Linkage Map of the Fragments of Herpesvirus Papio DNA

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Herpesvirus papio (HVP), an Epstein-Barr-like virus, causes lymphoblastoid disease in baboons. The physical map of HVP DNA was constructed for the fragments produced by cleavage of HVP DNA with restriction endonucleases *EcoRI*, *HindIII*, *SalI*, and *PvuI*, which produced 12, 12, 10, and 4 fragments, respectively. The total molecular size of HVP DNA was calculated as close to 110 megadaltons. The following methods were used for construction of the map; (i) fragments near the ends of HVP DNA were identified by treating viral DNA with lambda exonuclease before restriction enzyme digestion; (ii) fragments containing nucleotide sequences in common with fragments from the second enzyme digest of HVP DNA were examined by Southern blot hybridization; and (iii) the location of some fragments was determined by isolating individual fragments from agarose gels and redigesting the isolated fragments with a second restriction enzyme. Terminal heterogeneity and internal repeats were found to be unique features of HVP DNA molecule. One to five repeats of 0.8 megadaltons were found at both terminal ends. Although the repeats of both ends shared a certain degree of homology, it was not determined whether they were identical repeats. The internal repeat sequence of HVP DNA was found in the *EcoRI*-C region, which extended from 8.4 to 23 megadaltons from the left end of the molecule. The average number of the repeats was calculated to be seven, and the molecular size was determined to be 1.8 megadaltons. Similar unique features have been reported in EBV DNA (D. Given and E. Kieff, *J. Virol.* **28**:524-542, 1978).

Epstein-Barr virus (EBV) has been shown to be closely associated with Burkitt's lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis. Similar and related herpesviruses have been found in nonhuman primates, including baboon, gorilla, chimpanzee, orangutan, and African green monkey (1, 2, 5, 7-10, 18, 21-24).

The 594S/F9 cell line used in this study, which was originally derived from baboon lymphoma cells (22), produces herpesvirus papio (HVP). These cells were found to yield a limited amount of virus in culture. HVP has been found to show a great similarity to EBV (B95); it can infect B-cell lymphocytes and induce early antigen, membrane antigen, and viral capsid antigen, which then cross-react with EBV antigens, but nuclear antigen of this virus does not show cross-reaction with Epstein-Barr nuclear antigen (21-23).

Our previous studies indicated that treatment of 594S/F9 cells with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) enhances the yield of the virus (Y. S. Lee, A. Tanaka, R. Y. Lau, M. Nonoyama, and H. Rabin, *J. Gen. Virol.*, in press). HVP DNA is double stranded with a size of approximately 110 meg-

adaltons. It has a sedimentation value of 55S in a glycerol gradient and a buoyant density of about 1.718 g/cm³ in neutral cesium chloride (6; Lee et al., in press). DNA-DNA reassociation kinetics indicated that HVP DNA has about 40% homology with B95 DNA (Lee et al., in press). Blot hybridization studies confirmed the homology and further indicated that the homology of B95 DNA sequences to HVP DNA is not restricted to certain regions of the viral DNA (Lee et al., in press). Restriction enzyme digestion (*EcoRI*, *BamHI*, and *SalI*) of HVP DNA produced 9 to 15 fragments, with sizes ranging from 1.3 to 31 megadaltons (Lee et al., in press).

Knowledge of the molecular organization of HVP DNA is necessary to understand the expression and regulation of the HVP genome. Such information has been helpful in previous studies of simian virus 40, adenovirus, and other herpesviruses (3, 13, 20). In this communication, the linkage map of HVP DNA is presented. Construction of the linkage map was achieved by blot hybridization using individual ³²P-labeled restriction enzyme fragments as probes hybridizing to other digestion fragments on the blotting paper.

MATERIALS AND METHODS

Chemicals. Restriction endonucleases (*EcoRI*, *SalI*, and *HindIII*) were purchased from Bethesda Research Laboratories, Inc. Endonuclease *PvuI* and λ exonuclease were purchased from New England Biolabs. ^{32}P -labeled dCTP was from New England Nuclear Corp.

Cells and viral DNA preparations. The conditions for 594S/F9 cell culture which produces HVP, TPA induction of virus, virus purification, and viral DNA preparation were described previously (Lee et al., in press). The previous study showed that the restriction enzyme patterns of HVP DNA obtained by TPA induction are identical to those obtained from spontaneously produced virus.

Restriction endonuclease and λ exonuclease digests. The procedures for endonuclease digestion have been described previously (17). Briefly, 1 to 2 μg of HVP DNA was digested with restriction enzyme in 50 μl of *EcoRI* buffer (0.1 M Tris, pH 7.4, 0.05 M NaCl, and 0.01 M MgCl_2), *SalI* buffer (0.15 M NaCl, 0.06 M Tris, pH 7.9, 6 mM MgCl_2 , and 6 mM 2-mercaptoethanol), *PvuI* buffer (0.15 M NaCl, 0.06 M Tris, pH 7.4, 6 mM MgCl_2 , and 6 mM 2-mercaptoethanol), or *HindIII* buffer (0.05 M NaCl, 6 mM Tris, pH 7.5, and 6 mM MgCl_2). The required concentration of enzyme for complete digestion was determined by periodic titration of enzyme activity, using λ DNA. For the second enzyme digestion, each restriction fragment was isolated by electrophoresis, collected in a dialysis bag, precipitated with ethanol, and subsequently digested with a second enzyme. The exonuclease digestion conditions were as outlined by Hamlett et al. (12). Briefly, a DNA sample in a reaction mixture of 20 mM Tris (pH 7.8) and 3 mM MgCl_2 was incubated at room temperature (20°C) for various periods of time. The reaction was stopped by heating to 65°C for 5 min. Enzyme activity was checked by using T5 DNA (kindly provided by Nancy Hamlett, Towson State University, Baltimore, Md.).

