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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

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Viral DNA molecules were purified from a nontransforming and a transforming strain of Epstein-Barr virus. Each viral DNA was labeled in vitro and renatured in the presence of an excess of either one or the other unlabeled viral DNA. Both viral DNAs were also digested with the *Eco*R1 restriction endonuclease and subsequently labeled by using avian myeloblastosis virus DNA polymerase to repair either the *Eco*R1 nuclease-generated single-stranded ends of the DNAs or their single-stranded ends produced by a second digestion with exonuclease III after the first *Eco*R1 nuclease digestion. The results of these experiments support three general conclusions: (i) the DNAs of these two strains of Epstein-Barr virus share approximately 90% of their nucleotide sequences; (ii) both viral DNA populations are reasonably homogeneous; and (iii) both DNAs contain repetitions or inverted repetitions of some of their nucleotide sequences.

There are two common laboratory sources of Epstein-Barr virus (EBV): P3HR1 cells (9) and B95-8 cells (15). The former is a cloned line of Burkitt lymphoma cells (9), and the latter is a cloned line of cotton-top marmoset cells that were transformed with an infectious mononucleosis-derived virus (15). The virus released from P3HR1 cells will be called P3HR1 virus and that from B95-8 cells will be called B95-8 virus. Two lines of experiments have demonstrated that the populations of virus released by these two cell lines differ biologically. The virus-associated antigens induced by these virus populations in test cell lines differ (13, 15). In addition, Miller and Lipman and Menezes et al. have also shown that the B95-8 virus transforms human umbilical cord lymphocytes whereas the P3HR1 virus does not (14, 15). The source of the biological differences between P3HR1 and B95-8 viruses is not known; however, it may arise from either virus being composed of mixtures of defective viruses, for the viruses cannot be plaque purified.

Pritchett et al. have examined the DNA molecules isolated from these two virus populations and have concluded that, though they have the same molecular weight and buoyant density, B95-8 viral DNA lacks 15% of the sequences contained in P3HR1 viral DNA (19). We have further studied B95-8 and P3HR1 viral DNAs to determine whether each population is homogeneous and what is the nature of their sequence differences. We have used restriction endonuclease digestion and renaturation kinetics to analyze the viral DNAs. We find that seven of the eleven *Eco*R1 fragments of B95-8 viral DNA have the same molecular weight as do seven of the 12 *Eco*R1 fragments of P3HR1 viral DNA. These data along with those of renaturation kinetics indicate that B95-8 and P3HR1 viral DNAs share approximately 90% of their nucleotide sequences. The number and molar ratios of the DNA fragments indicate that these viral DNAs are both rather homogeneous and both contain repetitions and/or inverted repetitions as have been described for herpes simplex virus DNA (7, 24).

MATERIALS AND METHODS

Nucleotides. ^{3}H-labeled dCTP, ^{3}H-labeled dGTP, and α-^{32}P-labeled TTP were obtained from New England Nuclear Corp. ^{125}I-labeled dCTP was synthesized as described by Scherberg and Refetoff (21), using carrier-free Na^{125}I obtained from Amersham. Random penta- to octaoligodeoxynucleotides were prepared by pancreatic DNase digestion of calf thymus DNA (250 μg of DNase per 2 mg of DNA in 10 ml of 0.01 M Tris, pH 7.4, 0.005 M $MgCl_2$ incubated at 37 C for 2 h) followed by chromatographic separa-

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tion on DEAE-cellulose (0.85 by 50 cm) in urea (28). The pooled oligonucleotides were diluted and absorbed to DEAE-cellulose (1.2 by 20 cm), and the column was washed with water and eluted with 0.1 M HCl. The eluted oligonucleotides were neutralized and concentrated by lyophilization. Salt was removed from them by chromatography on Sephadex G-10 (0.85 by 52 cm) in water.

Enzymes. Avian myeloblastosis virus (AMV) DNA polymerase was purified from viremic chick plasma (kindly supplied by Dorothy and Joe Beard) according to the method of Kacian et al. (12). Exonuclease III was purified from *E. coli* K-12, using phase partition to remove nucleic acids (3), hydroxyapatite chromatography to remove salt and polyethylene glycol, and DEAE, phosphocellulose, and Sephadex G-150 chromatographies as described by Jovin et al. (11). *Eco*R1 endonuclease was generously provided by Ulf Pettersson and Gösta Winberg.

Cells. P3HR1 cells were grown in stationary suspension cultures in PRM1 1640 medium containing 5% fetal calf serum and antibiotics. When the cells had grown to stationary phase (5×10^5 to 10×10^5/ml), the cultures were placed at 34 C for 12 to 14 days. They were then stored at 4 C until the supernatant medium containing the virus was harvested (1 to 2 months).

B95-8 cells were adapted to grow in suspension in roller bottles in RPM1 1640 medium containing 5% calf serum and antibiotics. When the cells had grown to a stationary phase (10^6 to 2×10^6 cells/ml), they were stored at 37 C for 10 days and transferred to 4 C until the supernatant medium containing the virus was harvested.

Virus and viral DNA. Virus and viral DNAs were purified as described (1; B. Sugden, *in* H. Zur-Hausen and M. A. Epstein, ed., *Herpesviruses and Oncogenesis,* in press). Partially purified virus preparations were treated with DNase (50 μg/ml for 2 h) before viral DNA was extracted. All DNA samples were purified through two equilibrium centrifugation steps in CsCl followed by a velocity sedimentation step. Velocity sedimentation was carried out by using a 10 to 30% glycerol gradient in 1.0 M NaCl, 0.02 M Tris, pH 9.0, and 0.001 M EDTA in a Beckman SW27 nitrocellulose tube. The sample was centrifuged at 27,000 rpm for 200 min at 20 C. A parallel tube was treated identically and contained T6 DNA (105×10^6 daltons) as a molecular weight marker. EBV DNAs sedimented 40% of the tube length under these conditions, whereas T6 DNA sedimented 1 of 36 fractions faster. Only unit-length DNA molecules were collected. All sedimentation runs were collected in a closed system through 3- to 4-mm holes. At no time were the DNA samples precipitated with ethanol.

