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DNA of Epstein-Barr Virus

III. Identification of Restriction Enzyme Fragments That Contain DNA Sequences Which Differ Among Strains of Epstein-Barr Virus

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Previous kinetic and absorption hybridization experiments had demonstrated that the DNA of the B95-8 strain of Epstein-Barr virus was missing approximately 10% of the DNA sequences present in the DNA of the HR-1 strain (R. F. Pritchett, S. D. Hayward, and E. Kieff, J. Virol. **15**:556-569, 1975; B. Sugder, W. C. Summers, and G. Klein, J. Virol. **18**:765-775, 1976). The HR-1 strain differs from other laboratory strains, including the B95-8 and W91 strains, and from virus present in throat washings from patients with infectious mononucleosis in its inability to transform lymphocytes into lymphoblasts capable of long-term growth in culture (P. Gerber, Lancet **i**:1001, 1973; J. Menezes, W. Leibold, and G. Klein, Exp. Cell. Res. **92**:478-484, 1975; G. Miller, D. Coope, J. Niederman, and J. Pagano, J. Virol. **18**:1071-1080, 1976; G. Miller, J. Robinson, L. Heston, and M. Lipman, Proc. Natl. Acad. Sci. U.S.A. **71**:4006-4010, 1974). In the experiments reported here, the restriction enzyme fragments of Epstein-Barr virus DNA which contain sequences which differ among the HR-1, B95-8, and W91 strains have been identified. The DNA of the HR-1, B95-8, and W91 strains each differed in complexity. The sequences previously shown to be missing in the B95-8 strain were contained in the *Eco*RI-C and -D and *Hsu* I-E and -N fragments of the HR-1 strain and in the *Eco*RI-C and *Hsu* I-D and -E fragments of the W91 strain. The HR-1 strain was missing DNA contained in *Eco*RI fragments A and J through K and *Hsu* I fragment B of the B95-8 strain and in the *Eco*RI-A and *Hsu* I-B fragments of the W91 strain. The relationship of these data to the linkage map of restriction enzyme fragments of the DNA of the B95-8 and W91 strains (E. Kieff, N. Raab-Traub, D. Given, W. King, A. T. Powell, R. Pritchett, and T. Dambaugh, *In* F. Rapp and G. de-The, ed., *Oncogenesis and Herpesviruses III*, in press; D. Given and E. Kieff, submitted for publication) and the possible significance of the data are discussed.

In the 14 years which have elapsed since the discovery of Epstein-Barr virus (EBV), much has been learned about the agent and its association with human disease (for a review, see references 14, 15, and 27). Although rapid progress in the epidemiology and biology of EBV has provided an impetus for biochemical studies of the mechanisms involved in transformation, progress in chemical and genetic approaches has been made difficult by the unusual properties of the agent in tissue culture. The most significant of these problems has been the limitation in the host range of the virus to B lymphocytes (11, 24) and the observation that productive infection is a rare (4, 8, 22) or, in some instances, an infrequent outcome (3, 18-21). Largely because of these difficulties, and also because of the possible relationship of strain variation to the different diseases with which EBV has been associated, attention has focused on analysis of differences between isolates of EBV (3, 13, 18, 25). Quantities of virus, adequate for chemical analyses, can be obtained from three sources: from a Burkitt tumor cell line which has been grown in culture (the HR-1 line) (10), from marmoset lymphocytes infected in vitro with an infectious mononucleosis isolate (B95-8) (21), and from marmoset lymphocytes infected with another (Nyevu) Burkitt tumor isolate (W91) (18). Virus from the HR-1 line, EBV HR-1, can be used to superinfect Raji cells, thereby inducing the synthesis of early antigen (9), but this virus cannot transform normal lymphocytes to cells possessing capacity for long-term growth in vitro (17, 20). In contrast, virus from B95-8 cultures (17, 20), EBV B95-8, from W91 cultures, EBV W91

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(18), and from the throat washings of recently infected humans (2) cannot induce early antigen in Raji cells but can transform normal lymphocytes into cells capable of long-term replication in culture.

In previous studies no differences have been found between the complement-fixing, early, viral capsid, or neutralizing antigens of the B95-8 and HR-1 strains of EBV (17, 20, 21). Furthermore, the viral capsid and neutralizing antigens of the W91 (Nyevu) strain seem similar to those of other isolates (18). No differences were found among the molecular weights of the major structural polypeptides of the HR-1 and B95-8 strains (1). Comparative studies of the DNAs of the B95-8 and HR-1 strains indicated the following. (i) EBV B95-8 and HR-1 DNAs are linear, double-stranded molecules with the length and sedimentation properties indicative of a size of 100 $\times 10^6$ daltons (25). (ii) Kinetic and absorptive hybridization analyses with in vitro-labeled purified viral DNAs demonstrated that EBV B95-8 DNA is missing approximately 10 to 15% of the DNA sequences of EBV HR-1 DNA and that EBV HR-1 DNA contains at least 90 to 95% of the DNA sequences of EBV B95-8 DNA (25). In a second study, kinetic hybridization data suggested that B95-8 DNA might contain sequences which EBV HR-1 DNA lacked (29). No attempt was made in this study, however, to demonstrate that the residual labeled EBV B95-8 DNA which did not hybridize to EBV HR-1 DNA could hybridize to EBV B95-8 DNA. (iii) EBV B95-8 DNA is relatively enriched for DNA sequences with a buoyant density in neutral cesium chloride of 1.720 to 1.721 g/cm^{-3} (25). (iv) Incubation of EBV B95-8 DNA with *Sal* I, *Hsu* I, and *Eco*RI restriction endonucleases resulted in fragments separable on 0.4% agarose and ranging in molecular weight from 1×10^6 to 32×10^6 (6). All fragments were present in molar amounts. In contrast, incubation of EBV HR-1 DNA with *Sal* I, *Hsu* I, or *Eco*RI yielded fragments present in equimolar amounts and fragments present in submolar amounts (6). The molecular weights of some fragments of EBV HR-1 DNA were similar to those of EBV B95-8 DNA; others differed.