Nick translation and blotting hybridization. B95 DNA, HVP DNA, and DNA fragments were radioactively labeled in vitro by nick translation, using *Escherichia coli* DNA polymerase I (19, 25). DNA fragments were transferred to a nitrocellulose filter paper by Southern's method (28), and blotting hybridizations were carried out in 50% formamide as described by Dawid (4). Since most of the restriction fragments were stainable by ethidium bromide before blotting, identification of the hybridized fragments was done mainly by comparison with the original picture taken after ethidium bromide staining. For some ambiguous fragments, the hybridized paper was rehybridized to a ^{32}P -labeled HVP total probe (see Fig. 5b).

RESULTS

Restriction enzyme patterns of HVP DNA. HVP DNA isolated from purified virus was digested with restriction endonucleases. The cleavage products were separated by 0.4% agarose gel electrophoresis and then stained with ethidium bromide. Figure 1 shows the restriction enzyme patterns of HVP DNA digested with

FIG. 1. Gel electrophoresis patterns of restriction enzyme fragments of HVP DNA. One microgram of HVP DNA was digested with endonuclease *EcoRI*, *HindIII*, *SalI*, or *PvuI*. The digested DNA fragments were separated in 0.4% agarose gel by electrophoresis at 80 V for 16 h, stained with ethidium bromide, and photographed under UV light. The faint fragments not named here are due to partial digestion.

EcoRI, *HindIII*, *SalI*, and *PvuI*. The number of fragments produced by each enzyme was as follows: 12 for *EcoRI*; 12 for *HindIII*; 10 for *SalI*, and 4 for *PvuI*. The fragments smaller than 1 megadalton were not included. The molar ratio of the J fragment of *EcoRI* appeared to be less than 1. Subsequent studies have identified it as the terminal fragment which contains a heterogeneous fragment population. Most of the bands apparently had a molar ratio of unity; only a few bands showed a molar ratio of more than 2 (*SalI*-B and *HindIII*-B, -E, and -J bands). This

finding was confirmed by ^{32}P -labeled DNA studies and by densitometer tracings (data not shown). The estimated size of intact HVP DNA and the molar ratios of four restriction enzyme fragments are tabulated in Table 1. The total molecular weights of the restriction enzyme fragments, in megadaltons, were as follows: 110 for *EcoRI* digests; 106 for *HindIII*; 107 for *SalI*; and 110 for *PvuI*.

Terminal fragments of HVP DNA. Lambda exonuclease was used to determine the 5' end fragments of viral DNA. Since the physical maps of T5 phage and B95 EBV DNAs have been determined (11, 12), the proper condition for digesting terminal fragments of these two DNAs by λ exonuclease was established and applied to HVP DNA. Figure 2 shows the densitometer tracings of HVP DNA cleaved with *HindIII* (upper) and the profile of HVP DNA digested with λ exonuclease before the endonuclease digestion (lower). *HindIII*-A and -H fragments were found sensitive to λ exonuclease digestion, indicating that these two fragments are located at the terminal positions. The same digestion condition was applied to other restriction enzymes, *EcoRI*, *SalI*, and *PvuI*. The results showed *EcoRI*-A and -J, *SalI*-A and -C, and *PvuI*-A₁ and -A₂ to be located at the termini (data not shown). The K fragment of *EcoRI* probably is located next to the J fragment, since extensive λ exonuclease digestion also elimi-

FIG. 2. Lambda exonuclease digestion of HVP DNA. One microgram of HVP DNA was treated with lambda exonuclease and then subjected to *HindIII* endonuclease digestion. The DNA fragments were separated by 0.4% agarose gel electrophoresis, stained with 0.5 μg of ethidium bromide per ml, and photographed under UV light. The negative films were scanned with a densitometer (Beckman Instruments, Inc.). The top figure shows the restriction enzyme patterns of HVP DNA after digestion with *HindIII* endonuclease. The bottom figure shows the restriction enzyme patterns of HVP DNA which was first treated with λ exonuclease and then with *HindIII* endonuclease.