DNA:DNA hybridization on filters. DNA was immobilized on 47-mm-diameter nitrocellulose filters from which 6-mm-diameter filters were cut as described and soaked in Denhardt's solution (6, 10). Hybridization solutions of 0.2 ml contained 13 to 14 ng of labeled DNA, 50% formamide, 0.2 M sodium phosphate, pH 6.9, 0.75 M NaCl, 0.05% sodium dodecyl sulfate (SDS), and 0.0025 M EDTA. The initial $t_{1/2}$ (time for 50% of the DNA to renature) for the reannealing of DNA in solution was calculated, and hybridizations were continued for three to five times this value. The solution was removed each $t_{1/2}$ and heated to 85 C for 3 min to denature the DNA in solution and permit it to hybridize to the immobilized DNA. After hybridization, the filters were washed as described by Denhardt (6).

Repair synthesis using AMV DNA polymerase and oligonucleotides. Alkaline-denatured P3HR1 and B95-8 viral DNAs were used as templates for repair synthesis by AMV DNA polymerase. The repair reactions were carried out in a final volume of 0.075 ml containing 0.125 μg of denatured DNA, 5 μg of penta- to octaoligodeoxynucleotides, 0.13 mM each dATP and dTTP, 0.067 mM each [^{3}H]dCTP and [^{3}H]dGTP, 0.01 M $MgCl_2$, 0.02 M Tris, pH 8.0, 0.01 M β-mercaptoethanol, 0.03 M NaCl, 0.05 M sodium phosphate, pH 7.5 (from the polymerase), and sufficient AMV DNA polymerase to synthesize 20 ng of DNA per h under these conditions. The solutions were incubated at 15 C for 12 h and brought to 0.3% Sarkosyl and 0.06 M EDTA to stop the reactions. No acid-precipitable (<2%) material was synthesized in the absence of either denatured DNA or oligonucleotides. The products were purified by velocity sedimentation in alkaline sucrose gradients containing 5 to 20% sucrose, 0.1 M NaOH, 0.9 M NaCl, and 0.001 M EDTA. They were centrifuged in the Beckman SW50.1 rotor at 29,000 rpm for 18 h at 20 C. *Eco*R1-digested ^{14}C-labeled simian virus 40 was included as an internal molecular weight marker. The peak of radioactivity between 3 and 7.5*S* was pooled and precipitated with ethanol.

To calculate the specific activity of the product, it was assumed that the bulk of the unlabeled viral template DNA sedimented faster than the product (19). Our preparations of the AMV DNA polymerase contained no endonuclease activity as detected by conversion of SV40 form I to form II DNA. The synthesized DNA would then have a specific activity of 2×10^7 counts/min per μg. Only 70 to 80% of this DNA is rendered double stranded when renatured with a large excess of the template from which it was synthesized. The fraction that does not renature probably represents radioactive decay products, for it increases with time or upon concentration (e.g., freezing or ethanol precipitation).

Chemical iodination of double-stranded viral DNAs. Viral DNAs (0.3 to 0.5 μg) were dissolved in 0.05 ml of 0.1 M $NaO_2C_2H_3$, pH 4.2, 0.001 M KI, 0.5 mCi of $Na^{125}I$, and 0.05 M $TlCl_3$. The $TlCl_3$ was added last, and the solution (in a siliconized, sealed tube) was heated to 80 C for 20 min. The solution was cooled to 0 C, and 0.2 ml of 0.5 M sodium phosphate, pH 6.8, 0.01 M 2-mercaptoethanol, and 0.1 M KI was added to it. The solution was then heated to 60 C for 40 min to destroy unstable intermediates (5). The bulk of the free iodide was separated from the labeled DNA by chromatography on Sephadex G-75 (0.6 by 20 cm) in 0.001 M EDTA. The viral DNA was centrifuged to equilibrium in CsCl (36 h at 45,000 rpm in a Beckman SW65 rotor) to reduce backgrounds for hybridization (22), and 65 to 75% of it banded in the position of double-stranded, iodinated DNA whereas the remainder banded as sin-

gle-stranded, iodinated DNA (18). The double-stranded, iodinated DNA was finally purified by absorption to hydroxyapatite and elution with 0.4 M sodium phosphate, pH 6.8, and 0.4% SDS at 60 C. The above procedure was reproducible and yielded DNAs with specific activities of 10^6 to 3×10^6 counts/min per μg. The labeled DNAs were reduced in size for renaturation studies to fragments of approximately 500 nucleotides by controlled sonication.

DNA:DNA renaturation in solution. Viral DNAs labeled in vitro with AMV DNA polymerase and random oligodeoxynucleotides or by chemical iodination were renatured in the presence of a 100- or 400-fold excess of purified, fragmented (by sonication or alkaline hydrolysis) (27) homologous or heterologous viral DNAs or DNAs from cells that do not harbor EBV. Renaturation solutions contained 1 M NaCl, 0.2 M sodium phosphate, pH 6.8, 0.1% SDS, and 0.5 to 10 ng of labeled DNA in a volume of 0.1 to 0.15 ml. Aliquots (0.01 ml) were sealed in glass capillary tubes and incubated at 66 C for C_0t (C_0t = initial concentration of DNA in moles of phosphate/liter times time in seconds) values between 0.3 and 40 mol × s/liter (corrected to 0.12 M sodium phosphate). The same labeled viral DNAs were also renatured under the same conditions in 0.1 ml containing 750 μg of homologous EBV-negative cell DNAs or calf thymus DNA to C_0t values of 10^5 mol × s/liter. Some DNA samples (where noted) were renatured at 45 C in similar solutions containing 50% formamide. All samples were assayed for single- and double-stranded DNA content by hydroxyapatite chromatography (23). Different in vitro-labeled DNAs contained between 3 and 8% of their label that chromatographed as double-stranded DNA immediately after denaturation. All renaturation data were corrected for these presumptive hairpin structures.