The distinctive biological properties of the HR-1 strain could arise from the presence or absence of specific DNA sequences, as a consequence of differences in the arrangement of DNA sequences, or more simply by alteration in one or a few base pairs. The long-range objective of this series of experiments is to discriminate between these alternative possibilities. The experiments reported here were designed to determine which, if any, restriction enzyme fragments of the DNA of each of the strains contain sequences which are not present in the other strains. In conjunction with the mapping data available for EBV B95-8 and W91 DNAs (E. Kieff, N. Raab-Traub, D. Given, W. King, A. T. Powell, R. Pritchett, and T. Dambaugh, *In* F. Rapp and G. de-The, ed., *Oncogenesis and Herpesviruses III*, in press; D. Given and E. Kieff, submitted for publication), the data permit some tentative conclusions to be drawn about the relatedness between these strains.

MATERIALS AND METHODS

Cell culture and virus purification and extraction of viral DNA. Starter cultures of B95-8 and W91 cells were obtained from G. Miller, Yale University, New Haven, Conn., and those of HR-1 cells were from G. Klein, Karolinska Institutet, Stockholm, Sweden. Cells were grown at a density of 5×10^5 viable cells per ml in media consisting of RPMI 1640 supplemented with 10% fetal calf serum (both obtained from Grand Island Biological Co., Grand Island, N.Y.). Cultures were maintained at 35°C and refed every 7 days by the addition of 1/3 volume of media. At 3-monthly intervals cultures were switched between media containing tylocine (60 μg/ml; Grand Island Biological Co.) and spectinomycin (200 μg/ml; Upjohn Corp., Kalamazoo, Mich.) and media containing gentamicin (40 μg/ml; Schering Corp., Bloomfield, N.J.).

The procedures employed in purifying virus from the supernatant media of cell cultures have been described previously (1). Briefly, 7 days after feeding, supernatant media were harvested by aspiration from cultures which had been left unperturbed for at least 24 h. All subsequent steps were carried out at 4°C. The medium containing virus, debris, and some cells was centrifuged for 90 min at 9,000 rpm in a GS-3 rotor (Sorvall Corp., Newtown, Conn.). The pellet from 8 liters was suspended in 4 ml of 0.5 mM sodium phosphate (pH 7.4), homogenized with 25 strokes of a Dounce homogenizer, clarified by centrifugation for 10 min at 4,000 rpm in an SS34 rotor (Sorvall Corp.), layered onto a 5 to 30% (wt/vol) dextran gradient (dextran T10, Pharmacia Corp., Uppsala, Sweden), and centrifuged at 20,000 rpm for 1 h in an SW27 rotor (Beckman Corp., Palo Alto, Calif.). The single light-scattering band that formed midway between the top and bottom of the tube was removed, diluted with 0.5 mM sodium phosphate (pH 7.4), and centrifuged in an SW27 rotor for 90 min at 25,000 rpm. The virus pellet was resuspended in 0.1 M NaCl, 0.05 M Tris-hydrochloride, and 0.01 M EDTA, pH 7.4. Sodium dodecyl sulfate (BDH Laboratories, Poole, England) was added to a final concentration of 1% (wt/vol), and the mixture was incubated at 60°C for 2 min. The DNA solution was extracted twice with equal volumes of phenol and chloroform containing 2% (vol/vol) isoamyl alcohol and was exhaustively dialyzed against 0.3 M NaCl–0.01 M EDTA–0.05 M Tris-hydrochloride, pH 7.6.

Radioactive labeling of viral DNA. DNA was extracted from purified virus (W91, B95-8, or HR-1 strains) and was dialyzed against a solution containing 2 mM EDTA and 10 mM Tris-hydrochloride, pH 7.4. Solid CsCl (suprapure grade, E. Merck Co.) was added to a density of 1.710 g/cm^3 and the DNA was centri-

fuged for 48 h at 30,000 rpm and 20°C in a type 65 rotor (Beckman Corp.). Fractions of the gradient between 1.710 and 1.725 g/cm^3 were combined and shown to contain a single UV-absorbing band at 1.718 g/cm^3 in an analytical centrifuge (model E, Beckman Corp.). The DNA was labeled in vitro by nicked translation (12). Briefly, 0.1 to 0.2 μg of DNA was incubated at 37°C for 10 min in 300 μl of a solution containing 10^{-10} g of activated DNase (26), 50 μg of bovine serum albumin, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 50 mM Tris-hydrochloride, pH 7.4. Three unlabeled deoxynucleotide triphosphates, 10 μM each (P-L Laboratories, Milwaukee, Wis.), 4 μM of ^{32}P-labeled dCTP or dGTP (250 Ci/mmol) (Amersham/Searle, Arlington Heights, Ill.) and 5 U of *E. coli* DNA polymerase 1 (Boehringer-Mannheim Corp., Indianapolis, Ind.) were added, and the reaction was incubated at 16°C for between 4 and 6 h. When the specific activity of the labeled DNA was between 2×10^7 and 5×10^7 cpm/μg, the reaction was terminated by the addition of Sarkosyl to 1% and 0.1 M EDTA. The labeled DNA was separated from unincorporated deoxynucleotide by chromatography on a column (1 by 30 cm) of Sephadex G50 (Pharmacia Fine Chemicals) in 1 mM EDTA–10 mM Tris-hydrochloride, pH 7.4. Fractions containing labeled DNA were combined, mixed with 100 μg of calf thymus DNA (Sigma Chemical Corp., St. Louis, Mo.), adjusted to 1% (wt/vol) sodium dodecyl sulfate–0.1 M NaCl, extracted with phenol and chloroform containing 2% (vol/vol) isoamyl alcohol, and precipitated at −20°C after the addition of two volumes of ethanol.

