JOURNAL OF VIROLOGY, May 1986, p. 475–486
0022-538X/86/050475-12$02.00/0

Vol. 58, No. 2

Palindromic Structure and Polypeptide Expression of 36 Kilobase Pairs of Heterogeneous Epstein-Barr Virus (P3HR-1) DNA

HAL B. JENSON,[1,2]* MICHAEL S. RABSON,[1] AND GEORGE MILLER[1,2,3]

Departments of Epidemiology and Public Health,[1] Pediatrics,[2] and Molecular Biophysics and Biochemistry,[3] Yale University School of Medicine, New Haven, Connecticut 06510*

Received 17 October 1985/Accepted 30 January 1986

Among the Epstein-Barr virions (EBV) produced by the P3HR-1 (HR-1) cell line are a defective subpopulation with rearranged viral DNA designated heterogeneous DNA (het DNA). These defective virions are responsible for the capacity of HR-1 virus to induce early antigen in Raji cells and for *trans* activation of latent EBV in X50-7 cells. Virions with het DNA are independent replicons which pass horizontally from cell to cell rather than being partitioned vertically. We analyzed the structure and defined several polypeptide products of het DNA to understand these remarkable biologic properties. A 36-kilobase-pair (kbp) stretch of het DNA was cloned (as two *Eco*RI fragments of 20 and 16 kbp) from virions released from a cellular subclone of HR-1 cells. The unusual aspect of the 20-kbp fragment was the linkage of sequences of *Bam*HI-M and *Bam*HI-B', which are not adjacent on the standard EBV genome. The 16-kbp fragment was a palindrome in which at least two additional recombinations on each side of the palindrome had linked regions of the standard EBV genome which are not normally contiguous. The 20-kbp het DNA fragment was attached to at least one and possibly both ends of the 16-kbp het DNA fragment. We identified antigenic polypeptides produced in COS-1 cells after gene transfer of various cloned het DNA fragments. The 20-kbp fragment encoded a cytoplasmic antigen of about 95 kilodaltons (kDa). The 16-kbp fragment encoded antigens located in the nucleus, nuclear membrane, and cytoplasm. These were represented by several polypeptides, the most prominent of which were about 55, 52, and 36 kDa. The 36-kDa polypeptide was localized to a 2.7-kbp *Bam*HI fragment which had homology to standard *Bam*HI-W and *Bam*HI-Z. Another polypeptide of 50 kDa found in the nucleus was mapped to the 7.1-kbp *Bam*HI het DNA fragment which spans the *Eco*RI site linking the 20- and 16-kbp fragments of het DNA. Thus, HR-1 het DNA encodes several discrete polypeptide products, one or more of which could be responsible for the unusual biologic properties of the virus. The composition, regulation, and ultimately the expression of some of these products relative to standard EBV is probably altered by the genomic rearrangements of het DNA.

A population of Epstein-Barr viruses (EBV) diverse in biologic properties and genome structure is harbored by the P3HR-1 (HR-1) cell line, derived originally from Jijoye Burkitt lymphoma cells (22). In addition to the standard EBV genome, the virions produced by the P3HR-1 cell line contain heterogeneous viral DNA (het DNA) which differs in organization from the standard EBV genome (2, 11, 19, 21, 35, 40). het DNA has many features of defective viral DNA, i.e., only portions of the standard genome are represented and many rearrangements are present. Recently, it has been shown that het DNA is responsible for certain distinctive biologic properties of HR-1 virus (30). For example, one fragment of het DNA can activate expression of latent EBV genomes (9).

Assignment of biologic traits to standard or defective HR-1 virus has been made possible by subcloning cell lines from the parental HR-1 line. Cell subclones have been isolated which harbor only the standard HR-1 genome or the standard genome plus het DNA (21). The defective virus has not yet been propagated in the absence of standard virus, suggesting a reliance of defective virus on helper functions of the standard genome.

Neither the standard nor the defective virus from the HR-1 cell line can immortalize lymphocytes, most likely consequent to a large genomic deletion (3, 24, 27, 31, 32). The lack of immortalizing activity of parental HR-1 EBV is not due to interference by the defective virus since subpopulations lacking het DNA are also unable to immortalize lymphocytes (30).

A total of 10% or more of HR-1 cells which harbor both the standard and defective genomes spontaneously enter the viral replicative cycle (21). By comparison, cells which contain only the standard HR-1 genome have a low level of spontaneous virus production, usually less than 1%. Therefore, het DNA is likely to be responsible for spontaneous virus production by HR-1 cells. However, certain cell clones containing only standard HR-1 virus can be induced to produce and release large amounts of virus by treatment with butyrate or phorbol ester (42). These HR-1 cell clones are designated superinducible. Thus, het DNA is not required for chemical induction of the viral replicative cycle.

Cell cloning of the HR-1 line selects for subpopulations which contain only the standard EBV genome. When the parental HR-1 cell line was cloned, only 1 of 200 cell clones contained defective virus. When this rare cell clone was further subcloned, the defective genomes were lost (30). Thus, unlike the standard EBV genome, which is invariably partitioned to daughter cells, the defective genome is not readily maintained in dividing cells.

Incubation of the parental HR-1 line, or its rare cell clone which contains both standard and defective genomes, in the presence of EBV-neutralizing antibody eliminates the defective genomes from the culture while the standard genomes are unaffected by the antibody (28). Thus, the defective genome is encapsidated and maintained horizontally in the

* Corresponding author.

culture by cell-to-cell spread, unlike the standard genomes which are partitioned vertically to daughter cells.

Virions which contain standard and defective genomes mediate the ability of parental HR-1 EBV to induce early replicative antigens (EA) in Raji cells (15, 16, 20, 33). Populations consisting exclusively of standard virions do not initiate synthesis of replicative antigens in Raji cells. Whether these replicative products are encoded by the superinfecting standard or defective EBV genomes or by the endogenous Raji EBV genome is not known.

Similarly, virions which contain standard and defective genomes but not the standard genome alone activate complete expression of a latent immortalizing EBV present in a cell line designated X50-7 (30). Because the virus recovered is of the transforming phenotype and has restriction endonuclease polymorphisms of the endogenous EBV genome, it is clear that the defective HR-1 virus activates EBV in *trans*. In this experiment the superinfecting genome encompassing het DNA replicates, but the standard HR-1 genome, which is also present, does not.

The foregoing group of experiments indicate that the defective HR-1 viral genome contains a number of critical functions which mediate the biologic activity of HR-1 EBV. The defective genomes evidently contain one or more origins of viral DNA replication. Since they are encapsidated, they must contain packaging signals. Furthermore, they harbor one or more genes which code for products which act in *trans* to activate expression of latent EBV DNA. These defective HR-1 genomes are extremely valuable mutants for deciphering functional regions of the EBV genome.

The identifying characteristics of het DNA are digestion fragments which are heterogeneous in size and homology compared with those of the standard EBV genome. The present set of experiments was undertaken to define the structure of a 36-kilobase-pair (kbp) region of het DNA. This makes up the majority of het DNA in the defective HR-1 genome found in a cellular subclone enriched in defective sequences (21). We have begun to map functional regions of this defective genome by gene transfer of het DNA into eucaryotic cells.

(This work was presented in preliminary form at the Ninth International Herpesvirus Workshop, Seattle, Washington, August 1984.)

MATERIALS AND METHODS

Cells and cellular DNA. As a source of parental HR-1 virus we used the P3H3 line obtained in 1981 from W. Henle. A prototype cellular subclone lacking het DNA is clone HH514-16 (clone 16). Clone HH543-5 (clone 5) contains het DNA in addition to the standard HR-1 genome and exhibits the biologic properties of parental HR-1 virus (21, 30). Total intracellular DNA from clone 16, clone 5, or parental HR-1 cells was extracted by the procedure of Davis and Kingsbury (10). Homologies to specific EBV DNA sequences were detected by Southern blotting (38).

Plasmids. Digestion fragments of EBV DNA were cloned as *Eco*RI fragments into pACYC184 or as *Bam*HI fragments into pBR322. Bacterial plasmids were maintained in *Escherichia coli* DH1 (*recA*$^-$) (17). het DNA fragments were identified by their distinctive size and homologies compared with standard EBV DNA fragments (13). het DNA fragments were subcloned into pSV2-neo (39) by standard methods. Our nomenclature for the cloned het DNA fragment includes the restriction endonuclease which isolates the DNA fragment, the designation het, and an identifier based on size in kilobase pairs (e.g., *Eco*RI het 20, *Bam*HI het 2.7). Including the homology of the larger het DNA fragments proved cumbersome owing to multiple genomic rearrangements.

Restriction endonuclease site mapping. Two methods involving partial *Bam*HI digestion were used. In the first, unlabeled *Eco*RI het 16 was isolated from a 0.5% agarose gel and purified with Elutip-d (Schleicher & Schuell, Inc., Keene, N.H.) (36). This DNA was partially digested with *Bam*HI, and the products were separated by electrophoresis in 0.5% agarose and transferred to nitrocellulose. The Southern blot was probed with a fragment of het DNA isolated by agarose gel electrophoresis and labeled by nick translation (34).