***Eco*R1 digestion.** *Eco*R1 digestions were performed in 0.1-ml volumes containing 0.1 M NaCl, 0.02 M Tris, pH 7.5, 0.01 M $MgCl_2$, 0.001 M β-mercaptoethanol, and 10% glycerol to minimize possible contaminating nuclease activities. After incubating the DNA samples with *Eco*R1 at 37 C for 30 min, the solutions were heated to 70 C for 3 min to inactivate the enzyme.

Exonuclease III digestion and AMV DNA polymerase repair. The *Eco*R1 digestion DNAs were either repaired directly with AMV DNA polymerase and α-[^{32}P]TTP or further digested with exonuclease III and repaired with AMV DNA polymerase and [^{125}I]dCTP as described by Roberts et al. (20). The labeled products were then purified on Sephadex G-75 columns (0.8 by 25 cm) and precipitated with ethanol.

Gel electrophoresis. The labeled DNA fragments were analyzed in 0.4% agarose slab gels. The gels were prepared, the fragments were electrophoresed in the gels, and the gels were autoradiographed as described by Skare et al. (25).

RESULTS

Viral specificity of in vitro-labeled DNAs. Aliquots of ^{125}I-labeled EBV DNA fragments were hybridized to calf thymus DNA, to EBV-negative marmoset cell DNA and human cell DNA, and to EBV producer marmoset and human cell DNAs to test whether the labeled DNAs were EBV specific. The data in Table 1 indicate that the labeled species hybridized only to EBV producer cell DNAs and not to the homologous, EBV-negative cell DNAs. The labeled DNA was, therefore, virus specific within the sensitivity of this assay.

A second approach to determine the specificity of the labeled viral DNA was used. Viral DNAs labeled by repair synthesis with AMV DNA polymerase, random oligonucleotides, and [^{3}H]dCTP and [^{3}H]dGTP were renatured in the presence of a 10^6-fold excess of homologous cell DNAs (human or marmoset) or calf thymus DNA. These renaturation experiments were carried out to high C_0t values to detect possible homologies between unique cell DNA sequences and the labeled viral DNAs. Within experimental error (±5% due to counting error), the rate of renaturation of the viral DNAs in the presence of the homologous cell DNAs did not differ from that in the presence of calf thymus DNA (Fig. 1).

Reciprocal saturation renaturation kinetics. Purified viral DNA samples were labeled with AMV DNA polymerase and random penta- to octa-oligodeoxynucleotides as primers. The labeled viral DNAs were renatured with a 400-fold excess of the homologous DNAs that had served as templates for synthesis of the labeled material (Fig. 2). Of the labeled P3HR1 and B95-8 DNAs, 76 and 79%, respectively, were rendered double stranded as assayed by

TABLE 1. *^{125}I-labeled viral DNA hybridized to immobilized cell DNAs*[a]

Immobilized cell DNA	P3HR1 counts/min hybridized[b]	B95-8 counts/min hybridized[b]
Calf thymus	190	210
MLC (EBV-negative marmoset)	170	240
Molt 4 (EBV-negative human)	190	160
P3HR1	5,440	5,750
B95-8	4,300	4,525

[a] Aliquots of ^{125}I-labeled DNA fragments (60,000 to 70,000 counts/min) were hybridized to cell DNAs (5 μg/filter) from EBV-negative and homologous EBV producer cells as described in the text. Between 30 and 35% of the labeled DNA hybridized to the producer cell DNAs within the $3 \times t_{1/2}$ duration of this incubation.

[b] Each value represents the average of duplicate filters. Backgrounds are not subtracted.

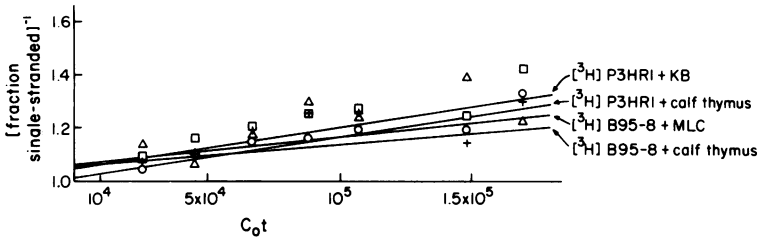

FIG. 1. *Renaturation of labeled viral DNAs (1,000 counts/min per point) in the presence of high concentrations (7.5 mg/ml) of homologous cell DNAs or calf thymus DNA. The reciprocal of the fraction of the labeled DNA remaining single stranded is plotted against the C_0t value in moles × seconds/liter. The slopes of the lines and their intercepts with the y axis have been calculated by using linear regression analysis. Symbols: ○, [^{3}H]P3HR1 + calf thymus DNAs; □, [^{3}H]P3HR1 + KB (human cell) DNAs; +, [^{3}H]B95-8 + calf thymus DNAs; △, [^{3}H]B95-8 + MLC (marmoset cell) DNAs.*

