fragments of the virus genome, derived from linear virion DNA of the B95-8 strain, and sequences corresponding to the terminal regions of virion DNA, derived from intracellular circular EBV DNA isolated from B95-8 cells, were cloned. Sizes of fragments were determined by agarose gel electrophoresis and their sum leads to an estimated molecular weight of 110 x 10^6 for virion DNA. Large Eco RI DNA fragments of special interest were also cloned in cosmids using another source of EBV DNA, that is, the circular viral DNA derived from Raji cells. In order to provide a set of overlapping sequences, all the 29 internal Bam HI fragments of B95-8 virion DNA were cloned in pBR322. The map location within the viral genome of each cloned DNA fragment was identified by hybridizing to blots of virion DNA cleaved with several different restriction endonucleases.

INTRODUCTION

The Epstein-Barr virus (EBV, for reviews, see 1,2) is carried in latent form by most human adults. This common herpesvirus is implicated in human disease, since it is the aetiological agent of infectious mononucleosis. Moreover, it has a strong association with two forms of tumour, poorly differentiated nasopharyngeal carcinoma and Burkitt's lymphoma, and at present it remains the best candidate for a virus playing a causative role in human malignant disease.

Studies on EBV have been seriously hampered by the absence of a tissue culture system that will allow lytic infection. The standard source of EBV has therefore been culture supernatants from certain virus-transformed lymphoid cell lines such as the

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B95-8 line (3), which contain small amounts of virus particles. Since only minute quantities of viral DNA may be obtained in this fashion, it is obvious that the cloning of EBV DNA as a series of distinct restriction endonuclease fragments which can be propagated in E.coli allows studies of the molecular biology of this virus in a more comprehensive way than has previously been possible.

EBV DNA, like other herpesvirus DNA molecules, has a molecular weight of about 10^8 and is cleaved into many fragments by all restriction enzymes currently available. Again, like the DNA of several other herpesviruses, it has a high guanine + cytosine content (about 58%). In general contrast to smaller DNA tumour viruses, EBV DNA contains a region of internal repeated sequences that make up about 20% of the viral genome (4). There are short direct repeat sequences at its termini which show no apparent homology with the internal repeats (5,6). Another difference between EBV and most of the smaller DNA tumour viruses is that multiple copies of complete virus genomes, replicating in concert with the host chromosomes, occur in non-integrated circular form in most virus-transformed cell lines.

In order to facilitate studies on EBV DNA, we have cloned restriction enzyme fragments of the viral genome and have isolated a complete library of cloned Eco RI fragments of the B95-8 strain of EBV. A set of overlapping fragments, obtained by Bam HI cleavage, has also been cloned. Moreover, several large Eco RI fragments derived from circular EBV DNA isolated from Raji cells have been cloned. This work was reported in part at the Cold Spring Harbor Herpesvirus meeting, 1979. While this project was being completed, studies similar to ours appeared from two other laboratories (7,8). The present report, however, is the first description of the isolation of a complete collection of overlapping genomic clones. It is also unique in its inclusion of the largest EcoRI fragment (which encompasses the internal repeating units) and the terminal repeat sequences present in intracellular EBV DNA from B95-8 cells.

MATERIALS AND METHODS

(a) Viral DNA. The EBV-transformed lymphoid cell lines

B95-8 and Raji were obtained from Dr. George Klein, Department of Tumor Biology, Karolinska Institute, Sweden. EBV particles were isolated from the spent medium of B95-8 cells, and virion DNA was extracted and purified essentially as described by Adams (9). Cell cultures were treated with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) according to zur Hausen et al. (10) to improve virus production. Covalently closed circular DNA preparations from B95-8 and Raji cells were isolated as described (11). These circular EBV DNA preparations were at least 90% pure and had host mitochondrial DNA as the only detectable contaminating material.

EBV DNA was radioactively labelled _in vitro_ by nick translation with _E.coli_ DNA polymerase I (Boehringer) in the presence of [α-^{32}P] labeled dCTP and TTP (Radiochemical Centre, Amersham) or [^{125}I]dCTP (12) according to Rigby et al. (13). The specific activity of the labelled DNA was 1-2 x 10^8 cpm/μg.