In another experiment the excised *Eco*RI-*Hin*dIII subfragments of *Eco*RI het 16 were labeled at the *Hin*dIII end with the Klenow fragment of DNA polymerase and [α-^{32}P]dCTP. The end-labeled DNA was partially digested, and the products were separated by agarose gel electrophoresis and visualized by autoradiography (37).

Molecular size markers for both methods were complete *Hin*dIII digests of lambda DNA.

Homology to standard EBV DNA. Cloned het DNA fragments were digested with either *Hin*dIII or *Bam*HI, and the subfragments were separated by agarose gel electrophoresis and transferred to nitrocellulose. Homology to standard EBV DNA was established by probing the Southern blot with nick-translated cloned *Bam*HI fragments of EBV DNA from EBV strain FF41 (13) and HR-1 subclone FF452-3, which contains only the standard HR-1 genome (32).

Gene transfer and antigen detection. het DNA fragments cloned in pSV2-neo were used to transfect COS-1 cells with DEAE-dextran followed by chloroquine (26). Antigens were detected by indirect antiimmunoglobulin or anticomplement immunofluorescence. Well-characterized human sera were the source of EBV antibodies. These sera were WC (EBNA1$^-$, EA$^+$, VCA$^+$) (12), GG49a (EBNA1$^+$, EA$^+$, VCA$^+$), and RM (EBNA1$^+$, EA$^-$, VCA$^+$) (EBNA1, Epstein-Barr nuclear antigen 1; EA, early antigen; VCA, viral capsid antigen). Extracts of COS-1 cells from five 100-mm tissue culture dishes transfected 3 days previously were analyzed by Western immunoblotting, using procedures previously described (4, 23, 25, 41).

RESULTS

Homologies between EBV sequences present in standard and het DNA genomes. We used three methods to determine the genomic composition of het DNA. In the first, intracellular DNA from HR-1 clone 16 (standard genome alone) and HR-1 clone 5 (standard plus defective genomes) was cleaved with *Eco*RI or *Bam*HI, and the Southern blots were probed with cloned standard EBV DNA *Bam*HI fragments. DNA restriction endonuclease fragments found in clone 5 but not in clone 16 were defined as het DNA. The homologies of these novel digestion fragments have been published previously (see reference 30, Table 5). An important finding from these experiments was that individual het DNA fragments exhibited homology to standard EBV fragments which are not adjacent on the genome.

In the second type of experiment, het DNA from HR-1 cell clone 5 was first inserted into chimeric plasmids as *Eco*RI or *Bam*HI fragments. These plasmids were then used to probe blots of intracellular DNA from clone 5 and clone 16 cells. A representative experiment is shown in Fig. 1. In this experiment the two large cloned *Eco*RI het DNA fragments, designated *Eco*RI het 20 and *Eco*RI het 16, were the probes.

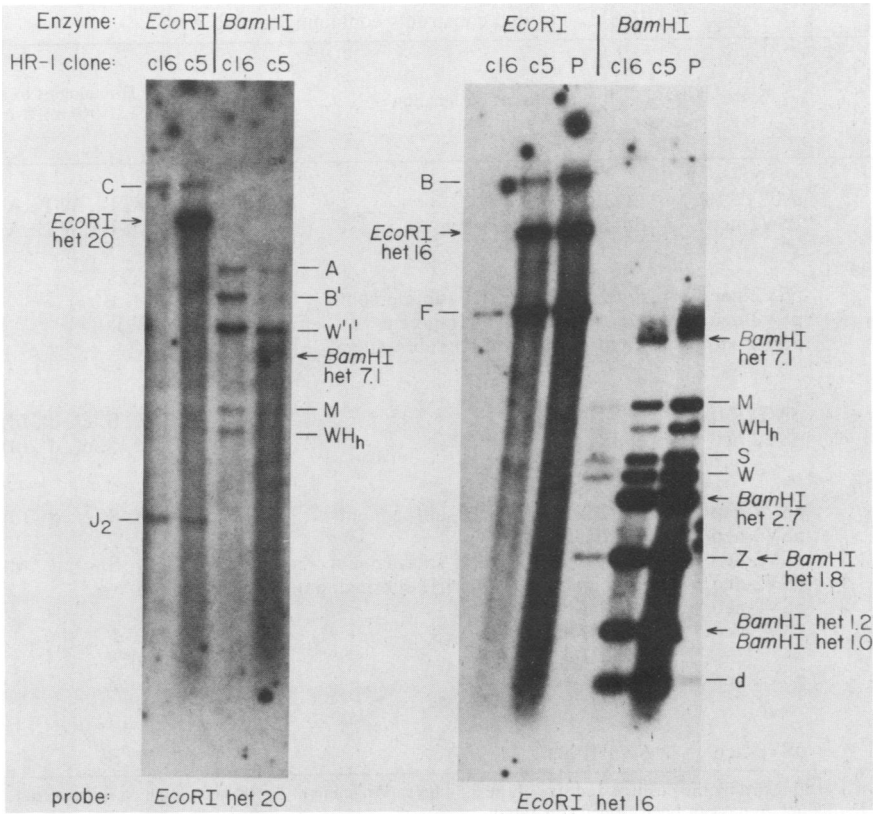

FIG. 1. Homology of cloned *Eco*RI het 20 and *Eco*RI het 16 to EBV DNA fragments present in clone 16 (c16), clone 5 (c5), and parental (P) HR-1 cells. Duplicate Southern blots containing both *Eco*RI and *Bam*HI digests of cellular DNA were probed with the cloned *Eco*RI het 20 or *Eco*RI het 16. Arrows indicate het DNA fragments which are seen in clone 5 and parental HR-1 DNA, but not in clone 16, which lacks het DNA. *Bam*HI het 1.8 comigrates with *Bam*HI-Z. The other letters indicate standard HR-1 DNA fragments which are homologous to the het DNA probe. WH_h indicates the HR-1 fusion fragment of *Bam*HI-W and *Bam*HI-H.

*Eco*RI het 20 identified a hypermolar 20-kbp DNA fragment in clone 5 cells which was absent in clone 16 cells. Similarly *Eco*RI het 16 had homology to a hypermolar 16-kbp *Eco*RI fragment present in clone 5 and parental HR-1 cells but missing from clone 16 cells. Thus, both large cloned *Eco*RI het DNA fragments were homologous to viral het DNA fragments of equivalent size found inside cells.

When the large cloned *Eco*RI het DNA fragments were used to probe *Bam*HI digests of intracellular DNA, they identified standard *Bam*HI fragments (present in clone 16 and in clone 5) as well as het DNA *Bam*HI fragments, found only in clone 5 (Fig. 1). For example, *Eco*RI het 20 identified *Bam*HI het 7.1 which we know from other studies to have homology to standard fragments *Bam*HI-M and *Bam*HI-B'. *Eco*RI het 16 had homology with many standard fragments as well as *Bam*HI het 7.1, *Bam*HI het 2.7, *Bam*HI het 1.8, *Bam*HI het 1.2, and *Bam*HI het 1.0.

The third type of experiment was to digest the recombinant *Eco*RI het DNA fragments with *Bam*HI or *Hin*dIII and probe Southern blots containing these digests with standard EBV *Bam*HI fragments (Table 1).

Structure of *Eco*RI het 20. Digestion of *Eco*RI het 20 with *Bam*HI yielded three subfragments of 5.1, 8.0, and 6.6 kbp. These subfragments had homology to standard *Bam*HI-M and *Bam*HI-B', *Bam*HI-W'I', and *Bam*HI-A, respectively. The latter two of the three *Bam*HI subfragments were identical in size and had identical homologies to subfragments of the standard *Eco*RI C fragment in the HR-1 genome. Therefore, the right end of *Eco*RI het 20 is comparable to standard EBV (HR-1) DNA represented by *Bam*HI-W'I' and *Bam*HI-A (Fig. 2). The rearrangement which distinguishes *Eco*RI het 20 as het DNA is the linkage of deleted portions of standard *Bam*HI-M and *Bam*HI-B' in a single *Bam*HI het DNA fragment.

Structure of *Eco*RI het 16. A similar approach proved difficult with *Eco*RI het 16 since the *Bam*HI digestion products appeared to be hypermolar and many were of identical size (Fig. 3). Densitometric analysis of the *Bam*HI digestion pattern corroborated the hypermolarity of all but one of the internal *Bam*HI subfragments of *Eco*RI het 16 (data not shown). Both *Bam*HI-*Eco*RI terminal subfragments of *Eco*RI het 16 were of identical size, 1.25 kbp, and both were homologous to standard *Bam*HI-M. Identically sized terminal subfragments were noted with a number of restriction endonucleases. For example, the terminal *Eco*RI-*Hin*dIII subfragments were of identical size, 5.8 kbp. This suggested that the structure of *Eco*RI het 16 might be palindromic.