TABLE 1. Size estimation of HVP DNA cleaved with restriction endonucleases

Frag- ment	<i>EcoRI</i>		<i>HindIII</i>		<i>SalI</i>		<i>PvuI</i>	
	Size ^a	Mo- lar ratio	Size	Mo- lar ratio	Size	Mo- lar ratio	Size	Mo- lar ratio
A	22.2	1	26.2	1	30.5	1	34	2
B	15.5	1	13.5	2	19.0	2	29	1
C	14.9	1	10.4	1	12.4	1	8.6	1
D	12.3	1	8.1	1	9.4	1	4.5	1
E	7.8	1	5.3	2	4.7	1		
F	7.2	1	4.1	1	4.0	1		
G	6.0	1	3.8	1	2.9	1		
H	5.7	1	3.6	<1	1.8	1		
I	5.2	1	2.9	1	1.7	1		
J	4.3	<1	2.1	2	1.4	1		
K	4.1	1	1.7	1				
L	2.5	1	1.5	1				
M	1.4 ^b	1 ^c	1.1 ^d	1 ^c				
N	1.3 ^b	1 ^c	1.0 ^d	1 ^c				

^a Size in megadaltons was calculated on a logarithmic scale, using *SalI* and *Sma* fragments of T5(+) DNA, intact λ DNA, and *EcoRI* fragments of λ DNA as reference DNA.

^b Detected from ^{32}P -labeled DNA fragments.

^c Deduced from the physical map.

^d Detected by Southern blot hybridization.

nated this fragment. To align each terminal restriction fragment of HVP DNA, *EcoRI*-A and -J were isolated and in vitro labeled with [^{32}P]dCTP. These labeled fragments were individually hybridized with *EcoRI*, *HindIII*, *SalI*, and *PvuI* fragments of HVP DNA that had been blot transferred to nitrocellulose paper. The labeled *EcoRI*-A fragment hybridized to *EcoRI*-A, *HindIII*-C,D and -H terminal, *SalI*-C terminal and -D, and *PvuI*-A₂ terminal fragments. Two fragments shown by dots will be discussed in the next section (Fig. 3b). The labeled *EcoRI*-J hybridized to *EcoRI*-J (and -K, since the K fragment was contaminated in the probe), *HindIII*-A, *SalI*-A (Fig. 3d), and *PvuI*-A₁ (not shown). Other heterogeneous bands shown in each blot will be discussed in the next section. Thus, *EcoRI*-A, *HindIII*-H, *SalI*-C, and *PvuI*-A₂ are the terminal fragments on one end (temporarily assigned at the right end), and *EcoRI*-J, *HindIII*-A, *SalI*-A, and *PvuI*-A₁ are the fragments on the other end.

Terminal heterogeneity. Terminal heterogeneity of the HVP DNA molecule was demonstrated by using some of the ^{32}P -labeled terminal fragments for hybridization to the blot of the homologous enzyme digest as well as the blot of other enzyme digests. When radioactive *EcoRI*-A hybridized to the blots of *EcoRI*, *HindIII*, *SalI*, and *PvuI* digests, radioactive bands of *EcoRI*-A, *HindIII*-C,D, and -H, *SalI*-C and -D, and *PvuI*-A₂ were seen, as previously discussed.

FIG. 3. Blot hybridization of *HindIII*-H, *EcoRI*-A, and *EcoRI*-J of HVP DNA to *EcoRI*, *HindIII*, *SalI*, and *PvuI* blots. 32 P-labeled *EcoRI*-A and -J (-K) and *HindIII*-H were prepared by nick translation (25) and were hybridized to *EcoRI*, *HindIII*, *SalI*, and *PvuI* fragments. The procedures for nick translation, gel electrophoresis, and blot hybridization are described in Materials and Methods. (a) Before hybridization, the digested DNA fragments were stained with ethidium bromide. The location of each fragment on this panel corresponds to that in (b). (b) Blot hybridization of *EcoRI*-A to *EcoRI*, *HindIII*, *SalI*, and *PvuI* of HVP DNA. (c) Blot hybridization of *HindIII*-H to *HindIII* fragments of HVP DNA. (d) Blot hybridization of *EcoRI*-J (and -K) to *EcoRI*, *HindIII*, and *SalI* of HVP DNA.

However, there were two extra fragments of 4.4 and 5.2 megadaltons found in the *HindIII* blot in addition to *HindIII*-H terminal fragment of 3.6 megadaltons (Fig. 3b, dots). When *HindIII*-H hybridized to the blot of *HindIII*, these two extra bands were again observed (Fig. 3c, dots) by radioautography. An increment of 0.8 megadaltons was obtained between *HindIII*-H and the 4.4-megadalton fragment and between the 4.4- and 5.2-megadalton fragments. *HindIII* fragments which had sizes similar to these, such as *HindIII*-E and -F, did not hybridize to *HindIII*-H or *EcoRI*-A. Therefore, these fragments are considered to be a terminal heterogeneity at the *HindIII*-H site. *HindIII*-H hybridization to its own blot also revealed other autoradiographic bands of 1.7, 2.5, 3.3, and 4.1 megadaltons (Fig.