(b) _Vectors_. _E.coli_ HB101, containing the plasmid pBR322 (14) was grown in L broth (1% tryptone, 0.5% yeast extract, 1% NaCl, medium adjusted to pH 7.2), and plasmid amplification was achieved by chloramphenicol treatment (15). Plasmid DNA was prepared according to Birnboim and Doly (16). The pBR322 derived small cosmids pHC79 (17) and Homer I (W. Chia, M.R.D. Scott and P.W.J. Rigby, unpublished) were carried in _E.coli_ HB101 and prepared in the same fashion. Vector DNAs were cleaved with either _Eco_ RI or _Bam_ HI under the standard conditions specified by the manufacturer. The cleaved DNA was in some cases treated with _E.coli_ alkaline phosphatase to prevent religation, phenol extracted, and ethanol precipitated before use (18).

(c) _Reagent enzymes_. Restriction endonucleases _Eco_ RI and _Bam_ HI were purchased from Boehringer and from Bethesda Research Laboratories. Each batch was tested for exonuclease contamination before use by screening for the ability of cleaved pBR322 DNA to be religated by T4 DNA ligase, as determined by agarose gel electrophoresis. _E.coli_ alkaline phosphatase (Worthington, chromatographically purified) was freed from contaminating traces of nucleases by heating at 95° for 5 min in 10mM Tris HCl (pH 8.0), 10mM $MgCl_2$, followed by incubation at 25° for 3 hr, application to a DEAE-cellulose column equilibrated with 10mM Tris HCl (pH 8.0), washing of the column with the same

buffer and elution of the enzyme with 0.2M NaCl, 10mM Tris HCl (pH 8.0). Phosphatase treatment of DNA was performed in 20mM NaCl, 10mM Tris HCl (pH 8.0) at 56°. T4 DNA ligase preparations were generous gifts from Drs. G. Magnusson, K. Murray and N. Smolar.

(d) Cloning of restriction fragments of viral DNA. Linear or circular EBV DNA (2μg) was digested with 5 units of *Eco* RI or *Bam* HI for 2 hr at 37° under standard conditions. The reaction mixture was phenol extracted, ethanol precipitated and air dried. To the precipitate was added 10μl of a solution containing 0.5μg cleaved vector DNA, 70mM Tris HCl (pH 7.6), 7mM $MgCl_2$, 0.7mM dithiothreitol, 0.1mM ATP and 1 unit T4 DNA ligase, and the mixture was incubated for 16 hr at 4°. Such recombinant DNA solutions were used in transformation or packaging experiments without further purification. For cloning of the *Eco* RI A, B, and C fragments, the restriction enzyme-digested EBV DNA was size fractionated by sucrose gradient centrifugation before use. Fractions containing the EBV DNA fragments were localized by analytical gel electrophoresis of aliquots, appropriate fractions were concentrated by ethanol precipitation, dissolved and ligated as described above.

In typical transformation experiments with pBR322 as vector, *E.coli* χ1776 (obtained from Dr. R. Curtiss) was grown in L broth supplemented with 100μg/ml diaminopimelic acid and 100μg/ml thymidine (supplemented L broth). Bacteria in the early logarithmic growth phase were collected, washed in 10mM NaCl, and suspended in 0.1 culture volume of 70mM $MnCl_2$, 30mM $CaCl_2$, 40mM Na-acetate (pH 5.6). After incubation at 0° for 20 min, the bacteria were pelleted and resuspended in 0.03 volume of the same buffer. Aliquots of this suspension (0.2ml) were mixed with 0.01ml of recombinant DNA solution (containing 0.1μg pBR322 DNA diluted with 10mM Tris HCl, 1mM EDTA, pH 8.0) and incubated at 0° for 1 hr. Supplemented L broth (1.8ml) was subsequently added, and after incubation at 37° for 30 min, the bacteria were plated on L agar containing diaminopimelic acid, thymidine, 100μg/ml ampicillin (and for *Eco* RI clones only, 10μg/ml tetracycline). After growth at 37° for 48 hr, individual colonies were picked into 150 μl broth wells in microtitre plates and grown at 37° for

40 hrs. Aliquots of each culture were transferred to nitrocellulose membranes on supplemented L agar plates (see above) and grown for colony hybridization (19,20). The remaining broth cultures in the wells were supplemented with 0.25 volume glycerol and stored at -70^{0}. Nick-translated, $[^{32}P]$ or $[^{125}I]$-labelled EBV DNA was used as probe in the colony hybridization experiments, and colonies containing plasmids carrying EBV DNA sequences were detected by autoradiography.