Separation of (labeled) DNA sequences which differ among strains of EBV. The strategy employed to separate labeled DNA which differed between each pair of the three strains of virus studied was as follows: 0.05 to 0.1 μg, or 1×10^6 to 3×10^6 cpm of ^{32}P-labeled EBV DNA was mixed with at least 50-fold excess of unlabeled DNA of a second strain contained in 2 mg of DNA extracted (16) from cells infected with the second strain. The mixture of ^{32}P-labeled viral and viral and cellular DNAs was denatured in 0.2 N NaOH at 20°C and incubated for between 4 and 6 Cot_{50} of the unlabeled viral DNA at 68°C in 0.5 ml of a solution consisting of 5 mM EDTA, 1.5 M NaCl, and 50 mM Tris-hydrochloride, pH 7.4. Single-stranded DNA, enriched for sequences which could not be driven into hybrid by excess unlabeled viral DNA of the heterologous strains, was separated from double-stranded DNA by chromatography on columns of hydroxyapatite (25) (Bio-Gel HTP, Bio-Rad Laboratories, Richmond, Calif.). The residual single-stranded DNA was dialyzed against a solution containing 10 mM EDTA, 0.1 M NaCl, and 50 mM Tris-hydrochloride, pH 7.4 and was precipitated at −20°C after the addition of two volumes of ethanol. The excess single-stranded DNA was hybridized again under identical conditions to excess unlabeled heterologous viral DNA so as to remove any sequences in common which might have escaped the first round of hybridization.

Identification of restriction enzyme fragments of viral DNA which contain sequences which differ among strains. A 5-μg amount of viral DNA was incubated for 2.5 h at 37°C in 0.5 ml of a solution consisting of 20 mM NaCl, 20 mM $MgCl_2$, 20 mM Tris-hydrochloride (pH 7.4), and 100 U of *Hsu* I restriction endonuclease per ml (purified as described previously) (6) or in a solution consisting of 10 mM $MgCl_2$, 50 mM NaCl, 100 mM Tris-hydrochloride, pH 7.4, and 100 U of *Eco*RI restriction endonuclease per ml (Bethesda Research Laboratories, Bethesda, Md.). The digestion was terminated by the addition of EDTA to 0.1 M, and the DNA was subjected to electrophoresis for 18 h at 25 V in a cylinder (1 by 28 cm) of 0.4% agarose (Seakem HGT, Marine Colloids, Rockland, Maine) in a solution consisting of 0.5 μg of ethidium bromide per ml, 36 mM Tris, 1 mM EDTA, and 30 mM NaH_2PO_4, pH 7.4. The gels were visualized under UV light and photographed with an MP4 camera (Polaroid Corp., Oak Brook, Ill.) using a Tiffin 21 filter and type 57 film (Polaroid). The gel was immersed in 0.5 N NaOH and 1.5 M NaCl for 30 min at 25°C and then in 3 M NaCl–0.5 M Tris-hydrochloride, pH 7, for 15 min. The DNA fragments were then transferred onto nitrocellulose sheets (HAWP, Millipore Corp., Bedford, Mass.) by passage of 20× SSC (SSC consisted of 0.15 M NaCl–0.015 M sodium citrate) through the gel and overlapping nitrocellulose into dry adsorbent cellulose filters (28). The nitrocellulose filter was peeled from the gel, cut in half lengthwise, notched in the upper right corner, washed in 2× SSC, and dried at 80°C for 2 h.

DNA fragments which contain sequences missing in a second strain of EBV were identified by incubating blots of fragments of the first strain with labeled single-stranded DNA of the same strain from which all of the sequences common to the two strains had been removed by hybridization and chromatography in hydroxyapatite as described above. The 3-mm-wide blot was incubated with labeled DNA and 2 mg of denatured calf thymus DNA (Sigma Chemical Corp.) for 24 h at 68°C in a sealed 5-ml pipette containing 1 ml of a solution consisting of 5 mM EDTA, 1.5 M NaCl, and 50 mM Tris-hydrochloride, pH 7.4. The other half of the same blot served as a control and was incubated under identical conditions with labeled single-stranded DNA of the same strain which had not previously been hybridized. The DNA-containing filters were then washed in 1× SSC for 4 h at 37°C, dried for 4 h under vacuum, and exposed to X-ray film (SB5, Eastman Kodak Co., Rochester, N.Y.) either at 20°C or with an intensifying screen (Cronex Lightning Plus, duPont Corp., Wilmington, Del.) at −70°C.

Kinetics of hybridization of W91 DNA with ^{32}P-labeled EBV HR-1 DNA. A 0.2-mg amount of DNA extracted from W91 or HR-1 cells or from calf thymus (Sigma Chemical Corp.) was mixed with between 1×10^{-3} and 2×10^{-3} μg of ^{32}P-labeled EBV HR-1 DNA (specific activity 1×10^7 to 5×10^7 cpm/μg) in 100 μl of 0.2 M NaOH. The mixture was neutralized with 1 M HCl and incubated in 10-μl portions of a solution consisting of 5 mM EDTA, 1.5 M NaCl, and 25 mM Tris-hydrochloride, pH 7.4, for intervals up to 18 h. After incubation, samples were frozen at −20°C. The procedures employed to assay the fraction of DNA remaining single stranded at each time have been described in detail previously (23).

RESULTS

Restriction enzyme fragments of EBV HR-1 and W91 DNA which contain DNA

not present in the B95-8 strain. The procedures used to prepare ^{32}P-labeled EBV HR-1 DNA, to remove sequences common to the B95-8 strain, and to identify EBV HR-1 DNA fragments which contain the HR-1-specific sequences are described above. Hybridization of the residual single-stranded ^{32}P-labeled EBV HR-1 DNA to filters containing separated *Eco*RI or *Hsu* I restriction enzyme fragments of EBV HR-1 DNA revealed hybridizations to *Eco*RI-C and -D (Fig. 1) and to *Hsu* I-E and -N fragments and to a minor heterogeneous *Hsu* I fragment smaller than fragment G (Fig. 2).