3c, arrows). These hybridizations were more obvious when 32 P-labeled terminal *EcoRI*-J (contaminated with fragment K) hybridized to the blots of *EcoRI*, *HindIII*, and *SalI* (Fig. 3d). It hybridized to *EcoRI*-J (and -K) and also to other multiple fragments, 5.1, 5.9, 6.7, and weakly 7.5 megadaltons (Fig. 3d, arrows), and slightly to *EcoRI*-A; to *HindIII*-A and its vicinity, the fragments detected by *HindIII*-H and by *EcoRI*-A as (Fig. 3b, c, dots) and 1.7-, 2.5-, and 3.3-megadalton fragments which were detected by *HindIII*-H hybridization (Fig. 3c, arrows); and to *SalI*-A and its vicinity and four smaller fragments of 1.8, 2.6, 3.4, and 4.2 megadaltons (Fig. 3d, arrows). The difference in size between the neighboring fragments of 4.3 (J), 5.1, 5.9, 6.7, and 7.5 megadaltons on the *EcoRI* blot was the same

as that between fragments of 1.7, 2.5, and 3.3 megadaltons of *Hind*III fragments and between fragments of 1.8, 2.6, 3.4, and 4.2 megadaltons on the *Sal*I blot (Fig. 3d, arrows). The fragments indicated by arrows in Fig. 3, therefore, should represent terminal heterogeneity with certain numbers of the repeat of 0.8 megadaltons. The smallest *Hind*III heterogeneous fragment of 0.9 megadaltons was run out of the gel in this experiment but recovered in a separate experiment.

The hybridization in the vicinity of *Hind*III-A and *Sal*I-A by *Eco*RI-J in Fig. 3d may have been due to the varying amounts of internal repeats within these fragments, as discussed in a later section, although the hybridization at the area of 8 to 15 megadaltons could still be due to the terminal heterogeneity. The fact that the terminal heterogeneity of *Eco*RI-J detected that of *Hind*III-H, which is located at the other end of the genome, and the terminal heterogeneity of *Hind*III-H in turn revealed that of *Hind*III-A terminal indicates that the terminal heterogeneities of both ends share a certain degree of sequence homology. The slight hybridization of *Eco*RI-J to *Eco*RI-A also was due to (the part of) the terminal repeat that *Eco*RI-A terminal contained. Thus, the alignment of terminal fragments and the terminal heterogeneity of HVP DNA may be summarized as in Fig. 4.

Linkage map of HVP DNA. The linkage map of HVP DNA was established mainly by blotting hybridization with isolated 32 P-labeled HVP DNA fragments to blots containing total fragments of HVP DNA generated by another restriction enzyme. The strategy for mapping HVP DNA was to start by aligning the terminal fragments of HVP DNA as illustrated in Fig. 4. The next step was to identify the fragments which are next to the terminal fragments. Two typical hybridization pictures are presented in Fig. 5, in which the hybridized fragments were identified by comparing the original fragment pattern in the gel obtained by ethidium bromide

staining (Fig. 5a) or by rehybridizing the same blot paper with a total probe of 32 P-labeled HVP DNA after completion of autoradiography for the fragment hybridization (Fig. 5b). Tables 2, 3, and 4 summarize all of the hybridization data.

Determination of end fragments was discussed in the previous section; *Eco*RI-J, *Hind*III-A, and *Sal*I-A were placed on one end. *Eco*RI-K was expected to be next to *Eco*RI-J from the λ exonuclease digestion pattern. Since *Hind*III-B₁ and *Eco*RI-C, -H, and -B, as well as *Hind*III-A and *Eco*RI-J and -K, hybridized to *Sal*I-A and vice versa, and since *Eco*RI-C hybridized only to *Hind*III-A and *Eco*RI-B hybridized only to *Hind*III-B₁, whereas *Eco*RI-H hybridized to both *Hind*III-A and -B₁, the linkage from the end fragment should be A-B₁ for *Hind*III and J-K-C-H-B for *Eco*RI, whereas *Sal*I-A stretches into the region of *Hind*III-B₁. Since *Eco*RI-B also hybridized to *Hind*III-E₁, a *Hind*III linkage of A-B₁-E₁ can be established which covers the J to B fragments of the *Eco*RI linkage. Since *Eco*RI-B hybridized to fragments F, G, and J as well as to B₁ (and A) of *Sal*I, whereas *Hind*III-B₁ but not -E₁ hybridized to *Sal*I-F, -G, and -J, the group of *Sal*I-F, -G, and -J should be placed next to *Sal*I-A. *Sal*I-B₁ should be located next to this group of fragments, since *Eco*RI-B and *Hind*III-E₁ and -E₂ also hybridized to *Sal*I-B₁. Therefore, the linkage of *Sal*I-A-(FGJ)-B₁ is established which extends to 60 megadaltons from the end. *Sal*I-F hybridized to *Pvu*I-D and to *Pvu*I-A₁, whereas *Sal*I-G hybridized to only *Pvu*I-D. Thus, *Sal*I-F-(GJ) and *Pvu*I-A₁-D should be the linkage arrangement. Furthermore, *Eco*RI-B hybridized to *Pvu*I-A₁, -D, and -B, indicating the linkage of A₁-D-B fragments of the *Pvu*I digest. *Sal*I-E and -I as well as -B₁, *Eco*RI-D and -G as well as -B, and *Hind*III-E₁, -E₂, -I, -J, -K, and -L were the fragments which hybridized to *Pvu*I-B. Among them, *Eco*RI-D hybridized only to *Sal*I-B₁ but *Eco*RI-G hybridized to both *Sal*I-B₁ and -E. Therefore, the linkage order should be A-F-(GJ)-B₁-E-1 for

FIG. 4. Alignment of terminal fragments of HVP DNA. *1, It has not been determined whether *Eco*RI-A and *Sal*I-C heterogeneity was not detected due to the insignificant addition of the small repeats to these fragments or the repeats contained cleavage site(s) for *Eco*RI and *Sal*I. *2, The size of the repeats is 0.8×10^6 daltons.