In experiments with cosmids as vectors, the religated recombinant DNA solutions were packaged in vitro into λ capsids, using ultraviolet irradiated E.coli BHB2673 and BHB2671 (both rec A) λ lysogens as source for the packaging mixture (21). Transduction of E.coli HB101 (rec A) with hybrid cosmids was then performed according to Hohn and Collins (17), and EBV DNA-containing cosmids were detected by colony hybridization as above. Because of the high efficiency of the transduction with hybrid cosmids, yielding many thousands of colonies of potential interest, only a minor proportion of the hybrid clones were analyzed.

(e) Characterization of cloned DNA fragments. Plasmids were isolated from chloramphenicol treated broth cultures of EBV DNA-carrying E.coli and cleaved with the appropriate restriction enzyme. The DNA fragments were separated by agarose gel electrophoresis in the presence of ethidium bromide. Blotting of gels and DNA hybridizations followed standard procedures, as described in detail elsewhere (22). In mapping experiments, cloned EBV DNA fragments were radioactively labeled by nick translation and hybridized against blots of EBV virion DNA cleaved with either Eco RI, Hind III, Bam HI, or Sal I, employing the miniblot modification of Southern's method (23).

Size determinations of isolated DNA fragments were performed by electrophoresis in agarose gels of different concentrations in the presence of several reference DNAs of known size. The size of each DNA fragment was estimated both from plots of log molecular weight against mobility (24) and from plots of molecular weight against 1/mobility (25).

The size references and their molecular weights were as follows:

phage λ DNA, 30.8 x 10^6 (26); adenovirus 2 DNA, 23.0 x 10^6 (27); *Sal* I fragments of adenovirus 2 DNA, 12.4 x 10^6, 5.7 x 10^6, 4.7 x 10^6, 0.2 x 10^6; *Eco* RI fragments of adenovirus 2 DNA, 13.5 x 10^6, 2.8 x 10^6, 2.4 x 10^6, 1.7 x 10^6, 1.4 x 10^6, 1.2 x 10^6; *Hpa* II fragments of polyoma virus DNA, 0.85 x 10^6, 0.67 x 10^6, 0.53 x 10^6, 0.42 x 10^6, 0.24 x 10^6, 0.23 x 10^6, 0.17 x 10^6, 0.07 x 10^6.

RESULTS

Mapping of restriction endonuclease fragments of EBV DNA.

Cleavage of linear EBV virion DNA, strain B95-8, with *Eco* RI yields 15 fragments, while cleavage with *Bam* HI produces 31 different fragments. Cloned EBV DNA fragments (see below) were nick translated and mapped by hybridization to blots of EBV DNA fragments obtained by *Hind* III, *Sal* I or *Eco* RI digestion of virion DNA, using published cleavage maps (28) for reference. Moreover, cloned large *Eco* RI fragments were cleaved into *Bam* HI subfragments and analysed by gel electrophoresis. The results (Fig. 1) are in general agreement with maps published by other groups (7,8,28). However, the small *Bam* HI fragment designated e and located between the E and Z fragments was not found by Dambaugh et al. (7), and fragment c was not identified by Skare and Strominger (8). Our results provide independently derived maps of all the *Eco* RI fragments and all internal *Bam* HI fragments of B95-8 EBV DNA.

Cloning of internal Eco RI fragments of EBV DNA.

Transformation of *E.coli* χ 1776 with pBR322/B95-8 EBV recombinant DNA molecules yielded about 500 colonies that contained EBV DNA sequences, as judged by colony hybridization experiments. Size determinations and mapping data showed that all internal EBV DNA fragments smaller than the *Eco* RI C fragment were represented in this collection. Cultures that contained only a single EBV *Eco* RI fragment were selected for this collection. Due to the discrimination against high-molecular weight plasmids in these transformation experiments, the largest EBV DNA fragments could not be cloned in this fashion.