The specificity of the residual, B95-8-adsorbed, ^{32}P-labeled EBV HR-1 DNA for fragments containing sequences missing in EBV B95-8 DNA is evident in this and subsequent experiments by comparing the autoradiogram (e.g., Fig. 1 and 2) of half of the blot which had been hybridized to ^{32}P-labeled EBV HR-1 DNA with the autoradiogram of the other half of the blot which was incubated with B95-8-absorbed, ^{32}P-labeled EBV HR-1 DNA. As expected, the hybridization of ^{32}P-labeled EBV DNA to fragments was in proportion to the amount of DNA in that fragment and was similar to the relative intensity of the fragment in the ethidium bro-

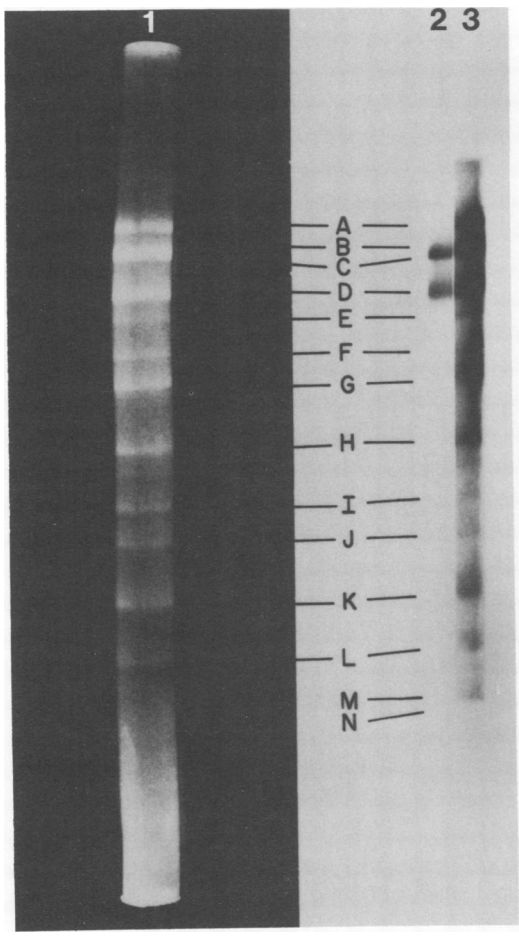

FIG. 1. *Identification of EcoRI fragment(s) of EBV HR-1 DNA which contain DNA sequences not present in DNA of the B95-8 strain. EBV HR-1 EcoRI fragments subjected to electrophoresis in a 0.4% agarose gel (slot 1) were transferred onto a nitrocellulose filter. One half of the filter was incubated with 80,000 cpm of ^{32}P-labeled EBV HR-1 DNA (slot 3). The other half of the filter was incubated with 35,000 cpm of the residual single-stranded ^{32}P-labeled EBV HR-1 DNA from which sequences homologous to EBV B95-8 DNA had been removed (slot 2).*

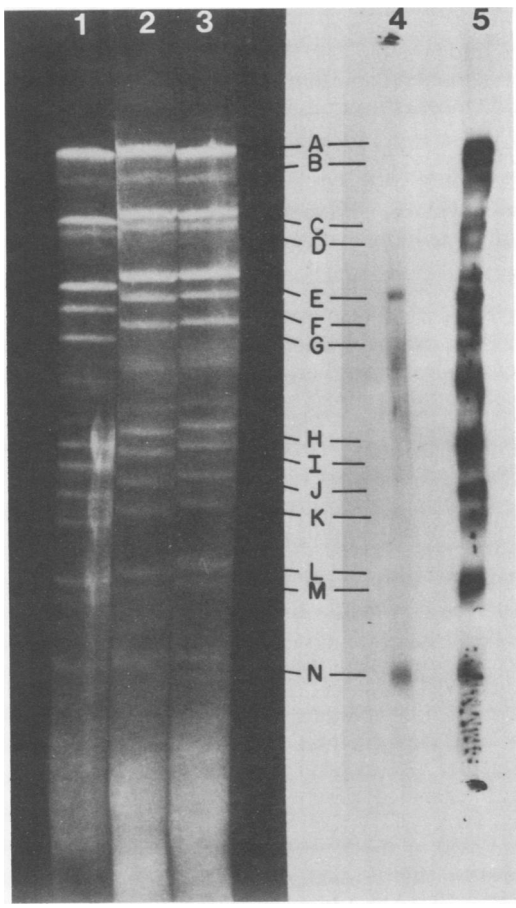

FIG. 2. *Identification of Hsu I fragment(s) of EBV HR-1 DNA which contain DNA sequences not present in the DNA of the B95-8 strain. EBV HR-1 DNA Hsu I fragments (slots 1 to 3) were transferred onto a nitrocellulose filter. One half of the filter was incubated with 80,000 cpm of ^{32}P-labeled EBV HR-1 DNA (slot 5). The other half of the filter was incubated with 45,000 cpm of the residual single-stranded ^{32}P-labeled EBV HR-1 DNA from which sequences homologous to EBV B95-8 DNA had been removed (slot 4).*

mide-stained gels from which the blots were made. In contrast, the B95-8-absorbed ^{32}P-labeled EBV HR-1 DNA hybridized to some fragments and not to other fragments which were present in equal or higher abundance.

To demonstrate that at least some of the HR-1 DNA which is missing from the B95-8 strain is present in another strain of EBV, the ^{32}P-labeled EBV HR-1 DNA from which sequences common to the B95-8 strain had been removed was hybridized to filters containing *Eco*RI or *Hsu* I restriction enzyme fragments of EBV W91 DNA. The results indicated that at least some of the HR-1-specific sequences are contained in EBV W91 DNA *Eco*RI fragment C (Fig. 3) and in *Hsu* I fragments D and E (Fig. 4). Some hybridization was also seen to *Eco*RI fragments A and possibly also B (Fig. 3). However, because fragments A and B are the largest fragments and the most efficient in hybridization to ^{32}P-labeled EBV HR-1 DNA, it is possible that the small amount of hybridization observed to fragments A and B in Fig. 3 is an artifact of incomplete removal of ^{32}P-labeled EBV HR-1 DNA sequences homologous to B95-8 DNA.