This hypothesis was tested by studying partial digestions of cloned *Eco*RI het 16 which had been purified by excision from an agarose gel. Duplicate Southern blots containing the *Bam*HI partial digestion products were separately probed with each of the 1.25-kbp *Bam*HI-*Eco*RI terminal subfragments of *Eco*RI het 16 (Fig. 4). Since these termini are identical in size and homology, they were isolated by digesting the plasmid pACYC184 carrying *Eco*RI het 16 only with *Bam*HI, leaving each of the *Bam*HI-*Eco*RI termini attached to an asymmetric arm of pACYC184. Identical *Bam*HI

TABLE 1. Plasmid constructs containing het DNA

Laboratory designation of clone	Vector	Insert designation	kbp	Homologies to standard EBV (*Bam*HI fragment)	Comparable fragment in standard HR-1 genome
*Eco*RI het 20					
pHH938-5	pACYC184	*Eco*RI het 20	20	M, B′, W′I′, A	No
pJJ537	PSV2-neo	*Eco*RI het 20	20	M, B′, W′I′, A	No
Subclones of *Eco*RI het 20					
pJJ967	pSV2-neo	Left *Eco*RI-*Bam*HI subfragment	5.1	M, B′	No
pJJ968	pSV2-neo	Middle *Bam*HI subfragment	8.0	W′I′	Yes
pJJ969	pSV2-neo	Right *Eco*RI-*Bam*HI subfragment	6.6	A	Yes
*Eco*RI het 16					
pJJ66-18	pACYC184	*Eco*RI het 16	16	M, S, c′, d′, (d)[a], Z, W	No
pJJ538	pSV2-neo	*Eco*RI het 16	16	M, S, c′, d′, (d)[a], Z, W	No
Subclones of *Eco*RI het 16					
pJJ858	pSV2-neo	Left *Eco*RI-*Hin*dIII subfragment	5.8	M, S, c′, d′, (d)[a], Z	No
pJJ860	pSV2-neo	*Hin*dIII het 4.7	4.7	W, Z	No
pJJ859	pSV2-neo	Right *Eco*RI-*Hin*dIII subfragment	5.8	M, S, c′, d′, (d)[a], Z	No
pJJ859-1.25	pSV2-neo	Terminal *Eco*RI-*Bam*HI subfragment	1.25	M	Yes
pKK106	pSV2-neo	*Bam*HI het 1.8	1.8	S	No
pJJ859-1.0	pSV2-neo	*Bam*HI het 1.0	1.0	c′, d′	No
—[b]		*Bam*HI-d	0.5	(d)[a]	Yes
pKK105	pSV2-neo	*Bam*HI het 2.7	2.7	W, Z	No
—[c]		*Bam*HI het 1.2	1.2	W	No
*Bam*HI het 7.1 (pKK104)	pSV2-neo	*Bam*HI het 7.1	7.1	M, B′	No

[a] The homology to *Bam*HI-d is established by the position and size of the 0.5-kbp subfragment in *Eco*RI het 16, which is identical to standard HR-1 *Bam*HI-d.
[b] This *Bam*HI fragment of *Eco*RI het 16 has not been cloned separately.
[c] *Bam*HI het 1.2 has not been cloned separately but is wholly contained within *Hin*dIII het 4.7.

partial digestion patterns were seen for both of the probes made from the left and right terminal digestion subfragments. Five *Bam*HI subfragments were duplicated surrounding a central 1.2-kbp *Bam*HI subfragment for a total of 11 *Bam*HI subfragments of *Eco*RI het 16. These 11 subfragments account for the molecular weight of 16 kbp. This experiment indicated that *Eco*RI het 16 is a large palindrome flanking a central 1.2-kbp *Bam*HI subfragment.

Because of this novel and unexpected finding, we substantiated the palindromic structure by comparison of the partial digestion products of each of the two *Hin*dIII-*Eco*RI terminal subfragments of *Eco*RI het 16. Each of these 5.8-kbp subfragments was cloned attached to an asymmetric *Hin*dIII-*Eco*RI arm of pACYC184. The het DNA from each of these clones was excised, labeled at the 3′ *Hin*dIII site, subjected to partial digestion with *Bam*HI, and visualized by autoradiography (Fig. 5). The results were identical for both of these large termini, confirming the restriction endonuclease map of *Eco*RI het 16 in a palindromic arrangement.

The next step was to identify homologies between stan-

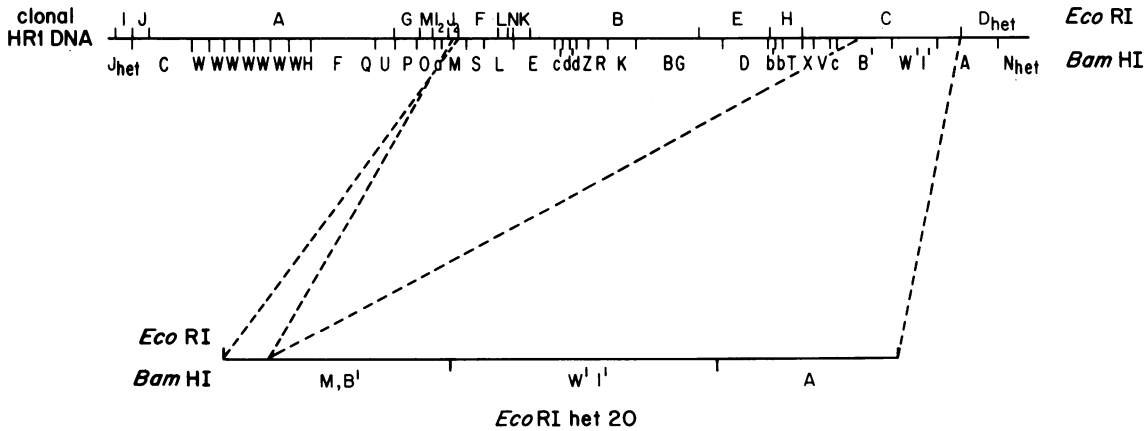

FIG. 2. *Bam*HI digestion map of *Eco*RI het 20. This het DNA fragment contains two subfragments, *Bam*HI-W′I′ and *Bam*HI-A, which are the same size as the comparable fragments in standard EBV DNA. The distinctive feature of *Eco*RI het 20 is found at the left end of the map where sequences of *Bam*HI-M and *Bam*HI-B′, which are not contiguous in the standard genome, are juxtaposed. The exact point of juncture of *Bam*HI-M and *Bam*HI-B′ sequences is unknown. The genomic map of the standard HR-1 genome is shown for comparison.

dard EBV DNA fragments and complete *Bam*HI digestions of the left, right, and center *Hin*dIII subfragments of *Eco*RI het 16. These results substantiated the palindromic structure, i.e., *Bam*HI subfragments of identical size in the left and right *Hin*dIII-*Eco*RI subfragments of *Eco*RI het 16 were homologous to the same standard EBV DNA fragments (Fig. 6).

Several features of *Eco*RI het 16 were noted in addition to the palindromic structure. *Eco*RI het 16 contained duplicated complete copies of three *Bam*HI het DNA subfragments: *Bam*HI het 2.7, *Bam*HI het 1.8, and *Bam*HI het 1.0. Furthermore, *Eco*RI het 16 contained as the terminal *Bam*HI-*Eco*RI subfragments a 1.25-kbp subfragment also found in *Bam*HI het 7.1. *Bam*HI het 7.1, *Bam*HI het 2.7, and *Bam*HI het 1.8 were cloned independently from HR-1 clone 5 virion DNA as het DNA fragments. A 1.2-kbp het DNA subfragment with homology to *Bam*HI W was found in the center of the palindrome. The extent to which the palindrome within this center *Bam*HI subfragment is perfect is under investigation by DNA sequence analysis. Two *Bam*HI subfragments of *Eco*RI het 16, of 1 and 0.5 kbp, were homologous to standard *Eco*RI-B but did not have homology to *Bam*HI-E, *Bam*HI-K, or *Bam*HI-Z. We do not have HR-1 fragments *Bam*HI-d, *Bam*HI-c', or *Bam*HI-d' cloned for use as probes but have investigated the 1-kbp subfragment by DNA sequencing (H. B. Jenson et al., manuscript in preparation). The 1-kbp subfragment correlates with HR-1 *Bam*HI-c' and *Bam*HI-d', with loss of the intervening *Bam*HI site owing to DNA base-pair changes. This difference distinguishes this subfragment as het DNA (32). The 0.5-kbp subfragment is identified by its position and size in *Eco*RI het 16, which is identical to standard HR-1 *Bam*HI-d.

Antigen expression after gene transfer with cloned fragments of het DNA. Antigens were sought by indirect anticomplement immunofluorescence in COS-1 cells which had been transfected with *Eco*RI het 20, *Eco*RI het 16, and subfragments cloned into pSV2-neo. The source of antibodies was human sera of defined reactivity to the classical EBV antigens.

An antigen located predominantly in the cytoplasm was induced by *Eco*RI het 20 (Fig. 7). Reaction with this antigen was brilliant with a serum from a patient with chronic active EBV infection and extremely high titers to EA and VCA (12); however, the antigen could also be detected by antisera which lacked antibody to EA. An antigen of similar morphologic and serologic characteristics was seen in COS-1 cells transfected with the 6.6-kbp *Bam*HI-*Eco*RI subfragment of *Eco*RI het 20 which has homology to standard *Bam*HI-A. A morphologically similar cytoplasmic antigen, also serologically classified as a VCA, was seen in COS-1 cells transfected with the comparable *Bam*HI-*Eco*RI region of *Bam*HI-A from EBV strain FF41. Antigens were not induced by the subfragments of *Eco*RI het 20 homologous to *Bam*HI-M and *Bam*HI-B' or *Bam*HI-W'I'.