Suitable vectors for cloning of large DNA fragments have been developed recently by insertion into pBR322 of the phage λ DNA sequence required for packaging *in vitro* (17). Recombinant

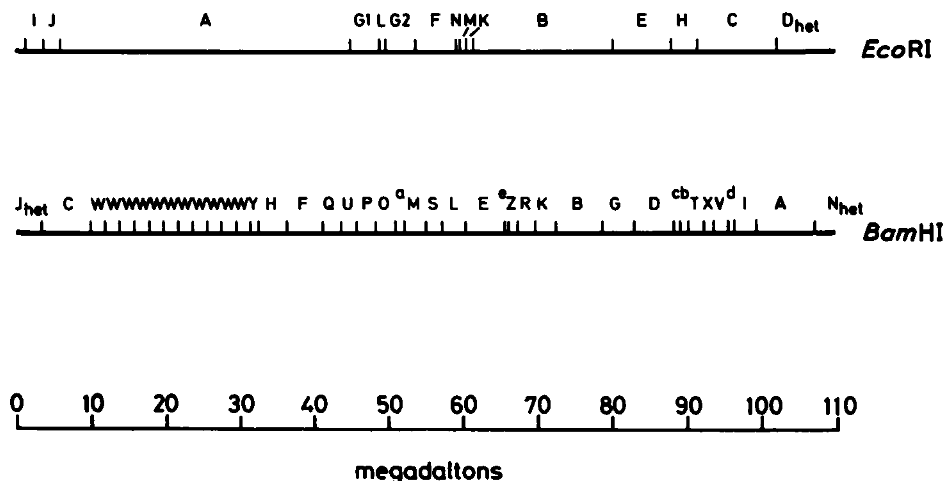

Figure 1

Restriction endonuclease cleavage maps of the B95-8 strain of EBV DNA. The relative positions of the terminal Bam HI J_{het} and N_{het} fragments are from Dambaugh et al. (7). The designations of the Eco RI fragments of EBV DNA adopted here are those of Given and Kieff (28). Two different nomenclatures have been published for the Bam HI fragments (7,8). Here we follow the nomenclature of Skare and Strominger (8) with one small fragment added.

DNA molecules containing a large insert of non-vector DNA are favoured in the packaging systems, and subsequent transduction experiments yield hybrid cosmids that replicate as plasmids in E.coli. In this fashion, the Eco RI A, B, and C fragments were cloned in the cosmid Homer I, completing the library of internal B95-8 EBV DNA Eco RI fragments. A gel electrophoretic analysis of these different cloned DNA fragments is shown in Fig. 2.

While the DNA sequences between different natural isolates of EBV DNA usually appear to be highly conserved, considerable sequence variability within the Eco RI C fragment has been found to occur among viral strains (22,29). Further, the B95-8 strain has a large deletion within this fragment (30). For this reason, we also cloned the corresponding fragment from another source, that is, from Raji circular EBV DNA. The vector employed was the