FIG. 5. Blotting hybridization of ^{32}P -labeled restriction enzyme fragments to *EcoRI*, *HindIII*, and *SalI* total fragments on nitrocellulose paper. (a) ^{32}P -labeled *HindIII*-C fragment was hybridized to *EcoRI*, *HindIII*, and *SalI* blot (left panel), and the hybridized fragments were identified by comparing to the original gel pattern stained with ethidium bromide (right panel) before the DNA fragments were transferred to nitrocellulose paper. (b) ^{32}P -labeled *EcoRI*-K was hybridized to *EcoRI*, *HindIII*, and *SalI* blot (left panel), and the hybridized fragments were identified by comparing with the hybridization picture of the same blot paper with total HVP DNA probe (right panel) after the completion of *EcoRI*-K fragment hybridization.

SalI fragments and J-K-C-H-B-D-G for *EcoRI* fragments. Among the *HindIII* fragments hybridized to *PvuI*-B, *HindIII*-E₂, -I, and -L hybridized to *EcoRI*-D, fragment K hybridized to *EcoRI*-D and -G, and fragment J₁ hybridized to *EcoRI*-G. Therefore, the linkage of *HindIII* fragments should be A-B₁-E₁-(E₂IL)-K-J₁. The hybridization of *EcoRI*-G to *HindIII* total DNA fragments revealed *HindIII*-M and -N as well as

-J₁ and -K, indicating that *HindIII*-J₁, -M, and -N are the group of fragments located in the *EcoRI*-G region. *SalI*-E hybridized to *EcoRI*-M as well as to *EcoRI*-G, thus placing this M fragment next to *EcoRI*-G. Since *SalI*-I hybridized to *EcoRI*-E and *HindIII*-B₂, *EcoRI*-G-M-E and *HindIII*-(J₁MN)-B₂ were determined. *EcoRI*-E hybridized to *SalI*-H and -B₂ as well as -I, and *SalI*-B₁ and B₂ in turn hybridized to *EcoRI*-E, -F, -L, and -I, as well as to the previously assigned fragments B, D, and G. Furthermore, *SalI*-H hybridized to *PvuI*-B and -C. Therefore, *SalI* linkage of I-H-B₂ and *EcoRI* linkage of M-E-(FLI) can be established. *SalI*-B₂ also hybridized to *PvuI*-C and -A₂ (terminal) and *HindIII*-B₂, -F, -G, and -J₂ among the fragments that have not been assigned. This establishes the complete linkage map of *PvuI* as A₁-D-B-C-A₂ as well as linkage relationship of *HindIII*-(J₁MN)-B₂-(FGJ₂). The linkage orders of *HindIII*-B₂-J₂-F-G and *EcoRI*-E-L-I-F were established by reciprocal hybridizations of each *EcoRI* and *HindIII* fragment as summarized in Table 2 and 3. *HindIII*-A to -G, *EcoRI*-J to -F, and *SalI*-A to -B₂ stretched to the same extent of 87 megadaltons, including some gaps as discussed in the following section. For the final part of linkage mapping of the terminal region, *HindIII*-H, *EcoRI*-A, and *SalI*-C were found to be terminal fragments as discussed in the previous section. *EcoRI*-A hybridized to *SalI*-C (terminal) and -D, indicating *SalI*-D-C linkage. *SalI*-C terminal hybridized to *HindIII*-C and -H (terminal), whereas *SalI*-D hybridized to *HindIII*-D, and *EcoRI*-A hybridized to all these three fragments of *HindIII*. Thus, *HindIII*-D-C-H is the order of the linkage. All of these fragments hybridized to *PvuI*-A₂ terminal. In addition, hybridization with radioactive *HindIII*-D revealed a small *EcoRI*-N which should be located next to *EcoRI*-A terminal. Thus, the total linkage of the fragments is A-B₁-E₁-(E₂-I-L)-K-(J₁MN)-B₂-J₂-F-G-D-C-H for *HindIII*, J-K-C-H-B-D-G-M-E-L-I-F-N-A for *EcoRI*, and A-F-(GJ)-B₁-E-I-H-B₂-D-C for *SalI*.

Double-digestion experiment. To confirm the linkage map and to make a fine alignment of the fragments, labeled or unlabeled HVP DNA was treated with an enzyme, and then the generated fragments were isolated from the gel after electrophoresis and further digested with a second enzyme. The results of these experiments (Table 5 and Fig. 6) are compatible with the linkage map shown in Fig. 9. The following points are particularly to be noted.