The antigens induced by *Eco*RI het 16 appeared on serologic grounds to be EAs, i.e., they were detected by sera containing antibodies to early and late (VCA) EBV antigens (12) but not by a human serum which contained antibodies only to the late products of EBV. A variety of morphologically different antigens (nucleus, nuclear membrane, or cytoplasm) were detected from *Eco*RI het 16. To attempt to separate these antigens, we expressed various subfragments of *Eco*RI het 16 in COS-1 cells (Fig. 7). A nuclear EA found predominantly in the nuclear membrane was expressed from *Bam*HI het 2.7, which has homology to *Bam*HI-W and *Bam*HI-Z. Digestion of *Bam*HI het 2.7 with *Hin*dIII resulted

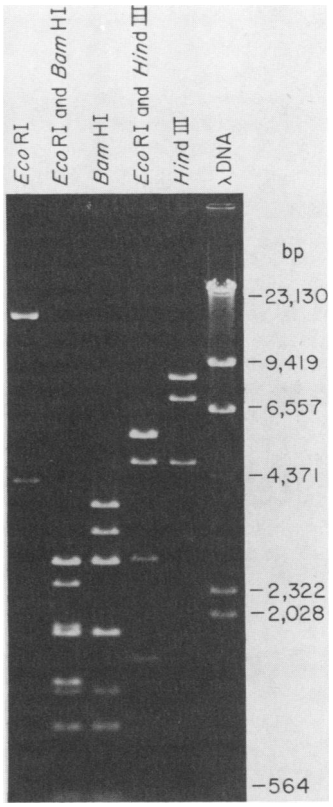

FIG. 3. *Bam*HI and *Hin*dIII restriction endonuclease subfragments of *Eco*RI het 16. The plasmid pJJ66-18, consisting of *Eco*RI het 16 inserted in the unique *Eco*RI site of pACYC184, was digested with *Eco*RI, *Bam*HI, and *Hin*dIII alone and in combination. Digestion with *Eco*RI and *Bam*HI compared with digestion with *Bam*HI alone shows the identical size (1.25 kbp) of the two *Bam*HI-*Eco*RI terminal digestion subfragments of *Eco*RI het 16. The two *Eco*RI-*Bam*HI subfragments of the vector pACYC184 (2.4 and 1.85 kbp) are each joined to a 1.25-kbp subfragment of *Eco*RI het 16 when *Bam*HI alone is used. Five sizes of internal *Bam*HI subfragments of *Eco*RI het 16 are seen, of 2.7, 1.8, 1.2, 1.0, and 0.5 kbp. Identical *Hin*dIII-*Eco*RI terminal digestion subfragments (5.8 kbp) are also seen with *Eco*RI and *Hin*dIII compared with *Hin*dIII alone. The two *Eco*RI-*Hin*dIII subfragments of the vector pACYC184 (2.8 and 1.5 kbp) are each joined to a 5.8-kbp subfragment of *Eco*RI het 16 when *Hin*dIII alone is used. A single internal *Hin*dIII subfragment of *Eco*RI het 16 is seen, of 4.7 kbp. Except for the center *Bam*HI subfragment of 1.2 kbp and the center *Hin*dIII subfragment of 4.7 kbp, all other *Bam*HI and *Hin*dIII digestion subfragments of *Eco*RI het 16 appear present in hypermolar quantity.

in loss of antigen expression. Accordingly, this antigen was not seen from either of the *Hin*dIII-*Eco*RI subfragments of *Eco*RI het 16. Therefore, the encoding region for this polypeptide presumably crosses this *Hin*dIII site. Another antigen, found in the nucleus and also characterized as an EA, was localized to *Bam*HI het 1.8, which has homology to *Bam*HI-S. A variety of nuclear and cytoplasmic antigen expression was seen from both of the *Hin*dIII-*Eco*RI subfragments of *Eco*RI het 16. Some of this antigen expression is likely to be from *Bam*HI het 1.8 which is wholly contained on each of these *Hin*dIII-*Eco*RI subfragments. The *Bam*HI het 7.1 which spans the *Eco*RI site between *Eco*RI het 20 and *Eco*RI het 16 induced a brilliant EA found in the nucleus and nuclear membrane. This antigen was not expressed from either of the *Bam*HI-*Eco*RI terminal subfragments of *Eco*RI het 20 or *Eco*RI het 16. Therefore, the

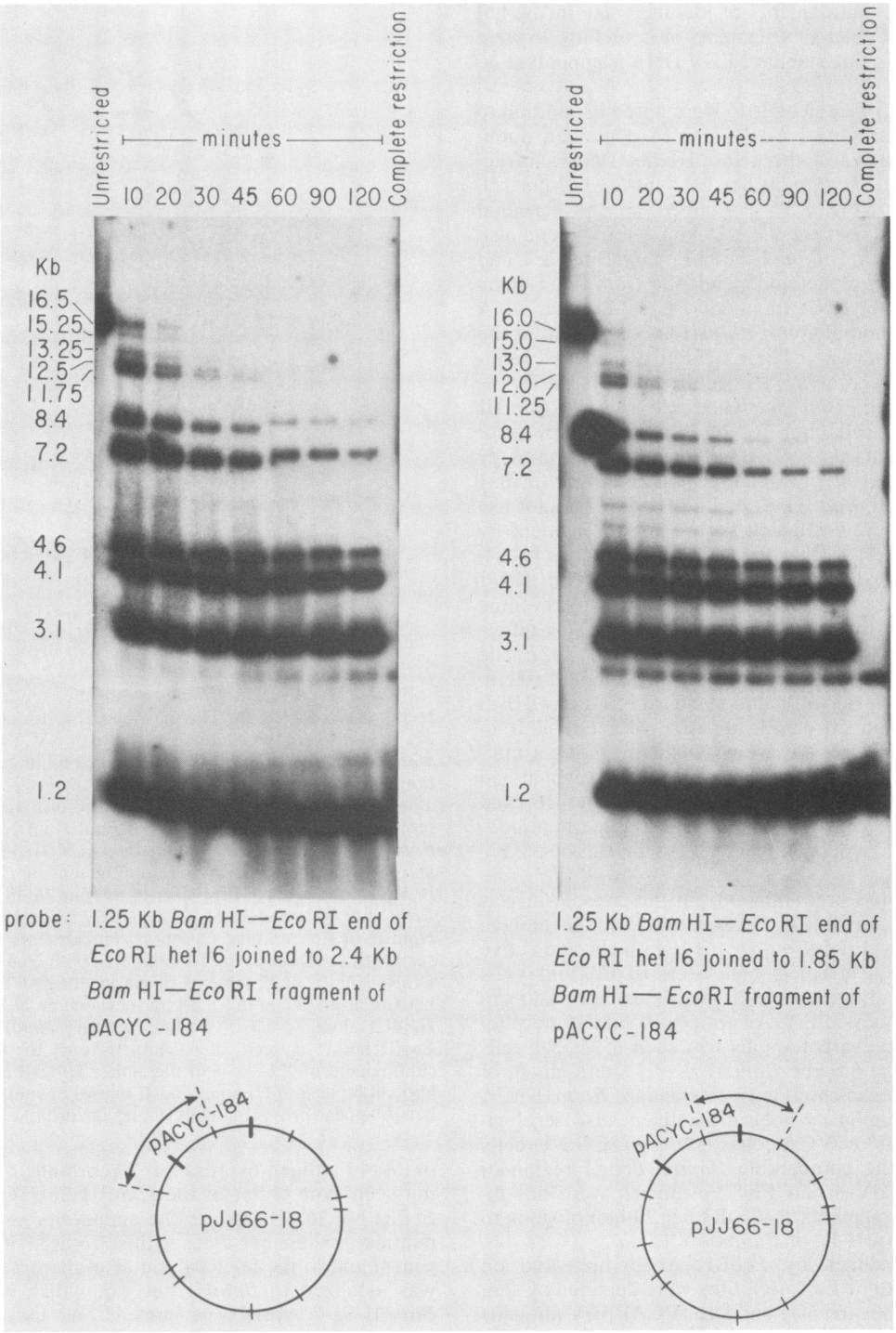

FIG. 4. Analysis of the structure of *Eco*RI het 16 by partial *Bam*HI digestion. *Eco*RI het 16 in pACYC184 was digested with *Eco*RI, and the het DNA fragment was eluted from an agarose gel. The fragment was digested with suboptimal amounts of *Bam*HI for various lengths of time. Duplicate Southern blots were prepared, each probed with one of the terminal *Bam*HI-*Eco*RI subfragments of *Eco*RI het 16. These identically sized terminal subfragments were isolated from a gel after digesting the pACYC184 plasmid carrying *Eco*RI het 16 with *Bam*HI only, leaving each terminal *Bam*HI-*Eco*RI subfragment linked to an asymmetric plasmid arm. The probe fragments were labeled by nick translation. Molecular size standards of lambda DNA digested with *Hin*dIII were included in duplicate on each blot. The pattern of partial digestion products is identical with each of the two terminal subfragments as the probe. This is indicative of a palindromic structure.