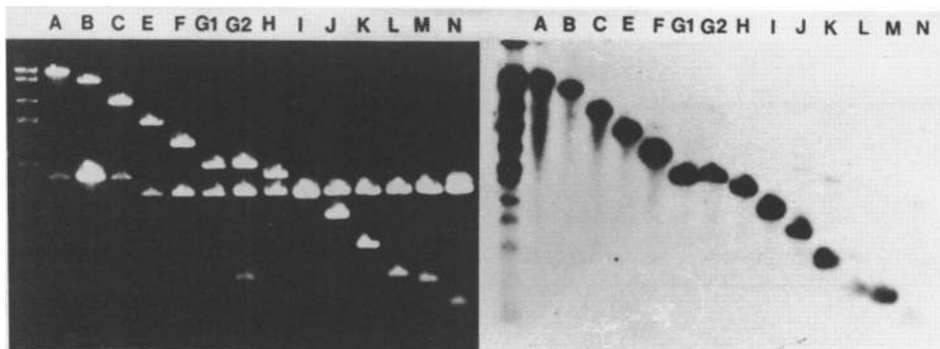

Figure 2

Electrophoretic analysis in a 0.4% agarose gel of internal Eco RI fragments of B95-8 EBV DNA, obtained after enzyme digestion of recombinant DNA molecules from E.coli. Panel A shows the fragments visualized by ethidium bromide fluorescence under ultraviolet light. The left-most lane shows the Eco RI cleavage pattern of EBV virion DNA, and the different clones A-N have then been ordered in decreasing size. The Eco RI fragments A-C were cloned with the Homer I cosmid as vector, while the E-N fragments were cloned with plasmid pBR322. Panel B shows an autoradiograph of the fragments after transfer to a nitrocellulose membrane, and hybridisation with ^{32}P labelled nick-translated EB virion DNA. The small DNA fragment present in addition to the G2 fragment in the same clone does not hybridize to EBV DNA. The very faint, slowly migrating bands discernable in some lanes are due to incomplete Eco RI digestion. The Eco RI B fragment clone occurs simultaneously with cloned multimers of the cosmid vector in the host bacteria, so the vector band is relatively strong in this lane.

pHC79 cosmid, and the fragment was identified by standard mapping procedures.

Cloning of Bam HI cleavage fragments of EBV DNA

Since the largest fragment of linear EBV DNA obtained after Bam HI cleavage has a molecular weight of about 8 x 10^6, all Bam HI fragments (with the exception of the terminal fragments) could be cloned using pBR322 as vector. We obtained a complete collection of the different internal Bam HI fragments of B95-8 EBV DNA by characterizing 600 different EBV DNA-positive clones, as described in the Methods section. The agarose gel electrophoresis patterns of a selection of these plasmids after Bam HI cleavage are shown in Fig. 3.

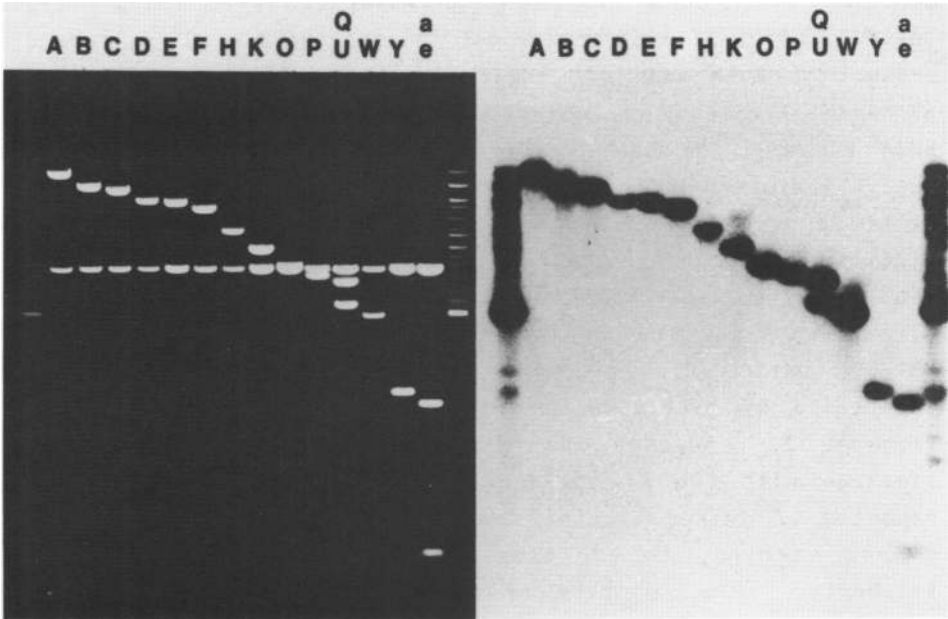

Figure 3

Electrophoretic analysis in a 0.8% agarose gel of Bam HI digested recombinant DNA molecules containing Bam HI cleavage fragments of B95-8 EBV DNA. The left- and right-most lanes show the Bam HI cleavage pattern of EB virion DNA and the lanes in between demonstrate the patterns of some selected clones ordered according to decreasing fragment sizes from A to e. The right panel shows the fragments after transfer to a nitrocellulose membrane and hybridisation with ^{125}I-labelled nick-translated EB virion DNA.

The EBV DNA Bam HI fragments provide overlapping sequences to those present in our complete library of Eco RI fragments. Similar clone banks of the Bam HI fragments of B95-8 EBV DNA have recently been obtained in two other laboratories (7,8). Several of the smaller Bam HI fragments in our collection are currently available only in bacteria that carry two different EBV DNA fragments simultaneously. Since these fragments can be easily separated by electrophoresis, recloning experiments have not yet been performed.