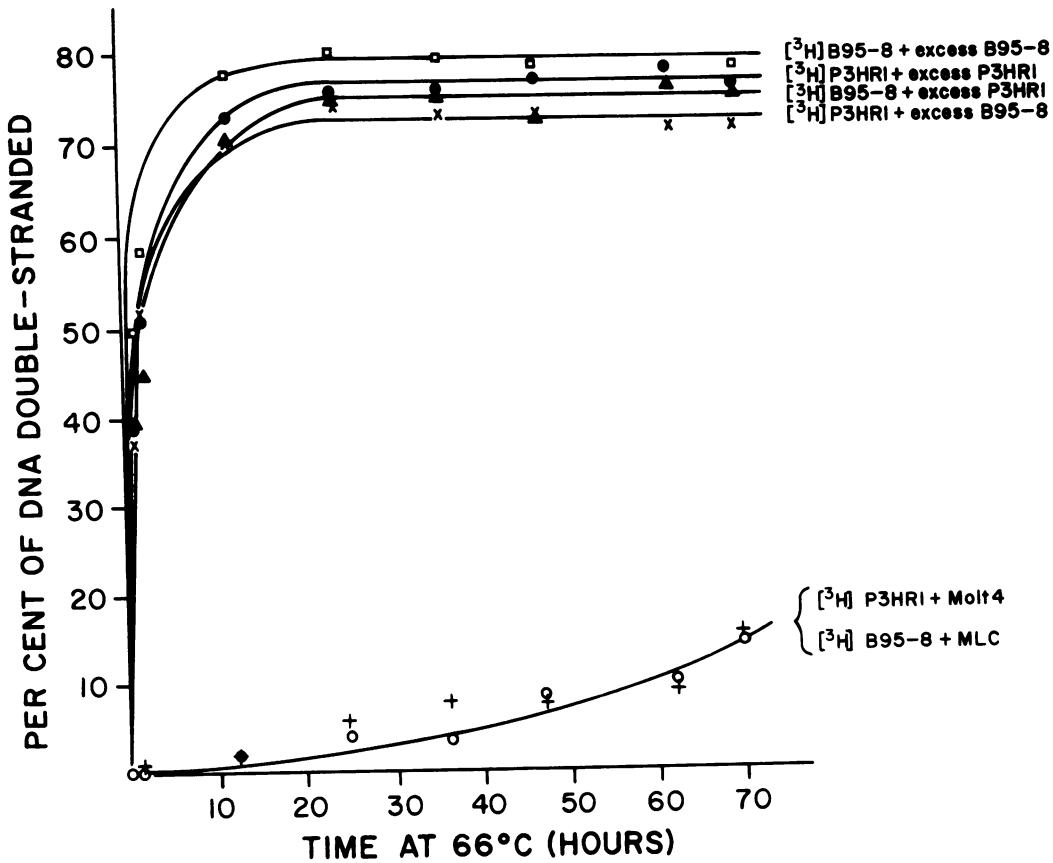

FIG. 2. *Reciprocal saturation renaturation kinetics of ^{3}H-labeled P3HR1 and B95-8 viral DNAs (1,000 counts/min per point) with 400-fold excess of purified homologous and heterologous viral DNAs. The average values of the percentage of DNA rendered double stranded at saturation (24 to 69.5 h) and their standard deviations (in parentheses) are: [^{3}H]P3HR1 + excess P3HR1 = 76% (1.2); [^{3}H]P3HR1 + excess B95-8 = 72% (0.9); [^{3}H]B95-8 + excess B95-8 = 79% (0.9); and [^{3}H]B95-8 + excess P3HR1 = 75% (1.2). Molt 4 is EBV-negative human cell DNA, and MLC is EBV-negative marmoset cell DNA. The rate of renaturation of the probes with these EBV-negative DNAs equals that with calf thymus DNA (data not shown).*

hydroxyapatite chromatography. Of the labeled sequences, 72 and 75%, respectively, were rendered double stranded when renatured with a 400-fold excess of the heterologous viral DNA. When these figures are corrected for complete renaturation of the probe DNAs by homologous DNAs, they indicate that for both P3HR1 and B95-8 labeled viral DNAs, 5% of the sequences that were rendered double stranded by the homologous DNA could not be renatured with the heterologous viral DNAs. We do not know that all of the nucleotide sequences of the virus DNAs were represented in the labeled products, so these observed 5% sequence differences of the labeled material indicate that no more than 95% of P3HR1 and B95-8 virus DNAs was homologous.

The saturation renaturation kinetics experiment was repeated by using viral DNAs chemically labeled with $Na^{125}I$ to avoid a possible labeling bias resulting from the enzymatic labeling technique used above. The chemically labeled DNAs were denatured and renatured in the presence of a 100-fold excess of either the sheared homologous or heterologous viral DNAs (Fig. 3). In the homologous renaturation reactions, 81% of P3HR1 DNA was rendered double stranded, whereas 84% of B95-8 DNA was rendered double stranded. In the heterologous renaturation reactions, 69% of labeled P3HR1 DNA formed duplexes with excess B95-8 DNA whereas 77% of labeled B95-8 DNA formed duplexes with excess P3HR1 DNA. In these experiments the isotopic label was introduced chemically into the DNAs and therefore probably uniformly. The differences in renaturation levels of the heterologous versus the homologous reactions should reflect differences in the nucleotide sequences of all of the P3HR1 and the B95-8 viral DNA molecules. Again, as with the enzymatically labeled DNAs, the homologous renaturation reactions plateaued when less than 100% of the labeled DNA was rendered double stranded.

Saturation renaturation kinetic studies with in vivo- and in vitro-labeled λ DNA. We investigated whether the low plateau figures for the homologous renaturation reactions of in vitro-labeled P3HR1 and B95-8 DNAs were peculiar to these animal viral DNAs or a result of the techniques we used to label them. Lambda phage DNA was labeled in vitro by chemical iodination exactly as were P3HR1 and B95-8 DNAs. In addition, λ phage DNA was labeled in vivo with [^{3}H]thymidine and was purified from purified virions. The two λ DNAs were sheared, denatured, and renatured in approximately a 100-fold excess of unlabeled DNA to C_0t values of 100 to 200 mol × s/liter. Of in vivo ^{3}H-labeled λ DNA, 96% formed duplexes with excess unlabeled λ DNA as assayed by hydroxyapatite chromatography. Only 86% of the in