Digestion of *EcoRI*-E with *SalI* endonuclease generated fragments of 1.4, 1.8, and 3.6 megadaltons, among which the 1.4-megadalton frag-

TABLE 2. Hybridization of ³²P-labeled *Eco*RI fragment of HVP DNA to blot of HVP DNA fragments

Probe	Blot hybridization			
	<i>Eco</i> RI	<i>Hind</i> III	<i>Sal</i> I	<i>Pvu</i> I
A	A	C, D, H, het ^a	C, D	A ₂
B	B	B ₁ , E ₁	A, B ₁ , F, G, J	A ₁ , B, D
C	C	A	A	A ₁
D	D	E ₂ , I, K, L	B ₁	B
E	E	B ₂	B ₂ , H, I	
F	F	F, G	B ₂	
G	G	J ₁ , K, M, N	B ₁ , E	B
H	H	A, B ₁	A	
I	I	B ₂ , J ₂ , F	B ₂	
J and K	A, J, and K, het	A, het	A, het	A ₁
K	K	A	A	A ₁
L	L	B ₂	B ₂	C

^a het, Terminal heterogeneity; see text and Fig. 3.TABLE 3. Hybridization of ³²P-labeled *Hind*III fragments of HVP DNA to blot of HVP DNA fragments

Probe	Blot hybridization			
	<i>Eco</i> RI	<i>Hind</i> III	<i>Sal</i> I	<i>Pvu</i> I
A	C, J, K, het ^a	A	A	A ₁
B ₁ , B ₂	B, E, H, I, L	B ₁ , B ₂	A, B ₂ , E, F, G, H, I, J	A ₁ , B, C, D
C	A	C	C	A ₂
D	A, N	D	D	A ₂
E ₁ , E ₂	D, B	E ₁ , E ₂	B ₁	B
F	F, I	F	B ₂	A ₂
G	F	G	B ₂	A ₂
H	A, het	H, het	C, het	A ₂
I	D	I	B ₁	B
J ₁ , J ₂	G, I	J ₁ , J ₂	B ₂ , E	A ₂ , B
K	D, G	K	B ₁	B
L	D	L	B ₁	B

^a See Table 2.TABLE 4. Hybridization of ³²P-labeled *Sal*I fragments of HVP DNA to blot of HVP DNA fragments

Probe	Blot hybridization			
	<i>Eco</i> RI	<i>Hind</i> III	<i>Sal</i> I	<i>Pvu</i> I
A	B, C, H, J, K, het ^a	A, B ₁	A	A ₁
B ₁ , B ₂	B, D, E, G, I, F, L	B ₂ , E ₁ , E ₂ , F, G, I, J ₂ , K, L	B ₁ , B ₂	A ₂ , B, C
C	A	C, H, het	C	A ₂
D	A	D	D	A ₂
E	G, M	J ₁	E	B
F	B	B ₁	F	A ₁ , D
G	B	B ₁	G	D
H	E	B ₂	H	B, C
I	E	B ₂	I	B

^a See Table 2.

ment was part of *Sal*I-I, the 1.8-megadalton fragment was *Sal*I-H, and the 3.6-megadalton fragment was the rest of *Eco*RI-E. However, there was a 1-megadalton discrepancy between the total size of these three fragments and the size of *Eco*RI-E indicating that there is a 1-megadalton gap not filled in the *Sal*I map of the

*Eco*RI-E region which should be placed between *Sal*I-H and -I (Fig. 6). Second, digestion of *Eco*RI-A with *Sal*I generated only *Sal*I-D and -C, and *Sal*I-D was not cleaved with *Eco*RI, indicating that the cleavage site of *Eco*RI-A is closely aligned with the cleavage site of *Sal*I-D. This could be verified from the fact that the size

TABLE 5. Further cleavage of *EcoRI*, *SalI*, and *PvuI* restriction fragments

Second digestion	Size (megadaltons) and product
<i>EcoRI</i> -A/ <i>SalI</i>	12.4, <i>SalI</i> -C; 9.4, <i>SalI</i> -D
<i>EcoRI</i> -B/ <i>SalI</i>	4.6; 4.1 <i>SalI</i> -F; 2.9, <i>SalI</i> -G; 2.5; 1.2, <i>SalI</i> -J
<i>EcoRI</i> -C/ <i>SalI</i>	14.9, <i>EcoRI</i> -C
<i>EcoRI</i> -D/ <i>SalI</i>	12.3, <i>EcoRI</i> -D
<i>EcoRI</i> -E/ <i>SalI</i>	3.6; 1.8, <i>SalI</i> -H; 1.4
<i>EcoRI</i> -F/ <i>SalI</i>	7.3, <i>EcoRI</i> -F
<i>EcoRI</i> -G/ <i>SalI</i>	3.5; 2.3
<i>EcoRI</i> -H/ <i>SalI</i>	5.7, <i>EcoRI</i> -H
<i>EcoRI</i> -I/ <i>SalI</i>	5.2, <i>EcoRI</i> -I
<i>SalI</i> -B ₁ , B ₂ / <i>EcoRI</i>	12.3, <i>EcoRI</i> -D; 7.3, <i>EcoRI</i> -F; 5.2, <i>EcoRI</i> -I; 4.6; 3.6; 2.5, <i>EcoRI</i> -L; 2.3
<i>SalI</i> -C/ <i>EcoRI</i>	12.4, <i>SalI</i> -C
<i>SalI</i> -D/ <i>EcoRI</i>	9.0; <i>SalI</i> -D
<i>SalI</i> -E/ <i>EcoRI</i>	3.5; 1.1
<i>SalI</i> -F/ <i>EcoRI</i>	4.1, <i>SalI</i> -F
<i>SalI</i> -G/ <i>EcoRI</i>	2.9, <i>SalI</i> -G
<i>SalI</i> -H/ <i>EcoRI</i>	1.8; <i>SalI</i> -H
<i>SalI</i> -I/ <i>EcoRI</i>	1.4
<i>PvuI</i> -C/ <i>SalI</i>	8.0
<i>PvuI</i> -D/ <i>SalI</i>	2.9, <i>SalI</i> -G; 1.0