Cloning of terminal DNA fragments

EBV DNA occurs mainly as non-integrated, covalently closed

circular EBV DNA molecules in transformed cells (31). Since several copies of a direct repeat sequence of 3×10^5 daltons are present at both ends of linear virion DNA (5,6), these circles presumably arise by recombination between terminal sequences. We have isolated EBV DNA circles from B95-8 cells by a procedure that allows them to be recovered conveniently from virus producer cells in more than 90% pure form (11). These B95-8 viral DNA circles are probably derived from the portion of the cell population not activated to virus production. Circular DNA was cleaved with Eco RI, and large DNA fragments were cloned in the pHC79 cosmid. Clones containing Eco RI fragment D or C were identified by hybridization with nick-translated [^{32}P] Bam HI fragment A. Several of these clones contained DNA that on cleavage with Eco RI yielded a fragment with about the size expected of joined termini from linear EBV DNA (11,28). Most clones carried, in addition to this fragment, smaller DNA fragments. The identity of the larger fragment as the one containing terminal EBV sequences was verified by mapping experiments, and clones containing only this fragment (and vector DNA) were chosen for our collection. The electrophoretic properties of the fragment, which largely corresponds to the Eco RI D fragment of the linear virion DNA, is shown in Fig. 4. By analogous procedures, the Eco RI fragment from circular Raji EBV DNA, which contains the terminal repeat sequences, was also cloned in the cosmid pHC79.

Sizes of the cloned EBV DNA fragments

The molecular weights of the cloned viral DNA fragments were determined as described in the Methods section. The results (average data from several experiments) are shown in Table 1. The Eco RI A fragment, which contains the internal repeat sequences, was further analysed by Bam HI cleavage and size determinations of these fragments were made. The results showed that the unique sequences of the Eco RI A fragment, comprised of the Bam HI F, H, Q, Y, and truncated C and U fragments, had a molecular weight of 18.3×10^6. Thus, the cloned Eco RI A fragment had $(33-18.3) \times 10^6$ daltons of internal repeat sequences, i.e., 7-8 copies of the Bam HI W fragment, which has molecular weight 2.0×10^6. Typical B95-8 EBV DNA molecules have

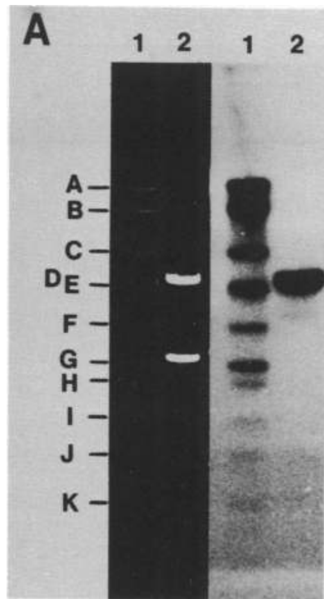

Figure 4

Characterisation of a recombinant DNA clone containing terminal EBV DNA sequences. Electrophoretic analysis in a 0.35% agarose gel of Eco RI cleaved B95-8 EBV DNA (lane 1) and cloned recombinant DNA (lane 2). The right panel shows the fragments after transfer to a nitrocellulose membrane and hybridisation with ^{125}I labelled EBV DNA. In a similar experiment (data not shown) this fragment was shown not to hybridise to the cloned Eco RI E fragment.

been reported to contain about 11 copies of this repeated sequence, but DNA molecules with ranges from 10 down to 2 copies also ocur in virion DNA preparations (4,28). Thus, the Eco RI A fragment cloned here was slightly smaller than a prototype A fragment. This was probably a result of the size limitation of the DNA that could be packaged in λ heads, causing selection for a cosmid that carried a relatively "small" A fragment in the *in vitro* packaging mixture. Since the internal repeat sequences appear identical to each other, as judged from restriction enzyme fine structure mapping (32, and M. Jones, unpublished results), the A fragment cloned here may be regarded as a representative source of viral sequences from this region of the genome.