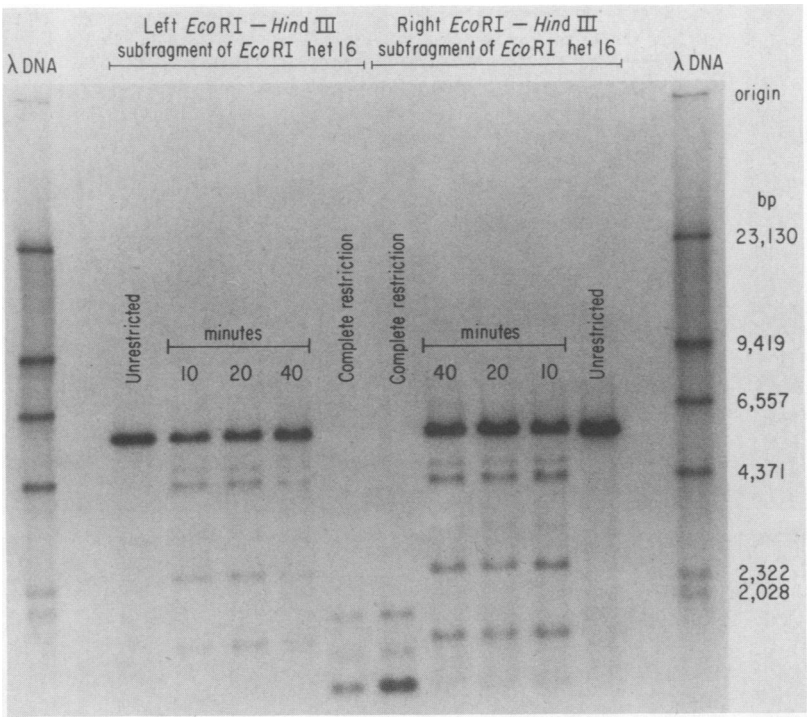

FIG. 5. Analysis of the structure of *Eco*RI het 16 with end-labeled DNA. The two identically sized *Eco*RI-*Hin*dIII terminal subfragments of *Eco*RI het 16 were cloned separately with an asymmetric plasmid arm to distinguish them. Each of the *Eco*RI-*Hin*dIII subfragments was excised from a gel, 3′ end labeled at the *Hin*dIII site, and subjected to partial and complete *Bam*HI digestion. The products were separated by electrophoresis and visualized by autoradiography. The pattern of complete and partial digestion is identical. The submolar bands, e.g., in the lanes of complete digest, are due to minimal internal labeling of the DNA.

encoding region likely spans the *Eco*RI site linking *Eco*RI het 20 and *Eco*RI het 16.

Polypeptides representing antigens expressed from het DNA. *Eco*RI het 20, *Eco*RI het 16, *Bam*HI het 7.1, and various subfragments cloned on pSV2-neo were transfected into COS-1 cells, and antigenic polypeptides were characterized by Western immunoblotting with the highly reactive polyvalent human serum WC (Fig. 8; Table 2).

The single antigen from *Eco*RI het 20 was detected as a prominent polypeptide of approximately 95 kilodaltons (kDa). The same polypeptide was expressed from the 6.6-kbp *Bam*HI-*Eco*RI subfragment of *Eco*RI het 20.

Several antigenic polypeptides were detected from *Eco*RI het 16, including prominent polypeptides of approximately 52 and 36 kDa. Less prominent polypeptides were identified at 42, 40, 28, and 19 kDa. Subfragments of *Eco*RI het 16 were used to localize the coding regions for some of these polypeptides.

The prominent 36-kDa polypeptide and the less prominent 28- and 19-kDa polypeptides were encoded by *Bam*HI het 2.7 but not from either of the *Hin*dIII-*Eco*RI subfragments of *Eco*RI het 16. This is consistent with the finding that digestion at the *Hin*dIII site of *Bam*HI het 2.7 resulted in loss of antigen production detectable by immunofluorescence.

Only one of the two *Hin*dIII-*Eco*RI subfragments showed appreciable antigen production detectable by Western immunoblotting, of approximately 63, 55, and 46 kDa. The basis for the apparent difference in polypeptide production between these otherwise apparently identical subfragments is unknown.

*Bam*HI het 7.1, which bridges *Eco*RI het 20 and *Eco*RI het 16, encoded a prominent polypeptide of approximately 50kDa. As in the immunofluorescence assay, this product was not seen from either *Eco*RI het 20 or *Eco*RI het 16.

DISCUSSION

Many rationales exist for studying the structure and expression of defective EBV genomes. The defective population we have been studying from HR-1 clone 5 cells holds clues to many important processes in the viral life cycle. het DNA is responsible, in part, for stimulating the replicative phase and disrupting latency (30). The het DNA replicates preferentially upon superinfection of the defective virus into cells harboring a latent EBV genome; therefore, it contains

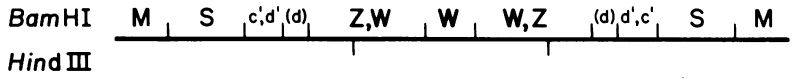

FIG. 6. *Bam*HI and *Hin*dIII digestion map of *Eco*RI het 16. The order of the *Bam*HI subfragments of *Eco*RI het 16 was determined from the sizes of the partial digestion products in Fig. 4 and 5. Homologies to standard EBV *Bam*HI fragments are shown above each het DNA *Bam*HI subfragment. The homology to *Bam*HI-d was assigned by the size and position of this subfragment in *Eco*RI het 16, which is identical to standard HR-1 *Bam*HI-d.

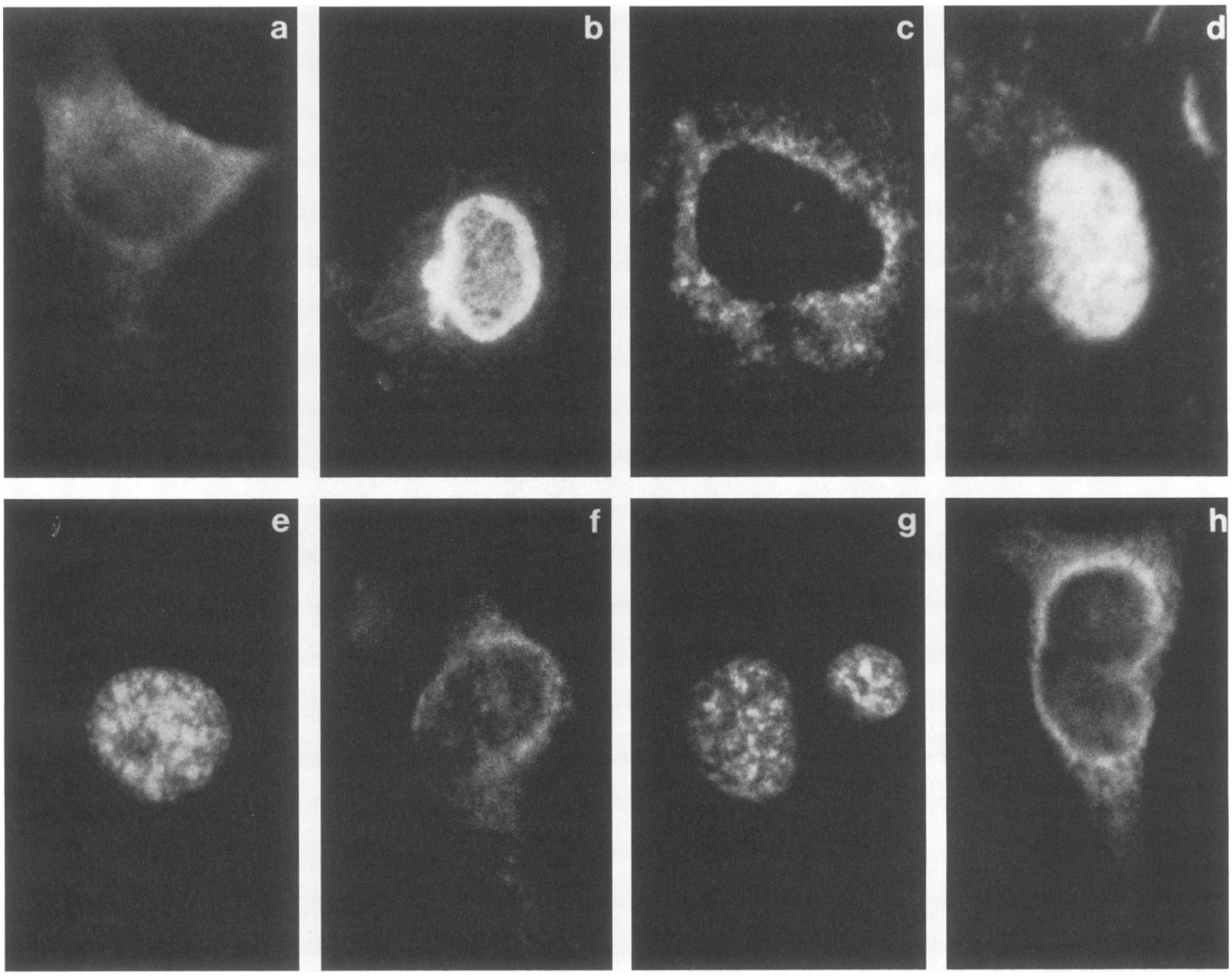

FIG. 7. Antigen expression in COS-1 cells after transfection with het DNA fragments cloned on pSV2-neo, detected by immunofluorescence with anticomplement and the highly reactive serum WC. (a) A cytoplasmic antigen is detected from *Eco*RI het 20 (pJJ537) and has been localized to the region with homology to standard EBV *Bam*HI-A. (b) A nuclear-nuclear membrane antigen is detected from *Bam*HI het 7.1 (pKK104), which has homology to *Bam*HI-B′ and *Bam*HI-M, including sequences homologous to standard *Eco*RI-F not present in *Eco*RI het 20. (c) A nuclear membrane antigen from *Bam*HI het 2.7 (pKK105), which has homology to *Bam*HI-W and *Bam*HI-Z. (d) A nuclear antigen from *Bam*HI het 1.8 (pKK106), which has homology to *Bam*HI-S. (e to h) A variety of morphologic antigens are detected from both of the *Eco*RI-*Hin*dIII subfragments of *Eco*RI het 16 (pJJ858 and pJJ859). (e and f) Antigens from the left *Eco*RI-*Hin*dIII subfragment (pJJ858); (g and h) antigens from the right *Eco*RI-*Hin*dIII subfragment (pJJ859).