FIG. 6. Relationship of *EcoRI* and *SalI* fragments of HVP DNA derived from Table 5.

of *EcoRI*-A was 22.2 megadaltons, which is close to the sum of *SalI*-C plus -D (21.8 megadaltons). Treatment of *EcoRI*-F with the *SalI* did not cleave the fragment. Digestion of *SalI*-B₁ and -B₂ with the *EcoRI* did not detect any extra molecule of significant size, and the expected fragments *EcoRI*-D, -F, -I, and -L and the fragments of 4.6-megadalton (part of *EcoRI*-B), 3.6 megadaltons (part of *EcoRI*-E), and 2.3 megadaltons (part of *EcoRI*-G) were generated. Therefore, the ends of *SalI*-B₂ and *EcoRI*-F should be aligned closely, thus leaving a gap close to the size of the *EcoRI*-N fragment between *SalI*-B₂ and -D.

To align *PvuI*-C and -D within the linkage map, these two unlabeled fragments were redigested with *SalI* enzyme and newly generated fragments were detected by ethidium bromide staining after electrophoresis through 0.75% agarose gel. *PvuI*-C generated an 8.0-megadalton fragment which belonged to the *SalI*-B₂ region in the map (see Fig. 9). This indicates that the one end of the 8.6-megadalton *PvuI*-C locates at

0.6 megadalton from the end of *SalI*-B₂. *PvuI*-D generated *SalI*-G and a 1-megadalton fragment by the second digestion with *SalI*. This 1-megadalton fragment belonged to *SalI*-F, since *SalI*-F hybridized to this fragment. This indicates that the 4.5 megadaltons of *PvuI*-D can be aligned to 34.5 megadaltons from the left end (or 1 megadalton away from *SalI*-G) and contains *SalI*-G and part (0.5 megadalton) of *SalI*-J. This also establishes the linkage of *SalI*-F-G-J. From this alignment, the sizes of *PvuI*-A₁ and *PvuI*-A₂ are calculated as 34.5 and 33.4 megadaltons, respectively, based on the total size of 110 megadaltons of the *EcoRI* physical map.

Internal repeat sequences of HVP DNA. Although the cleavage of B95 DNA with *Bam*HI endonuclease yields approximately 12 copies of 2-megadalton fragments (14, 26, 29), the restriction enzyme cleavage of HVP DNA with *EcoRI*, *SalI*, *Hind*III, *PvuI*, and *Bam*HI did not reveal any highly repeated fragments. Another set of restriction enzymes such as *Hpa*I, *Kpn*I, *Sst*II, *Xba*I, and *Xho*I, which generated smaller fragments of EBV DNA, was tested. All of the five enzymes tested generated a reiterated fragment of approximately 1.8 megadaltons (Fig. 7). An average molar ratio of 7 is expected from the densitometry determination. These repeat fragments hybridized to *Hind*III-A and *EcoRI*-C, (Fig. 8).

DISCUSSION

HVP and EBV share common characteristics: (i) both viruses are closely associated with lymphoblastic disease; (ii) they immortalize B lymphocytes; (iii) the virus antigens cross-react with each other. Furthermore, a previous report showed that both viral DNAs share 40% homology which is dispersed throughout the viral genomes (Lee et al., in press). All of these features may suggest that EBV and HVP have evolved from a common ancestry. Thus, functional and genomic studies of HVP will help an understanding of the oncogenic property of EBV and may provide a system of genetic analysis between the two viruses. Such an analysis of EBV has not been performed so far due to the technical limitations. The linkage mapping of HVP DNA has been conducted to make future genetic studies between the two viruses possible.

To establish an overall picture of the linkage map of HVP DNA, fragments smaller than 1 megadalton which migrated off the gel under the present conditions were not considered. These fragments will be integrated into the map after they are cloned in bacteria. Thus, the true size of HVP DNA would be slightly higher than the values obtained by summation of the frag-

FIG. 7. Gel electrophoresis of HVP DNA digested with another set of restriction endonucleases. Two micrograms of HVP DNA was digested with *Xba*I, *Xba*I, *Sst*II, *Kpn*I, and *Hpa*I endonucleases individually. The digested products were separated by 0.8% agarose gel electrophoresis, stained with ethidium bromide (0.5 μ g/ml), and photographed under UV light with Kodak gelatin filter (23A). *Hind*III fragments of lambda DNA were used as markers.