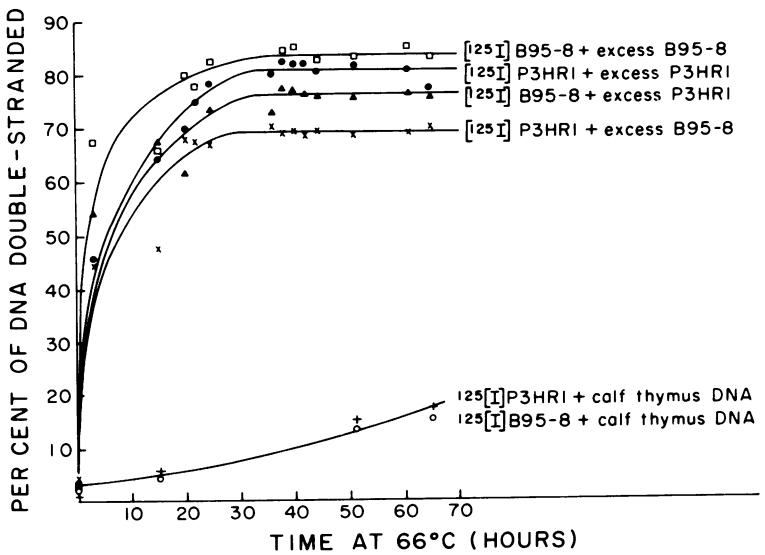

FIG. 3. *Reciprocal saturation renaturation kinetics of [^{125}I]P3HR1 and B95-8 viral DNAs (800 counts/min per point) that were labeled chemically, denatured, and renatured with a 100-fold excess of purified homologous or heterologous viral DNAs in solutions containing 50% formamide at 45 C. The average values of the percentage of labeled DNA rendered double stranded at saturation (38 to 65 h) and their standard deviations (in parentheses) are: [^{125}I]P3HR1 + excess P3HR1 = 81% (1.8); [^{125}I]P3HR1 + excess B95-8 = 69% (0.6); [^{125}I]B95-8 + excess B95-8 = 84% (1.1); [^{125}I]B95-8 + excess P3HR1 = 77% (0.8).*

vitro-iodinated λ DNA formed duplexes with excess unlabeled λ DNA, again as assayed by hydroxyapatite chromatography. We conclude that the low plateau values of the homologous renaturation reactions of in vitro-labeled P3HR1, B95-8, and λ viral DNAs are a result of the in vitro-labeling conditions, which yield some labeled species that cannot renature to form stable duplexes when assayed by hydroxyapatite chromatography.

Restriction nuclease cleavage patterns of labeled P3HR1 and B95-8 viral DNAs. Purified P3HR1 and B95-8 viral DNAs were digested with *Eco*R1 endonuclease and usually further digested with exonuclease III, and the single-stranded ends were repaired with AMV DNA polymerase. Lambda phage DNA was simultaneously digested and labeled in the same way. Between 40,000 and 150,000 counts/min of each of the labeled DNAs was analyzed by electrophoresis in 0.4% agarose gels (Fig. 4). P3HR1 viral DNA was cut into 12 fragments with molecular weights greater than 10^6, whereas B95-8 viral DNA was cut into 11 such fragments (no search was made for fragments with lower molecular weights). Seven fragments of P3HR1 and B95-8 DNAs co-migrated in the same slab gel, indicating that they had the same molecular weights (Fig. 4b). Increasing *Eco*R1 digestion by increasing the quantity of enzyme four- and eightfold did not alter the pattern in Fig. 4a, indicating that those fragments represent complete digestion products.

The pattern in Fig. 4b was obtained by first digesting the DNAs with exonuclease III, followed by repair synthesis, and completed with *Eco*R1 digestion. All fragments were labeled, indicating that there are internal nicks in the DNAs that serve as sites for exonuclease III digestion and subsequent repair synthesis. However, the known λ DNA fragments were labeled in nearly molar quantities when *Eco*R1 digestion preceded exonuclease III treatment (see Table 2), which indicates that internal labeling in this case does not dominate the labeling of the ends of the DNA fragments.

The molecular weights of viral DNA fragments were determined by using λ *Eco*R1 fragments and partial digestion products as internal molecular weight markers (Fig. 5 and 6). The molecular weights of the λ phage *Eco*R1 fragments were taken from Thomas and Davis (26), and the molecular weights of the λ partial digestion products were calculated from the same data (26). The molar ratios of the *Eco*R1 fragments of EBV DNAs were determined by cutting up the dried gels and counting the iodinated fragments in a gamma counter, and the molar ratios of the ^{32}P-labeled fragments were determined by means of Cerenkov radiation in a liquid scintillation counter. A total of 40,000 to 150,000 counts/min was applied to the gels; after correction for differing background counts in different gels, 60 to 80% of the counts were recovered in the peaks, the counts per gel were summed, one peak was set arbitrarily equal to unity, and all others were normalized to it. Three gels containing samples digested and labeled at different times were averaged to provide the molar ratios presented in Table 2. Analysis of the autoradiographs with a Joyce Loebl microdensitometer gave peak distributions similar to those obtained by counting the dried gels directly.