one or more origins of viral DNA replication. Since it is passed cell to cell and encapsidated it evidently contains signals for packaging into mature virions. It does not seem to partition to daughter cells; this may be the result of its ability to disrupt latency or it may be due to the lack of signals required for partitioning. Since it does not immortalize and does not establish a latent infection, EBV with het DNA is an interesting potential candidate for a live virus vaccine (29).

Structure of het DNA. The 36-kbp stretch of het DNA which we analyzed makes up the majority of het DNA which has been identified from HR-1 clone 5. Many unusual features are evident by comparison to EBV DNA isolated from standard genomes. (i) A number of intramolecular recombination events of the standard EBV genome seem to have occurred. We can define at least three new recombinations present in clone 5 het DNA. They are between *Bam*HI-M and *Bam*HI-B′, *Bam*HI-W and *Bam*HI-Z, and *Bam*HI-S and a region of *Eco*RI-B very close to the *Bam*HI site between *Bam*HI-E and *Bam*HI-c′. (ii) There have been extensive deletions of the genome. The het DNA fragments identified represent only about 25% of the total EBV genome. Many regions of the standard genome are not represented at all. Furthermore, there are several smaller deletions within fragments which are part of het DNA. For example, *Bam*HI het 1.8, which has homology only to *Bam*HI-S, is 1.6-kbp smaller than standard *Bam*HI-S. (iii) Reorientation of het DNA fragments relative to the standard EBV genome has occurred. For example, the orientation of *Bam*HI-M sequences in *Bam*HI het 7.1 vis-à-vis the *Bam*HI-B′ sequences is opposite to the relative orientation of these two fragments in the standard genome (see below). (iv) DNA base-pair changes exist between the standard HR-1 genome and comparable regions of het DNA. The most obvious example is in *Bam*HI het 1.0 in which two base-pair changes abolish the *Bam*HI site. Many other base-pair differences are

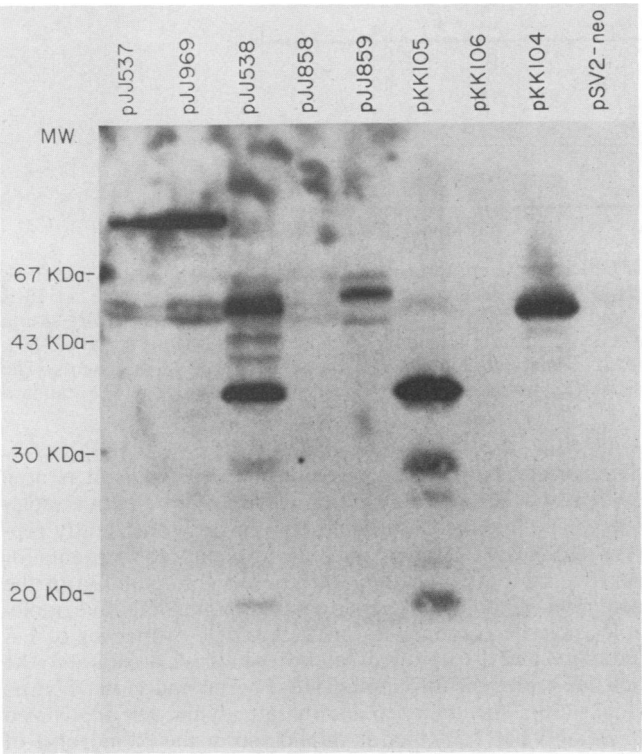

FIG. 8. Western immunoblot of polypeptide antigens expressed in COS-1 cells after transfection with cloned het DNA fragments (Table 2). A single 95-kDa polypeptide is detected from*Eco*RI het 20 (pJJ537) and from the *Bam*HI subfragment with homology to *Bam*HI-A (pJJ969). Several polypeptides are detected from *Eco*RI het 16 (pJJ538), the strongest signals being the 52- and 36-kDa polypeptides. Other polypeptides are detected from the *Eco*RI-*Hin*dIII subfragments of *Eco*RI het 16 (pJJ858 and pJJ859), but the 36-kDa polypeptide is lost. *Bam*HI het 2.7 (pKK105) reveals three polypeptides of 36, 28, and 19 kDa which are also seen with *Eco*RI het 16 (pJJ538). No polypeptide is detected from *Bam*HI het 1.8 (pKK106). A 50-kDa polypeptide not present on either *Eco*RI het 20 or *Eco*RI het 16 is localized to *Bam*HI het 7.1 (pKK104). MW, Molecular weight.

seen by comparison of het DNA to standard HR-1 sequences (Jenson et al., in preparation). (v) Large stretches of sequences have been duplicated. The most dramatic example of duplication is the large palindrome in *Eco*RI het 16. (vi) Presumably concatemerization of a defective DNA monomer takes place to fulfill requirements of a headful packaging mechanism. This hypothesis has not been tested experimentally.

*Eco*RI het 16 illustrates all of these anomalies. Sequence analysis in the center of the palindrome will provide fine structural details including the extent of the palindrome within the center 1.2-kbp *Bam*HI subfragment (Jenson et al., in preparation). This palindrome is represented in virion DNA. A 16-kbp het DNA fragment was found intracellularly in HR-1 clone 5 cells and not in clone 16 cells (Fig. 1). Furthermore, this 16-kbp fragment in intracellular DNA had identical homologies to those of the cloned fragment. Finally, *Bam*HI het DNA subfragments of *Eco*RI het 16 were also independently isolated directly from HR-1 clone 5 virion DNA.

Several findings substantiate the premise that *Eco*RI het 20 attaches to one and possibly both ends of *Eco*RI het 16 (Fig. 9). The left terminus of *Eco*RI het 20 has homology to *Bam*HI-M as do both termini of *Eco*RI het 16. The sequences of *Bam*HI-M found in *Eco*RI het 20 have homology to *Eco*RI-J_2 (middle sequences of *Bam*HI-M), and the sequences of *Bam*HI-M found in *Eco*RI het 16 have homology to *Eco*RI-F (right-hand sequences of *Bam*HI M) (Fig. 1). These correspond to sequences on either side of the rightward *Eco*RI site in standard HR-1 *Bam*HI-M (Fig. 2). Because the sequences of *Bam*HI-M in *Eco*RI het 20 correspond to the middle of *Bam*HI-M, but proceeding in a leftward direction from *Eco*RI J_2, there must be an inversion of sequences *Bam*HI-M and *Bam*HI-B'/W'I'/A in *Eco*RI het 20. To further substantiate the joining of *Eco*RI het 20 to *Eco*RI het 16, a cloned 7.1-kbp *Bam*HI het DNA fragment which spans this *Eco*RI site had homology to both *Eco*RI het 20 and *Eco*RI het 16 (Fig. 1). This fragment when cleaved with *Eco*RI gave rise to a 1.25-kbp subfragment identical in size to the *Bam*HI-*Eco*RI terminal subfragments of *Eco*RI het 16. Therefore, the *Eco*RI site cleaving the two large *Eco*RI het DNA fragments is comparable to the right *Eco*RI site of *Bam*HI-M in the standard HR-1 genome. This rearrangement placed sequences of *Bam*HI-M and *Bam*HI-B' in opposite orientation in het DNA compared with the standard EBV genome (Fig. 2).

Much work remains to be done on the structure of the het DNA in HR-1 clone 5 cells. We do not yet know the composition of the entire het DNA molecule, nor how the monomer units are assembled. Sequencing of het DNA is needed at recombination points. These studies, particularly of the center of the palindrome, may provide some clues regarding how the molecules were generated.