To confirm that EBV W91 DNA contains DNA not present in the B95-8 strain, EBV W91 DNA was labeled in vitro, incubated twice with EBV DNA contained in B95-8 cell DNA, and chromatographed after each hybridization on a column of hydroxyapatite to remove double-stranded DNA. The residual single-stranded DNA hybridized specifically to EBV W91 DNA *Hsu* I fragment E (Fig. 5).

Kinetic and absorptive hybridization experiments were employed to determine whether EBV HR-1 DNA contained sequences absent from the W91 strain. The results were as follows. (i) Analysis of the kinetics of hybridization of ^{32}P-labeled EBV HR-1 DNA in the presence of excess unlabeled W91 DNA (Fig. 6) indicated that W91 DNA contains more than 90% (94 ± 6%, mean ± standard deviation of five separate kinetic experiments) of the sequences of EBV HR-1 DNA. (ii) After two cycles of hybridization of ^{32}P-labeled EBV HR-1 DNA with a 50-fold excess of EBV DNA in W91 cell DNA and removal of the homologous DNA on hydroxyapatite, the residual single-stranded ^{32}P-labeled EBV HR-1 DNA (approximately 1% of the starting counts) possessed equal homology to each of the separated restriction enzyme fragments of EBV HR-1 DNA. This result would be expected either if sequences homologous to W91 DNA had been incompletely removed or if small regions of nonhomology were distributed equally among all of the fragments.

Restriction enzyme fragments of EBV B95-8 and W91 DNAs which contain DNA not present in the HR-1 strains. To determine whether EBV B95-8 DNA contains sequences not present in the DNA of the HR-1 strain and to identify which restriction enzyme fragments of EBV B95-8 DNA contain these

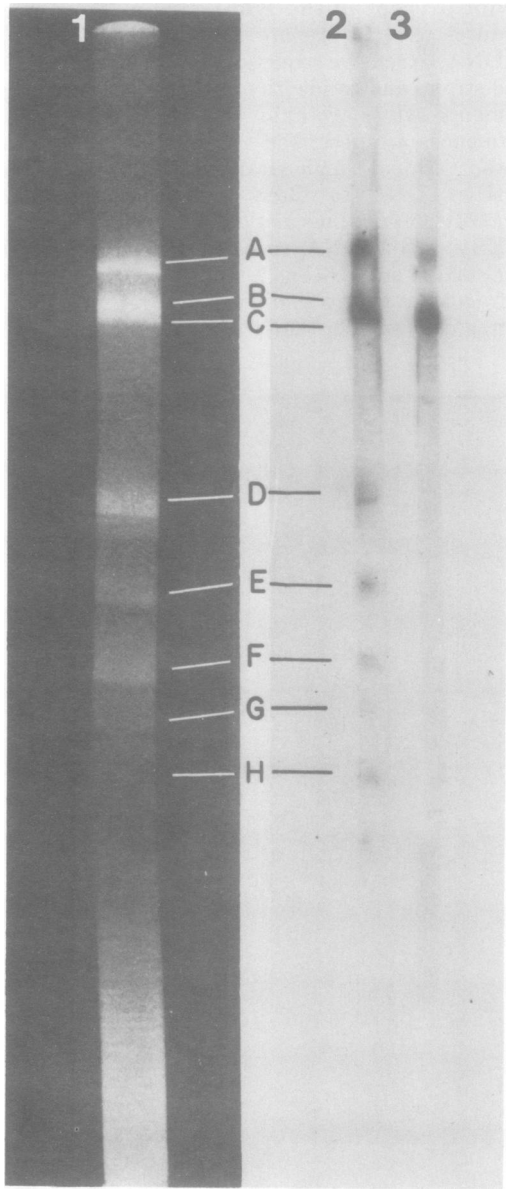

FIG. 3. *Identification of EcoRI fragments of EBV W91 DNA which contain DNA sequences homologous to EBV HR-1 DNA sequences not present in the DNA of the B95-8 strain. EBV W91 DNA EcoRI fragments (slot 1) were transferred onto a nitrocellulose filter. One half of the filter was incubated with 30,000 cpm of ^{32}P-labeled EBV HR-1 DNA (slot 2). The other half of the filter was incubated with 45,000 cpm of the residual single-stranded ^{32}P-labeled EBV HR-1 DNA from which sequences homologous to EBV B95-8 DNA had been removed (slot 3).*