ments listed in Table 1. Based on the sensitivity to λ exonuclease digestion, *Eco*RI-A and -J, *Hind*III-A and -H, and *Sal*I-A and -C, and *Pvu*I-A₁ and -A₂ fragments of HVP DNA have been identified as the terminal fragments, which have been confirmed and aligned by blot hybridization with each terminal fragment (Fig. 3). Heterogeneous bands were shown by hybridizing *Eco*RI-J to *Eco*RI, *Hind*III, and *Sal*I blots. The *Eco*RI blot revealed four clear bands of 4.3, 5.1, 5.9, and 6.7 megadaltons, in addition to the contaminating K fragment. The *Hind*III blot showed hybridization to *Hind*III-A and its vicinity and additional hybridization to 1.7-, 2.5-, and 3.3-megadalton fragments (Fig. 3d, arrows) and 3.6-, 4.4-, and 5.2-megadalton fragments (Fig. 3d, dots). The *Sal*I blot indicated two heterogeneous regions hybridized, one in a high-molecular-weight region including *Sal*I-A and the other in a low-molecular-weight region including 1.8-,

FIG. 8. Blotting hybridization of 32 P-labeled *Kpn*I repeat fragment of HVP DNA to HVP DNA fragments digested with either *Eco*RI or *Hind*III endonuclease. (a) Before hybridization, the digested DNA fragments were stained with ethidium bromide. The location of each fragment on this panel corresponds to that in (b). (b) Autoradiogram of the blot hybridization obtained from the same filter with 32 P-labeled *Kpn*I probe.

2.6-, 3.4-, and 4.2-megadalton fragments. Some of the hybridization to *Hind*III-A and *Sal*I-A was contributed by *Eco*RI-K. The sizes of *Eco*RI-J and three strongly hybridized bands

FIG. 9. Summary of the linkage map of HVP DNA. *, The type of heterogeneity at *PvuI* end has not been determined.

were 4.3, 5.1, 5.9, and 6.7 megadaltons, showing 0.8-megadalton increments to the next larger fragment. An identical stepwise increment was observed among the four hybridized bands of small size in the *SalI* blot, 1.8, 2.6, 3.4, and 4.2 megadaltons, as well as in the *HindIII* blot, 1.7, 2.5, and 3.3 megadaltons. The smallest *HindIII* fragment of 0.9 megadalton was not observed in this experiment but was detected in the repeated experiment. Therefore, these fragments may represent a heterogeneous end with a different number of terminal repeats on the *EcoRI*-J side. The heterogeneity observed in the high-molecular-weight region of *HindIII* and *SalI* blots hybridized by *EcoRI*-J may be due to the varying amounts of internal repeats within these fragments which contain homology to *BamHI*-V of EBV DNA (Y. S. Lee, A. Tanaka, R. Y. Lau, M. Nonoyama, and H. Rabin, manuscript in preparation).

The other side of terminal heterogeneity is shown by hybridization with *HindIII*-H terminal to *HindIII* total fragments (Fig. 3c), showing 3.6- (H), 4.4- and 5.2-megadalton fragments, again with an 0.8-megadalton stepwise increment. These fragments are also shown by hybridization with *EcoRI*-A terminal (Fig. 3b, dots). Since *EcoRI*-J detected these bands as well and *HindIII*-H detected the other end of heterogeneity (Fig. 3c, arrows), a certain sequence homology should exist between the terminal repeats of both ends of the molecule.

Another highly repeated region with an average of seven repeats was revealed by various restriction enzymes (Fig. 7). This repeat is located within *EcoRI*-C and *HindIII*-A (Fig. 8) and hybridizable to *BamHI*-V of EBV DNA (manuscript in preparation), which exists in an average of 12 repeats in the *EcoRI*-A region of EBV DNA (11, 13). The topographical location of this repeat in the *EcoRI*-C region is similar to that of the *BamHI*-V repeat in the *EcoRI*-A region of EBV DNA, thus suggesting an evolutionary relationship between these two viruses and functional importance of these repeats in viral replication or transformation.

In the *EcoRI* map, fragment N has been assigned next to the terminal A fragment by hybridization to *HindIII*-D. The reciprocal hybrid-

ization could not be performed since *EcoRI*-N could not be isolated in adequate quantities to make a probe. The same-size gap in the *SalI* map corresponding to the location of *EcoRI*-N between the D and B fragments has not been filled.

We have not identified fragments in the *HindIII* map which should be assigned to the gap(s) in the region of fragments *E2*, I, and L. It is possible that a few small fragments of less than 1 megadalton may have been overlooked in this study. The groups of *HindIII*-*E2*, -I, and -L and *HindIII*-J₁, -M, and -N have not been arranged relative to each other because each fragment within the group hybridized only to the same fragment of other enzyme digests. To determine these linkages, it is necessary to use other restriction enzyme fragments.

Thus, we have presented an overall physical map of *EcoRI*, *HindIII*, *SalI*, and *PvuI* with the unique feature of repeat sequences (Fig. 9). Small gaps remain to be filled, and two groups of fragments have yet to be arranged. The next important question will be whether a colinear relation can be seen between EBV and HVP DNA.

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ADDENDUM

Similar studies have been conducted by E. Kieff and his collaborators (15, 16), and we received their manuscripts at the time of submission of this study.

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