TABLE 1 Sizes of cloned fragments of B95-8 EBV DNA

Eco RI fragment	Molecular weight ($\times 10^{-6}$)	Bam HI fragment	Molecular weight ($\times 10^{-6}$)
A	33	A	7.6
B	18.5	B	6.3
C	11.3	C	6.2
D_{end} [a]	8.3	D	5.3
E	7.9	E	5.1
F	5.5	F	4.8
G1	4.0	G	4.2
G2	4.0	H	3.8
H	3.4	I	3.6
I	2.6	K	3.2
J	2.0	L	3.2
K	1.4	M	3.0
L	0.8	O	2.8
M	0.7	P	2.7
N	0.4	Q	2.5
		R	2.3
		S	2.2
		T	2.2
		U	2.1
		V	2.1
		W	2.0
		X	1.35
		Y	1.20
		Z	1.15
		a	1.10
		b	0.80
		c	0.70
		d	0.68
		e	0.31

[a] D_{end} refers to the fragment cloned from the circular form of B95-8 EBV DNA.

The sum of the sizes of the Eco RI fragments in Table 1 is 103.8×10^6. An average sized Eco RI A fragment has about three more repeat units than the one cloned here, i.e. a total of eleven copies of the Bam HI W fragment per EBV DNA molecule. We therefore estimate the molecular weight of the linear B95-8 EBV genome to be about 110×10^6. Consideration of the sizes of the Bam HI fragments yields a similar result. This is in good agreement with size estimates from other laboratories (8,29).

DISCUSSION

A library of clones of all the Eco RI fragments of strain B95-8 EBV DNA has been obtained, from which we could estimate the molecular weight of the EBV genome. The use of both cosmids and plasmids as cloning vectors enabled us to obtain a complete bank of EBV Eco RI fragments, including for the first time, the A fragment which contains an intact internal repeat region. Even though this sequence contains a large number of repeating units, it seems to be reasonably stable when carried in an E.coli recA strain.

We have isolated circular EBV DNA directly from B95-8 cells and used such molecules (after Eco RI cleavage) as a source of the sequences present at the termini of linear virion DNA. Dambaugh et al. (7) cloned a corresponding fragment from circular EBV DNA present in a cord blood leukocyte line transformed with B95-8 derived virus particles. However, EBV DNA molecules present in such cord blood lines usually are of considerably reduced size (33), and in view of the loss of viral sequences that occurs it is unclear if cloned DNA of such origin would be representative of the B95-8 parental DNA. Since B95-8 EBV DNA contains a deletion within the Eco RI C fragment (30), the corresponding fragment was also cloned from EBV DNA from the Raji strain. Agarose gel electrophoresis shows this fragment to have a molecular weight of about 18 x 10^6 and compared to B95-8 DNA, it is more representative of EBV DNA isolated from cells of various origins (30).

In cloning DNA resulting from digestion with two different enzymes, Eco RI and Bam HI, we have obtained sets of overlapping sequences which span the entire EBV genome. Bam HI clone banks have been obtained by Dambaugh et al. (7) and by Skare and Strominger (8). However each of these groups overlooked a small Bam HI fragment which corresponded to e and c respectively (see Figure 1).

The size, complexity and scarcity of EBV DNA has made it difficult to study at the molecular level, so that its biological and physical definition has lagged considerably behind that of the other DNA tumour viruses. The availability of large quantities of pure and defined fragments of EBV DNA now however,

allows a number of detailed molecular studies. For example, the DNA sequence of a region within the Eco RI J fragment coding for two early transcripts has been completed (J.R.A. and L.R., in preparation) and sequencing of the internal repeat region is underway (M. Jones, unpublished). The clones described here are also currently being used in a large scale effort to sequence the complete EBV genome (B.G. Barrell, personal communication). Cloned EBV DNA fragments have been used as radioactive probes in studies on EBV sequence complexity (11), in mapping studies (34), in the characterisation of viral transcripts in transformed cells (J.R.A. and L.R., in preparation) and in probing for integrated viral sequences (M. Anvret, C. Carlsson, E.B. and T.L., unpublished data).

EBV, more perhaps than any of the other DNA viruses, may play a role in human malignant disease. Our rationale in undertaking the studies described in this paper was to make available for ourselves and others, materials for probing further into the molecular details of this important virus and its mode(s) of action within the human cell.

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