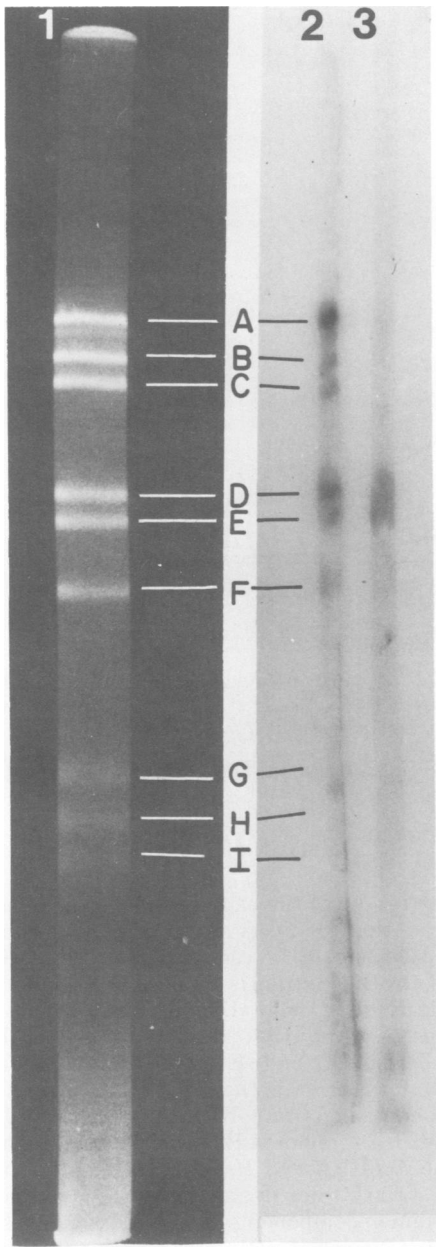

FIG. 4. *Identification of Hsu I fragments of EBV W91 DNA which contain DNA sequences homologous to the EBV HR-1 DNA sequences not present in the DNA of the B95-8 strain. EBV W91 DNA Hsu I fragments (slot 1) were transferred onto nitrocellulose filters. One half of the filter was incubated with 30,000 cpm of ^{32}P-labeled EBV HR-1 DNA (slot 2). The other half of the filter was incubated with 45,000 cpm of the residual single-stranded ^{32}P-labeled EBV HR-1 DNA from which sequences homologous to EBV B95-8 DNA had been removed (slot 3).*

sequences, EBV B95-8 DNA was labeled in vitro and subjected to two cycles of hybridization with 50- to 100-fold excess of EBV DNA from HR-1 cells. Approximately 2% of the starting EBV B95-8 DNA eluted as single-stranded DNA from the first hydroxyapatite column. Incubation of the labeled single-stranded DNA with additional HR-1 DNA failed to increase the rate of hybrid-

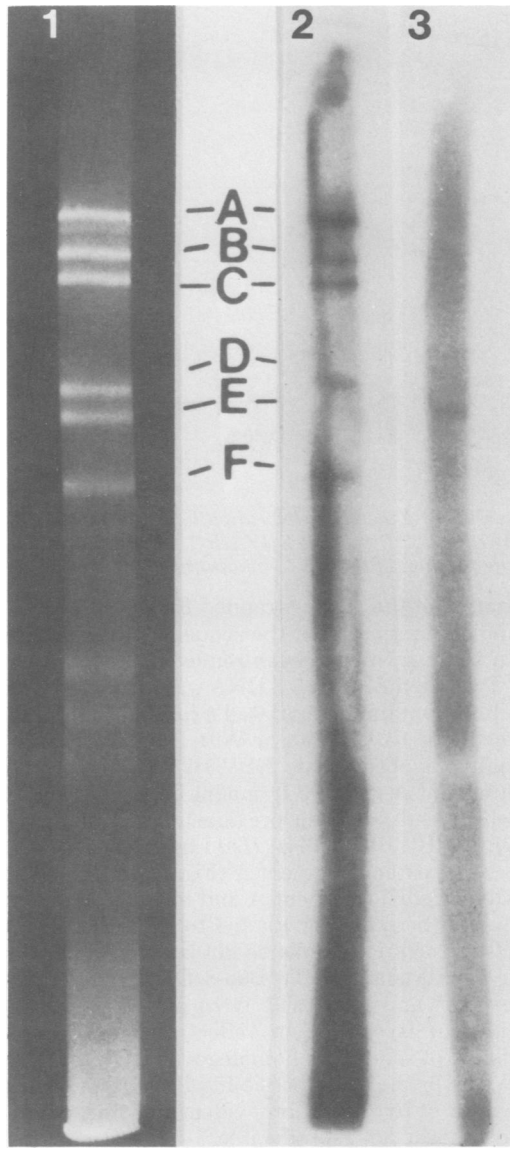

FIG. 5. *Identification of the Hsu I fragments of EBV W91 DNA which contain sequences not present in the DNA of the B95-8 strain. EBV W91 DNA Hsu I fragments (slot 1) were transferred onto a nitrocellulose filter. The filter was incubated with 100,000 cpm of ^{32}P-labeled EBV W91 DNA from which those sequences homologous to EBV B95-8 DNA had been removed and exposed to X-ray film with a Cronex screen for 3 days (slot 3). A matching filter was incubated with 30,000 cpm of ^{32}P-labeled EBV B95-8 and exposed to X-ray film with a Cronex screen for 3 days (slot 2).*

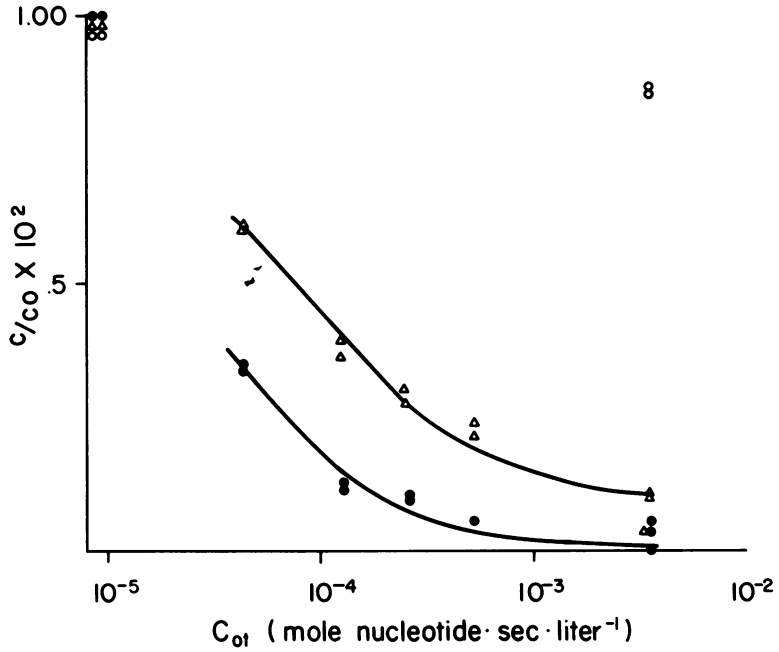

FIG. 6. *Kinetics of hybridization of ^{32}P-labeled EBV HR-1 DNA with EBV homologous DNA in HR-1 or W91 cells. ^{32}P-labeled EBV HR-1 DNA was incubated with HR-1 (●), W91 (△), or calf thymus DNA (○) and the fraction of the DNA remaining single-stranded was determined as described in the text.*