Comparison of HR-1 clone 5 het DNA with parental HR-1 defective DNA. Others have analyzed defective DNA in the parental HR-1 strain. Heller et al. (19) looked at defective DNA in parental HR-1 virions which consisted of a mixture of standard and defective genomes. Cho et al. (6) analyzed P3HR-1 defectives which were preferentially replicated

TABLE 2. Summary of antigen and polypeptide expression in COS-1 cells after gene transfer with cloned het DNA fragments

Laboratory designation of clone	Insert designation	kbp	Location	kDa
pJJ537	*Eco*RI het 20	20	Cytoplasm	95
pJJ969	Right *Bam*HI-*Eco*RI end of *Eco*RI het 20	6.6	Cytoplasm	95
pJJ538	*Eco*RI het 16	16	Nucleus, nuclear membrane, cytoplasm	52, 42, 40, 36, 28, 19
pJJ858	Left *Hin*dIII-*Eco*RI end of *Eco*RI het 16	5.8	Nucleus, cytoplasm	Not seen
pJJ859	Right *Hin*dIII-*Eco*RI end of *Eco*RI het 16	5.8	Nucleus, cytoplasm	63, 55, 46
pKK105	*Bam*HI het 2.7	2.7	Nuclear membrane	36, 28, 19
pKK106	*Bam*HI het 1.8	1.8	Nucleus	Not seen
pKK104	*Bam*HI het 7.1	7.1	Nucleus, nuclear membrane	50

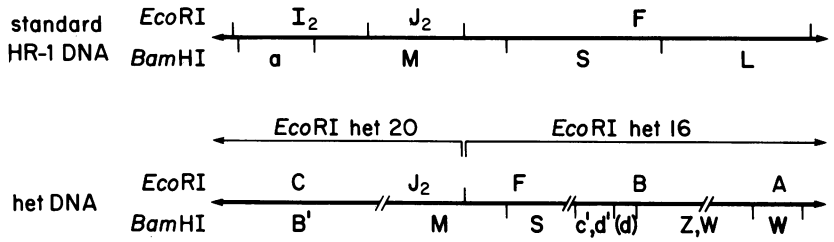

FIG. 9. Linkage of *Eco*RI het 20 and *Eco*RI het 16. One end of *Eco*RI het 20 and both ends of *Eco*RI het 16 are homologous to *Bam*HI-M. The region of *Bam*HI-M found in *Eco*RI het 20 is homologous to *Eco*RI-J_2 (Fig. 1). The region of *Bam*HI-M found in *Eco*RI het 16 is homologous to *Eco*RI-F (Fig. 1). The *Eco*RI site between *Eco*RI het 20 and *Eco*RI het 16 is comparable to the right-hand *Eco*RI site in *Bam*HI-M. The // marks indicate the three intramolecular recombination events which have joined four noncontiguous regions of EBV DNA. Letters in the line diagram of het DNA indicate homologies to standard *Eco*RI (above) and *Bam*HI (below) EBV DNA fragments. The homology of HR-1 *Bam*HI-d is established by the position and size of the 0.5-kbp subfragment in *Eco*RI het 16, which is identical to standard HR-1 *Bam*HI-d.

upon infection of BJAB cells. Further analysis was carried out on recombinant DNA clones of defective DNA harvested from Raji cells which were superinfected at high multiplicity with parental P3HR-1 virus (7).

There are both similarities and differences between the previously published descriptions of the structure of defective DNA in the parental HR-1 strain and our analysis of HR-1 clone 5 defective DNA. Many equivalent regions of EBV DNA are represented. For example, the region which is contained in *Eco*RI het 20 from the right end of the standard genome was found to be invariant. The defective fragment designated *Eco*RI-C1 by Cho et al. (7) appears identical to *Eco*RI het 20. Similar but not identical regions from the middle of the EBV genome have also been described. Heller et al. (19) and Cho et al. (6) both detected sequences of *Bam*HI-R to be represented. We did not find homology to *Bam*HI-R in *Eco*RI het 20, *Eco*RI het 16, or in any het DNA fragment identified in HR-1 clone 5 (30). Cho et al. (6) indicated that *Bam*HI-R is linked to *Bam*HI-W, whereas in the het DNA of clone 5 only *Bam*HI-Z was joined to *Bam*HI-W. Furthermore, the linkage of sequences of *Bam*HI-R to sequences of *Bam*HI-Z indicated by Cho et al. (6) is specifically excluded in HR-1 clone 5 het DNA by DNA sequencing which confirms the recombination of *Bam*HI-Z only to *Bam*HI-W (Jenson et al., in preparation).

All defectives so far described contain regions homologous to *Bam*HI-C, *Bam*HI-M, *Bam*HI-S, and *Bam*HI-Z. Heller et al. (19) and Cho et al. (6) detected homology to standard *Bam*HI-C in the same *Eco*RI het DNA fragment containing these other sequences. In HR-1 clone 5 het DNA a fragment with homology to *Bam*HI-C is found, but located on a separate *Eco*RI fragment of 2.8 kbp (30). We detected weak hybridization of standard *Bam*HI-C to *Eco*RI het 16 which is due to the homology which exists between *Bam*HI-C and *Bam*HI-W as the result of the first internal repeat (1, 5, 18). Furthermore, the sequences of *Eco*RI het 16 with homology to *Bam*HI-W do not contain any DNA sequences which are unique to *Bam*HI-C (Jenson et al., in preparation). Previous studies have shown that the defectives contain multiple intramolecular recombinations and abnormalities of orientation by comparison with the standard genome. However, neither of the two other groups studying HR-1 defective DNA have detected a palindrome.

The differences between our study and those of Cho et al. (6) and Heller et al. (19) most likely lie in the material under study. We looked at het DNA from a single-cell subclone of HR-1 cells in which one population of defective EBV DNA is enriched. Both other groups have examined a mixture of HR-1 defective DNA either in the parental line or in the defective viruses which replicate in BJAB cells. It is now evident that HR-1 cells contain a family of defective molecules. The finding that certain regions are consistently represented in het DNA may indicate stringent requirements for survival or that some sequences select for retention of the defective genome. However, only certain defective molecules may be responsible for the biologic properties of EA induction and disruption of latency which we attribute to the het DNA present in parental HR-1 virus and clone 5 virus stocks (30). The ability to disrupt latency has been localized to *Bam*HI het 2.7 which is duplicated in the palindrome of *Eco*RI het 16 (9). This biologically active *Bam*HI subfragment, with homology to standard *Bam*HI-W and *Bam*HI-Z, may only be present in a minor proportion of HR-1 defective DNA since it was not detected by Heller et al. (19) or Cho et al. (6).

Polypeptide expression and biologic activity of defective DNA. Two of the antigens we saw have been described previously by Cho et al. (7), who carried out gene transfer into BHK cells with cloned fragments of defective DNA. These were the antigen of *Eco*RI het 20, which reacts with EA^- and VCA^+ sera, and the EA encoded by *Bam*HI het 7.1. The 95-kDa VCA polypeptide of *Eco*RI het 20 is localized entirely within the right-hand *Bam*HI-*Eco*RI subfragment having homology to *Bam*HI-A. This region of *Eco*RI het 20 contains no apparent rearrangements. A morphologically similar antigen is localized within the comparable *Bam*HI-*Eco*RI subfragment of *Bam*HI-A of EBV strain FF41. The encoding region for this polypeptide may be comparable to the B95-8 EBV BALF4 reading frame which is contained entirely within the *Bam*HI-*Eco*RI subfragment and encodes a polypeptide with a calculated molecular weight of 95.6 kDa (1). The encoding region of the EA of *Bam*HI het 7.1, which spans the *Eco*RI site between HR-1 *Eco*RI-J_2 and *Eco*RI-F, is comparable to BMLF1 of EBV strain B95-8 (1). The protein of this encoding region of B95-8 has a calculated molecular size of 49.7 kda, equivalent to our experimental molecular size in het DNA of 50 kDa. Cho et al. (8) localized a morphologically similar 60-kDa EA to this region of defective HR-1 DNA. Whether these antigens of HR-1 clone 5 are, in fact, identical to previously described antigens must await comparison of the polypeptides induced by different cloned standard and defective EBV DNA fragments.

We described three additional antigens which had not been recognized previously. These are a prominent nuclear EA of 36 kDa from *Bam*HI het 2.7, a nuclear antigen from *Bam*HI het 1.8, and a cytoplasmic antigen from the *Hin*dIII-*Eco*RI subfragments of *Eco*RI het 16. Our ability to recog-

nize these antigens may possibly have been due to more sensitive assays (i.e., cloned DNA on a simian virus 40 vector expressed in COS-1 cells) or to the presence of rearrangements which are unique to clone 5 het DNA (e.g., *Bam*HI het 2.7). The polypeptide products of *Bam*HI het 2.7 are of particular interest because of the capacity of this subfragment to activate latent EBV by gene transfer (9). Polypeptide antigen expression detectable by immunofluorescence from this subfragment was lost after digestion with *Hin*dIII. An open reading frame is found in *Bam*HI het 2.7 which crosses the *Hin*dIII site (Jenson et al., in preparation). This reading frame, although different in DNA sequence and length from B95-8 EBV, is roughly comparable to BZLF1 of B95-8 EBV (1). *Bam*HI-Z has recently been shown to encode an immediate early mRNA (M. Biggin and P. Farrell, personal communication). Therefore, *Bam*HI-Z is a region likely to be important in the regulation of latency and initiation of the replicative cycle.

There is no doubt that knowledge of the structure of het DNA is important in understanding its biologic attributes, but exactly how the structure relates to expression is at this time speculative. For example, het DNA must contain origins of DNA replication, but clues to the location of these origins in the form of long stretches of tandem duplications, such as occur in herpes simplex defective DNA, have not been found (14).