DISCUSSION

We have analyzed the viral DNAs of two "strains" of EBV: a non-immortalizing virus released from P3HR1 cells, and an immortalizing virus released from B95-8 cells. The low levels of virus released by these cell lines necessitated our labeling the viral DNAs in vitro in order to detect them. This in vitro labeling introduced a level of uncertainty in the analysis of the DNAs that would be obviated had we been able to label the DNAs in vivo. The labeled DNAs are virus specific as shown by hybridization of the labeled species to immobilized EBV-positive and -negative cell DNAs (Table 1) and by renaturation kinetic experiments performed with a large excess of homologous cell DNAs (fig. 1). These latter experiments do not exclude up to 5% of the labeled viral nucleotide sequences being homologous to unique DNA sequences of the respective host cells. If there exist any sequences in the labeled viral DNA homologous to the host DNAs, it is likely they form part of the viral DNA molecules and are not co-purifying, contaminating cell DNAs be-

FIG. 4. *(a) Viral DNAs were digested with EcoR1, repaired with AMV DNA polymerase + [^{32}P]TTP, and electrophoresed in a 0.4% agarose gel, which was dried and autoradiographed. Schematic drawings of the patterns are also presented. Symbols: L, λ DNA fragments; P, P3HR1 DNA fragments; B, B95-8 DNA fragments; 1, p, b, schematic drawings of the corresponding digestion patterns. (b) Viral DNAs were digested with exonuclease III, repaired with AMV DNA polymerase + [^{125}I]dCTP, digested with EcoR1, and electrophoresed in a 0.4% agarose gel, which was dried and autoradiographed. (Some EcoR1 partial digestion products are present.) The fragments of P3HR1 and B95-8 that co-migrate are marked with parallel lines to the right of the gel. Symbols: L, λ DNA fragments; P, P3HR1 DNA fragments; B, B95-8 DNA fragments.*

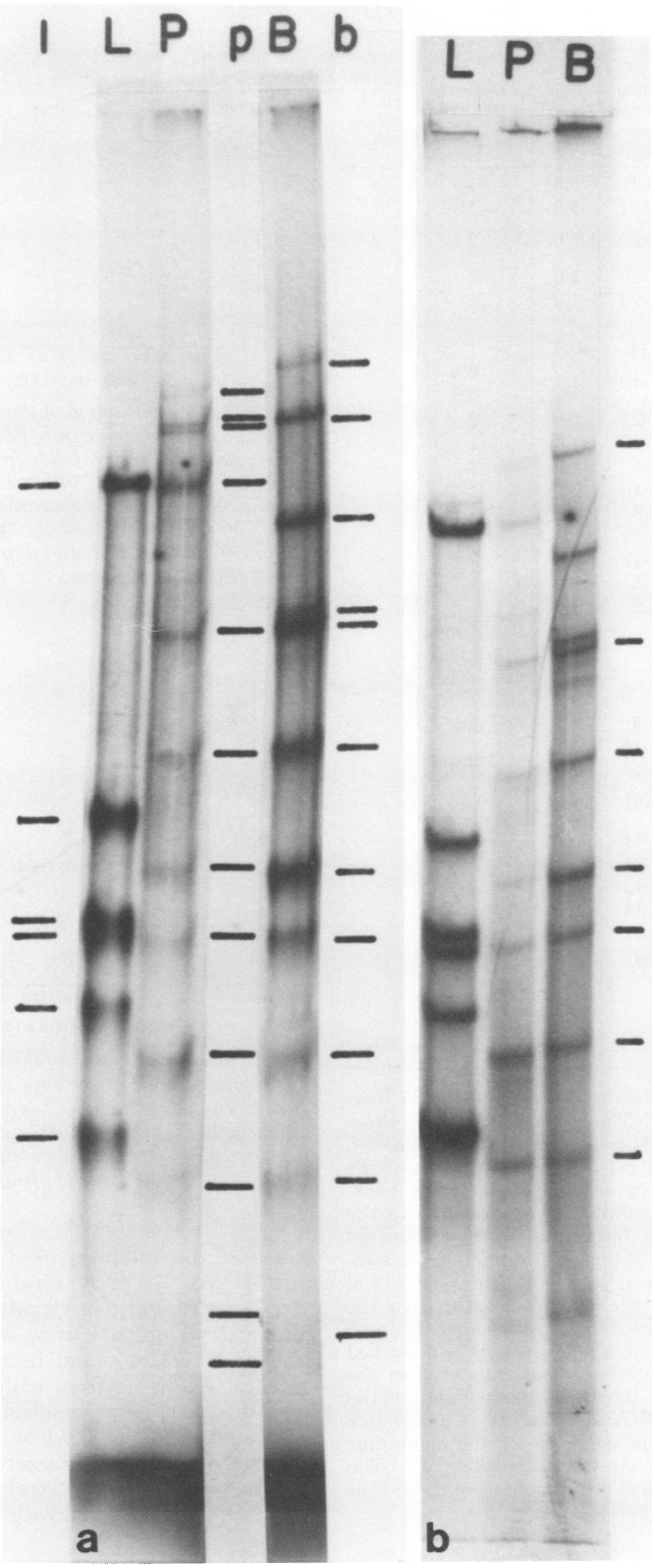
I L P pB b
a
L P B
b

TABLE 2. *Molar ratios of viral DNA fragments*[a]

Virus DNA fragments	Mol wt $\times 10^{-6}$	Range of % of counts/min per fragment	Avg of observed ratios	Suggested molar ratios	Known molar ratios
λ	13.7	10–17	0.8	1	1
	4.75	12–21	1.0	1	1
	3.73	33	2.0	2	2
	3.48				
	3.02	14–20	1.0	1	1
	2.13	15–23	1.2	1	1
P3HR1	24	2–4	0.4	1	
	20*	6–10	1.1	0.5	
	18.8			0.5	
	13.2	7–21	1.9	2	
	8.0*	10–13	1.4	1	
	5.6*	7–8	1.0	1	
	4.2*	4–10	0.9	1	
	3.6*	4–8	0.8	1	
	2.7*	19–26	2.9	3	
	2.1*	16–22	2.4	2	
	1.5	7–8	1.0	1	
	1.3	6–8	0.9	1	
				Total mol wt = 106×10^6	
B95-8	28	2–3	0.3	1	
	20*	5–11	0.8	1	
	12	5–12	0.9	1	
	8.6	15–21	2.0	1	
	8.0*			1	
	5.6*	8–14	1.2	1	
	4.2*	20–21	2.3	2	
	3.6*	7–13	1.1	1	
	2.7*	8–13	1.1	1	
	2.1*	4–12	0.9	1	
	1.4	6–9	0.9	1	
				Total mol wt = 100×10^6	

[a] The molecular weights for λ DNA *Eco*R1 fragments are taken from Thomas and Davis (26); those for P3HR1 and B95-8 are derived from Fig. 3 and 4. The range for these gels of the percentage of the counts per minute in each fragment after backgrounds have been subtracted is presented in the second column. The backgrounds have been set equal to the average of the baseline counts per minute on both sides of each fragment. From 60 to 80% of the cpm applied to each gel is recovered in the fragments. The observed molar ratios are calculated from the average of three separate gels as described in the text. The suggested molar ratios reflect the need for the sum of the molecular weights of the fragment for each DNA to equal $100 \times 10^6 \pm 10\%$ (see Discussion). The accuracy of the molecular weight estimates is on the order of ±10%. The starred P3HR1 and B95-8 fragments are those that co-migrate in 0.4% agarose gels.