ization of the single-stranded EBV B95-8 DNA, indicating that all of the sequences homologous to HR-1 DNA had been removed. The residual ^{32}P-labeled EBV B95-8 DNA was incubated with filters containing separated *Eco*RI or *Hsu* I fragments of EBV B95-8 or W91 DNA. Hybridization was observed to EBV B95-8 DNA *Eco*RI fragment A and to a fragment between J and K which had not been previously identified (Fig. 7); to EBV B95-8 DNA *Hsu* I fragment B and to a lesser extent fragment A (Fig. 8); to EBV W91 DNA *Eco*RI fragment A and to lesser extent B and D (hetero) (Fig. 9); and to EBV W91 DNA *Hsu* I fragment B. We do not know whether the hybridization to EBV B95-8 DNA *Hsu* I fragment A and EBV W91 DNA *Eco*RI-B and -D (hetero) is specific or reflects incomplete removal of sequences homologous to HR-1 DNA. We favor the latter possibility because the small extent of hybridization evident in some experiments was not reproducible.

To confirm that EBV W91 DNA contains DNA not present in the HR-1 strain, ^{32}P-labeled EBV W91 DNA was twice hybridized to 50- to 100-fold excess of EBV DNA in HR-1 cell DNA and chromatographed on hydroxyapatite to remove double-stranded DNA each time. The residual single-stranded DNA which contained less than 2% of the initial ^{32}P-labeled EBV W91 DNA was incubated with a filter containing fragments of EBV B95-8 DNA. The autoradiogram (Fig. 10) revealed hybridization to EBV B95-8 *Hsu* I fragment B and to a lesser extent to fragment E.

DISCUSSION

The results of the experiments reported here indicate that the DNA of the HR-1, B95-8, and W91 strains of EBV each differ in complexity. Thus, the B95-8 strain is missing DNA contained in the *Eco*RI-C and -D and *Hsu* I-E and -N fragments of the HR-1 strain and in the *Eco*RI-C and *Hsu* I-D and -E fragments of the W91 strain. Furthermore, the HR-1 strain is missing DNA contained in *Eco*RI fragments A and J to K and *Hsu* I fragment B of the B95-8 strain and in the *Eco*RI-A and *Hsu* I-B fragments of the W91 strain. Other fragments which could contain minor components of DNAs which differ between strains of EBV were identified in some experiments but not in others. The data available to date suggest that the W91 strain contains all of the sequences present in both the B95-8 and HR-1 strains.

In another series of experiments (Kieff et al., *in* Rapp and de-The, *Oncogenesis and Herpesviruses III*, in press; Given and Kieff, submitted for publication), a linkage map of the restriction enzyme fragments of the DNA of the B95-8 and W91 strains was derived by hybridizing labeled fragments produced by treating EBV DNA with one restriction enzyme with filters containing separated products of another restriction en-

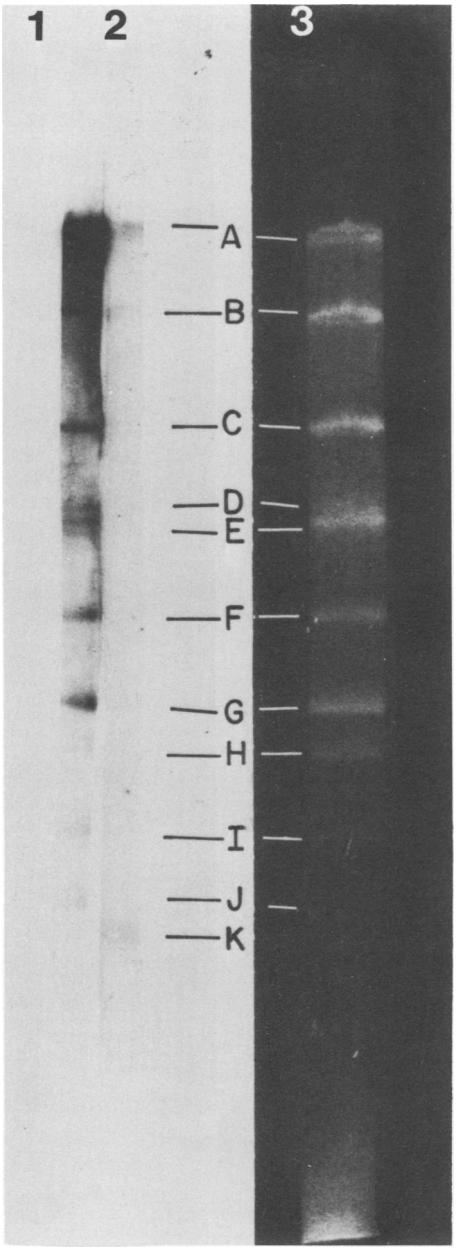

FIG. 7. *Identification of EcoRI fragments of EBV B95-8 DNA which contain DNA sequences not present in DNA of the HR-1 strain. EBV B95-8 DNA fragments (slot 3) were transferred onto a nitrocellulose filter. One half of the filter was incubated with 20,000 cpm of ^{32}P-labeled EBV B95-8 DNA (slot 1). The other half of the filter was incubated with 25,000 cpm of the residual single-stranded ^{32}P-labeled EBV B95-8 DNA from which those sequences homologous to EBV HR-1 DNA had been removed (slot 2).*

zyme. Two points should be made about the relationship of the two sets of data. (i) The mapping data and the comparative size of the fragments of EBV B95-8 and W91 DNAs indicate that the major difference in size and linkage of the fragments of EBV B95-8 and (W91) DNAs is that the B95 *Eco*RI-C fragment is approximately 8 megadaltons smaller than the homologous *Eco*RI-C fragment of the W91 strain and that the *Hsu* I-D fragment of the B95-8 strain is approximately 8 megadaltons smaller than the