Some of the genomic rearrangements are responsible for the capacity of virus with het DNA to disrupt latency and to induce viral replication. Gene transfer experiments have shown that *Bam*HI het 2.7 but not *Bam*HI het 7.1 or *Bam*HI het 1.8 can activate expression of the latent genome in D98/HR-1 cells (9). The capacity of *Bam*HI het 2.7 to activate latent genomes is probably contingent upon the intramolecular recombination which generated this rearranged piece of DNA. How such a genome rearrangement might confer new biologic properties upon het DNA is a matter for future study. The rearrangement may generate new reading frames encoding proteins which do not have a counterpart in standard EBV. A more likely possibility is that the rearrangements cause altered regulation of expression of a standard EBV protein, i.e., by locating encoding regions of the standard genome which are normally quiescent close to strong constitutive promoters. Thus, the rearrangement would result in the production of a standard EBV polypeptide which is under different control in the defective genome.

ACKNOWLEDGMENTS

We thank Duncan Fischer, Elizabeth Grogan, and Lee Heston for helpful discussions.

This work was supported in part by Public Health Service grants AI-07210, AI-14741, CA-12055, and CA-16038 from the National Institutes of Health and by grant MV-173F from the American Cancer Society. H.B.J. is supported by Physician Scientist Award AI-00651 from the National Institutes of Health.

LITERATURE CITED

1. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.
2. **Bornkamm, G. W., H. Delius, U. Zimber, J. Hudewentz, andM. A. Epstein.** 1980. Comparison of Epstein-Barr virus strains of different origin by analysis of the viral DNAs. J. Virol. **35**:603–618.
3. **Bornkamm, G. W., J. Hudewentz, U. K. Freese, and U. Zimber.** 1982. Deletion of the nontransforming Epstein-Barr virus strain P3HR-1 causes fusion of the large internal repeat to the DS_L region. J. Virol. **43**:952–968.
4. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
5. **Cheung, A., and E. Kieff.** 1981. Epstein-Barr virus DNA. X. Direct repeat within the internal direct repeat of Epstein-Barr virus DNA. J. Virol. **40**:501–507.
6. **Cho, M.-S., G. W. Bornkamm, and H. zur Hausen.** 1984. Structure of defective DNA molecules in Epstein-Barr virus preparations from P3HR-1 cells. J. Virol. **51**:199–207.
7. **Cho, M.-S., L. Gissmann, and S. D. Hayward.** 1984. Epstein-Barr virus (P3HR-1) defective DNA codes for components of both the early antigen and viral capsid antigen complexes. Virology **137**:9–19.
8. **Cho, M.-S., K.-T. Jeang, and S. D. Hayward.** 1985. Localization of the coding region for an Epstein-Barr virus early antigen and inducible expression of this 60-kilodalton nuclear protein in transfected fibroblast cell lines. J. Virol. **56**:852–859.
9. **Countryman, J., and G. Miller.** 1985. Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. Proc. Natl. Acad. Sci. USA **82**:4085–4089.
10. **Davis, D. B., and D. T. Kingsbury.** 1976. Quantitation of the viral DNA present in cells transformed by UV-irradiated herpes simplex virus. J. Virol. **17**:788–793.
11. **Delius, H., and G. W. Bornkamm.** 1978. Heterogeneity of Epstein-Barr virus. III. Comparison of a transforming and a nontransforming virus by partial denaturation mapping of their DNAs. J. Virol. **27**:81–89.
12. **Edson, C. M., L. K. Cohen, W. Henle, and J. L. Strominger.** 1983. An unusually high-titer human anti-Epstein Barr virus (EBV) serum and its use in the study of EBV-specific proteins synthesized *in vitro* and *in vivo*. J. Immunol. **130**:919–924.
13. **Fischer, D. K., G. Miller, L. Gradoville, L. Heston, M. W. Weststrate, W. Maris, J. Wright, J. Brandsma, and W. C. Summers.** 1981. Genome of a mononucleosis Epstein-Barr virus contains DNA fragments previously regarded to be unique to Burkitt's lymphoma isolates. Cell **24**:543–553.
14. **Frenkel, N.** 1981. Defective interfering herpesviruses, p. 91–120. *In* A. J. Nahmias, W. R. Dowdle, and R. F. Schinazi (ed.), The human herpesviruses. An interdisciplinary perspective. Elsevier Science Publishing, Inc., New York.
15. **Fresen, K.-O., M.-S. Cho, and H. zur Hausen.** 1978. Heterogeneity of Epstein-Barr virus. IV. Induction of a specific antigen by EBV from two transformed marmoset cell lines in Ramos cells. Int. J. Cancer **22**:160–165.
16. **Fresen, K.-O., B. Merkt, G. W. Bornkamm, and H. zur Hausen.** 1977. Heterogeneity of Epstein-Barr virus originating from P3HR-1 cells. I. Studies on EBNA induction. Int. J. Cancer **19**:317–323.
17. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
18. **Hayward, S. D., L. Nogee, and G. S. Hayward.** 1980. Organization of repeated regions within the Epstein-Barr virus DNA molecule. J. Virol. **33**:507–521.
19. **Heller, M., T. Dambaugh, and E. Kieff.** 1981. Epstein-Barr virus DNA. IX. Variation among viral DNAs from producer and nonproducer infected cells. J. Virol. **38**:632–648.
20. **Henle, W., G. Henle, B. A. Zajac, G. Pearson, R. Waubke, and M. Scriba.** 1970. Differential reactivity of human serums with early antigens induced by Epstein-Barr virus. Science **169**:188–190.
21. **Heston, L., M. Rabson, N. Brown, and G. Miller.** 1982. New Epstein-Barr virus variants from cellular subclones of P3J-HR-1 Burkitt lymphoma. Nature (London) **295**:160–163.
22. **Hinuma, Y., M. Konn, J. Yamaguchi, D. J. Wudarski, J. R. Blakeslee, Jr., and J. T. Grace, Jr.** 1967. Immunofluorescence and herpes-type virus particles in the P3HR-1 Burkitt lymphoma

cell line. J. Virol. **1**:1045–1051.
23. **Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder.** 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Tech. **1**:3–8.
24. **King, W., T. Dambaugh, M. Heller, J. Dowling, and E. Kieff.** 1982. Epstein-Barr virus DNA. XII. A variable region of the Epstein-Barr virus genome is included in the P3HR-1 deletion. J. Virol. **43**:979–986.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
26. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. **11**:1295–1308.
27. **Menezes, J., W. Leibold, and G. Klein.** 1975. Biological differences between Epstein-Barr virus (EBV) strains with regard to lymphocyte transforming ability, superinfection and antigen induction. Exp. Cell Res. **92**:478–484.
28. **Miller, G., L. Heston, and J. Countryman.** 1985. P3HR-1 Epstein-Barr virus with heterogeneous DNA is an independent replicon maintained by cell-to-cell spread. J. Virol. **54**:45–52.
29. **Miller, G., L. Heston, M. Rabson, J. Countryman, and H. Jenson.** 1985. Epstein-Barr viruses that do not immortalize lymphocytes or remain latent, p. 319–325. *In* R. A. Lerner, R. M. Chanock, and F. Brown (ed.), Vaccines 85. Molecular and chemical basis of resistance to parasitic, bacterial, and viral diseases. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Miller, G., M. Rabson, and L. Heston.** 1984. Epstein-Barr virus with heterogeneous DNA disrupts latency. J. Virol. **50**:174–182.
31. **Miller, G., J. Robinson, L. Heston, and M. Lipman.** 1974. Differences between laboratory strains of Epstein-Barr virus based on immortalization, abortive infection and interference. Proc. Natl. Acad. Sci. USA **71**:4006–4010.
32. **Rabson, M., L. Gradoville, L. Heston, and G. Miller.** 1982. Non-immortalizing P3J-HR-1 Epstein-Barr virus: a deletion mutant of its transforming parent, Jijoye. J. Virol. **44**:834–844.
33. **Rabson, M., L. Heston, and G. Miller.** 1983. Identification of a rare Epstein-Barr virus variant that enhances early antigen expression in Raji cells. Proc. Natl. Acad. Sci. USA **80**: 2762–2766.
34. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase. J. Mol. Biol. **113**: 237–251.
35. **Rymo, L., and S. Forsblom.** 1978. Cleavage of Epstein-Barr virus DNA by restriction endonucleases EcoRI, HindIII, and BamI. Nucleic Acids Res. **5**:1387–1402.
36. **Schmitt, J. J., and B. N. Cohen.** 1983. Quantitative isolation of DNA restriction fragments from low-melting agarose by Elutip-d affinity chromatography. Anal. Biochem. **133**:462–464.
37. **Smith, H. O., and M. L. Birnstiel.** 1976. A simple method for DNA restriction site mapping. Nucleic Acids Res. **3**:2387–2398.
38. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
39. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
40. **Sugden, B., W. C. Summers, and G. Klein.** 1976. Nucleic acid renaturation and restriction endonuclease cleavage analyses show that the DNAs of a transforming and a nontransforming strain of Epstein-Barr virus share approximately 90% of their nucleotide sequences. J. Virol. **18**:765–775.
41. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
42. **zur Hausen, H., F. J. O'Neill, U. K. Freese, and E. Hecker.** 1978. Persisting oncogenic herpesvirus induced by tumour promoter TPA. Nature (London) **272**:373–375.