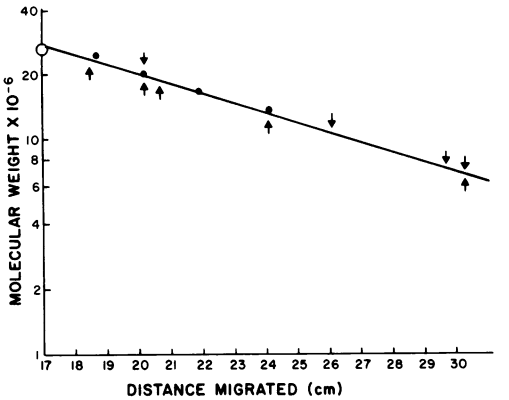

FIG. 5. *Determination of the molecular weights of the large EcoR1 fragments of P3HR1 and B95-8 viral DNAs using EcoR1 λ DNA fragments and partial digestion products and intact T7 DNA as molecular weight markers in 0.4% agarose gel electrophoresis at 70 V for 72 h. Symbols: ●, Largest EcoR1 λ fragment and partial digest products; ○, T7; ↓, B95-8 EcoR1 fragments; ↑, P3HR1 EcoR1 fragments.*

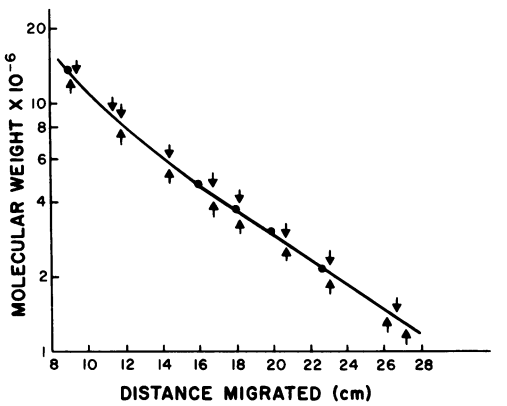

FIG. 6. *Determination of the molecular weights of the smaller EcoR1 fragments of P3HR1 and B95-8 viral DNAs using EcoR1 λ DNA fragments as molecular weight markers in 0.4% agarose gel electrophoresis at 100 V for 22 h. Symbols: ○, EcoR1 λ fragments; ↓, B95-8 EcoR1 fragments; ↑, P3HR1 EcoR1 fragments.*

cause of the methods used for purifying the viral DNAs. All of the viral DNA preparations were treated while in virions with DNase, and after extraction they were sedimented to equilibrium two times and finally sedimented in glycerol gradients from which only viral unit length molecules were selected.

When a 100- or 400-fold excess of purified viral DNAs was used to accelerate the rate of renaturation of labeled viral DNAs, the homologous DNAs renatured with the labeled probes

to a greater extent (5 to 14%) than did the heterologous DNAs (Fig. 2 and 3). These figures of 5 to 14% are calculated by correcting the plateau values of the homologous renaturation reactions of P3HR1 and B95-8 DNAs to the 96% value found for the renaturaion of in vivo-labeled λ DNA. The plateau renaturation values for the homologous reactions are significantly different from the plateau values for the heterologous reactions in the experiments presented in both Fig. 2 and 3: the respective mean values differ by more than twice the value of the standard error of their difference. These results demonstrate that P3HR1 and B95-8 viral DNAs share between 86 and 95% of their nucleotide sequences.

The in vitro-labeled DNAs present one general difficulty in the renaturation kinetics experiments. Only 76 to 84% of the labeled species formed duplexes with large excesses of homologous DNAs when assayed by hydroxyapatite chromatography. It was demonstrated that this problem arises from the in vitro labeling conditions; 96% of the in vivo-labeled λ DNA formed duplexes with excess λ DNA, whereas only 86% of in vitro chemically iodinated λ DNA formed duplexes with excess unlabeled DNA. One possible explanation for this discrepancy is that the in vitro-labeled DNAs contain radioactive species that as a result of decay or depurination (during the chemical labeling) are too small to form stable duplexes under the conditions used for hydroxyapatite chromatography (30).

*Eco*R1 endonuclease digestion of the two viral DNAs yielded 12 fragments greater than 10^6 daltons for P3HR1 and 11 such fragments for B95-8 DNA. The estimated molar ratios of the fragments are influenced by any non-randomness of the in vitro labeling. For λ phage DNA this method of labeling gives ratios that vary by 20% from the known values (see Table 2). The heaviest fragments of both P3HR1 and B95-8 are consistently present in less than 1 mol per mol of viral DNA, but are not removed by increasing endonuclease digestion. These large fragments would be the most sensitive to shear and if near the center of the intact viral molecules would be readily lost during our digestion procedures. Prichett et al. (19) have shown that the molecular weights of both P3HR1 and B95-8 viral DNAs equal 100×10^6 as determined by electron microscopy and neutral sucrose velocity sedimentation analysis. We have suggested probable molar ratios for the *Eco*R1 fragments based on our results and the requirement that the total molecular weights equal 100×10^6. This requirement has led to our interpretation that the largest *Eco*R1 fragments of both P3HR1 and B95-8 viral DNAs are probably present in molar quantities. If, for example, the largest *Eco*R1 fragment of B95-8 viral DNA were an artifactually resistant partial digestion product, the observed molar ratios for the remaining 10 fragments would lead to a molecular weight of 72 or 144×10^6 for B95-8 virus DNA. These estimates are both beyond the range of error of our molecular weight determinations (±10%). The requirement that the molecular weight of the viral DNAs be 100×10^6 also leads to our proposing that the high-molecular-weight doublets of *Eco*R1 fragments of P3HR1 viral DNA are probably present in half-molar quantities. Similar findings have been presented by Skare et al., who have shown that two *Eco*R1 fragments of plaque-purified herpes simplex virus type 1 are present in less than molar quantities (25). An alternative proposal that meets the molecular weight requirement for P3HR1 viral DNA is that one of its three largest *Eco*R1 fragments is a partial digestion product that is artifactually resistant to *Eco*R1. This alternative proposal would lead to a molecular weight of 101 or 107×10^6 for P3HR1 viral DNA, which is consistent with the accuracy of our molecular weight determinations. Our need to use in vitro labeling to detect the fragments limits the accuracy of estimating their molar ratios. Therefore, the interpretations presented, which are both compatible with the data, can be resolved only by further experiments.

Several conclusions can be drawn from the *Eco*R1 digestion patterns of P3HR1 and B95-8 viral DNAs in spite of uncertainties in the molar ratios of the fragments. (i) Some of the fragments of both viral DNAs are present in greater than molar quantities. This finding indicates that there may exist sequence repetitions or inverted sequence repetitions in both DNAs as has been found for herpes simplex DNA by Sheldrick and Berthelot (24) and by Grafstrom et al. (7). A second, but less appealing, explanation for this finding is that those fragments present in greater than molar quantities represent fragments of slightly different molecular weights that are not resolved by our gel electrophoresis and are present in molar amounts. (ii) These endonuclease digestion patterns suggest that P3HR1 and B95-8 viral DNAs are each reasonably homogeneous because the heterogeneous, highly repeated fragments characteristic of high-multiplicity-passaged, defective herpes simplex (29) are not present. This finding might seem surprising because, where studied, the bulk of EBV DNA is found to be present as a covalently closed, supercoiled, extrachromosomal molecule with 50 copies on the average present per cell (2, 17). If

EBV DNA existed in this physical state in P3HR1 and B95-8 cells, it is possible that after several hundred cell generations defective DNA molecules would accumulate, which would be packaged. We have not found evidence to support this possibility. The *Eco*R1 digestion patterns are consistent with EBV replicating in an orderly fashion in which defective molecules of the type formed during high-multiplicity passage of herpes simplex are not favored during prolonged passage of the producer cells. (iii) The differences in the *Eco*R1 digestion patterns give another estimate of the extent of sequence homologies between the two DNAs. *Eco*R1 endonuclease recognizes a hexanucleotide sequence in bihelical DNA (8). It is likely that when one recognition site is lost in two closely related DNAs, only a single nucleotide has changed. Eleven B95-8 or 12 P3HR1 fragments are formed by 10 or 11 recognition sites, respectively, indicating that *Eco*R1 is sampling 60 or 66 nucleotides in these DNAs. Thus, four and five fragments are different between the viral DNAs, indicating that only four to five nucleotides are different per 60 to 66 nucleotides, which means that P3HR1 and B95-8 viral DNAs share 92 to 94% of their sequences. This finding agrees well with the extent of homology found by saturation renaturation kinetics. The calculation using the *Eco*R1 recognition site is based on the assumption that the recognition sequence is randomly distributed in EBV DNA. Botchan et al. have shown that the sequence is, at least, distributed randomly among the nonsatellite DNA of mice (4). The relatedness of these viral DNAs is underscored in a second manner by their having seven *Eco*R1 fragments that co-migrate upon electrophoresis in agarose gels. No deletion or addition mutations have occurred in these seven fragments of P3HR1 and B95-8 viral DNAs that are large enough to be detectable by our gel electrophoresis techniques. The most reasonable estimate for the extent of nucleotide sequence homology between P3HR1 and B95-8 viral DNAs from our data is the mean of the estimates obtained from all of the renaturation and the endonuclease digestion experiments. The mean of these data indicates that P3HR1 and B95-8 viral DNAs share approximately 92% of their nucleotide sequences.

Our conclusion that P3HR1 and B95-8 viral DNAs share approximately 92% of their sequences is somewhat at variance with the interpretation of Pritchett et al. that B95-8 virus represents a mutant of P3HR1 virus in which 15% of the P3HR1 viral DNA sequences have been deleted and a second set of 15% of P3HR1 viral DNA sequences have been repeated so that the molecular weights of the viral DNAs remain the same (19). The *Eco*R1 digestion patterns of both viral DNAs are not compatible with B95-8 having a single, contiguous deletion and substitution of other P3HR1 viral nucleotide sequences. However, the patterns could be generated by B95-8 virus containing several noncontiguous deletions and substitutions of P3HR1 viral DNA sequences. Pritchett et al. have based their conclusions in part on renaturation kinetics using total producer cell DNA to drive in vitro-labeled viral DNAs (16). Their and our results both are subject to the difficulties of using in vitro-labeled DNAs as a probe in renaturation kinetics. A second difficulty they may have encountered arises from their using total-cell DNA to drive their labeled viral DNA probes. If their labeled P3HR1 viral DNA preparation contained any host DNA sequences, then homologous cell DNA would renature with all of the preparation whereas the heterologous B95-8 cell DNA would not. We have circumvented this difficulty by using only purified viral DNAs (unit length before shearing) to drive both homologous and heterologous purified, in vitro-labeled viral DNAs in renaturation experiments.

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