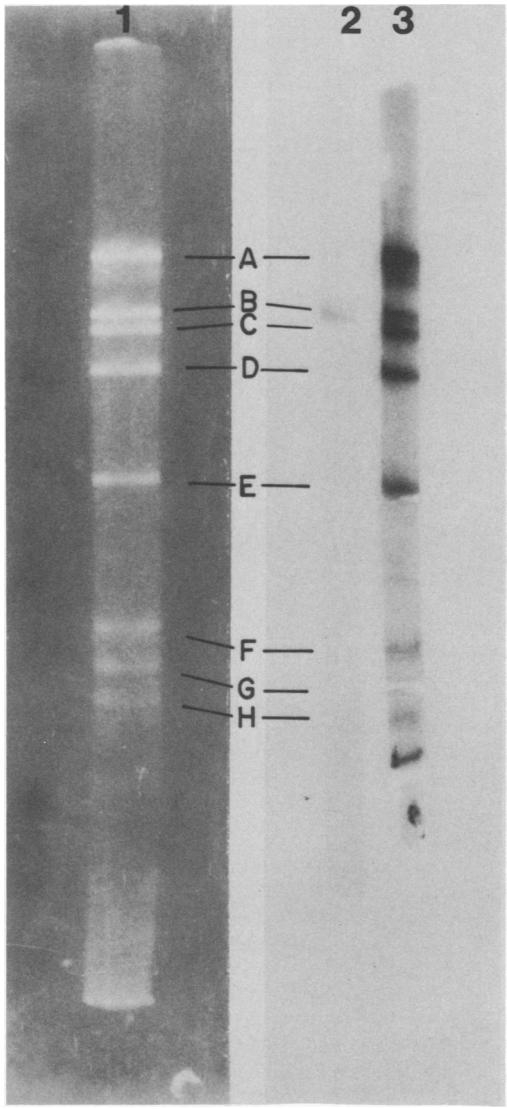

FIG. 8. *Identification of Hsu I fragments of EBV B95-8 DNA which contain DNA sequences not present in DNA of the HR-1 strain. EBV B95-8 DNA Hsu I fragments (slot 1) were transferred onto a nitrocellulose filter. One half of the filter was incubated with 20,000 cpm of ^{32}P-labeled B95-8 DNA (slot 3). The other half of the filter was incubated with 20,000 cpm of the residual single-stranded ^{32}P-labeled EBV B95-8 DNA from which those sequences homologous to EBV (HR-1) DNA had been removed (slot 2).*

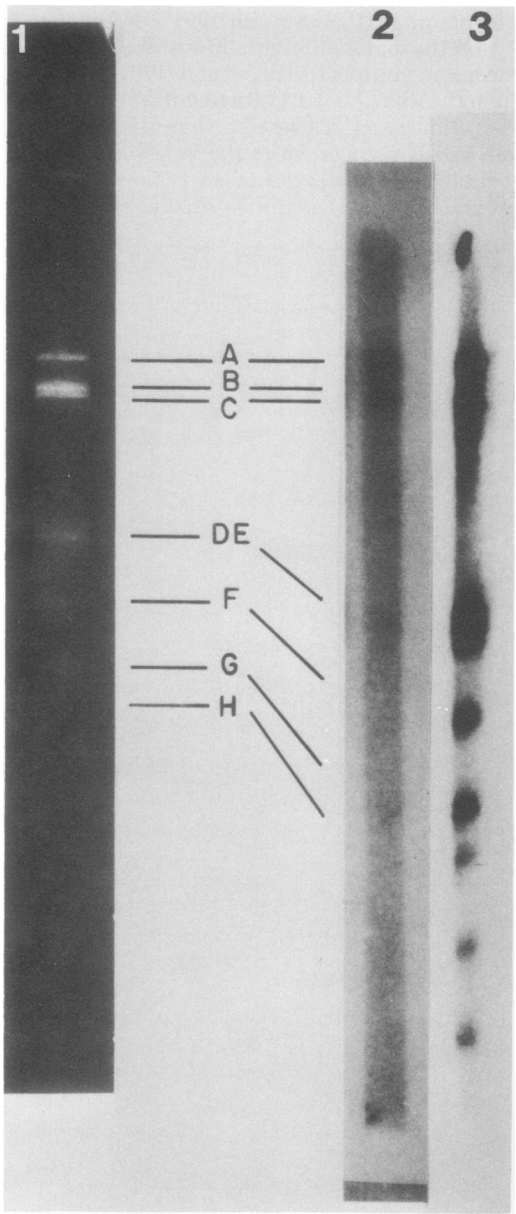

FIG. 9. *Identification of EcoRI fragment(s) of EBV (W91) DNA which contain DNA homologous to EBV B95-8 DNA sequences not present in the DNA of the HR-1 strain. EBV W91 DNA EcoRI fragments (slot 1) were transferred onto a nitrocellulose filter. One half of the filter was incubated with 140,000 cpm of ^{32}P-labeled EBV B95-8 DNA (slot 3). The other half was incubated with 190,000 cpm of the residual single-stranded ^{32}P-labeled EBV B95-8 DNA from which those sequences homologous to EBV HR-1 DNA had been removed (slot 2).*

two fragments (termed D and E in this study and D1 and D2 in Given and Kieff [submitted for publication]) of the W91 strain. It is apparent from the absorptive hybridization data reported here that both the HR-1 and the W91 strains contain DNA sequences not present in the B95-8 strain which hybridize to the *Eco*RI-C and *Hsu* I-D and -E fragments of W91 DNA. The sequences deleted from the B95-8 strain must

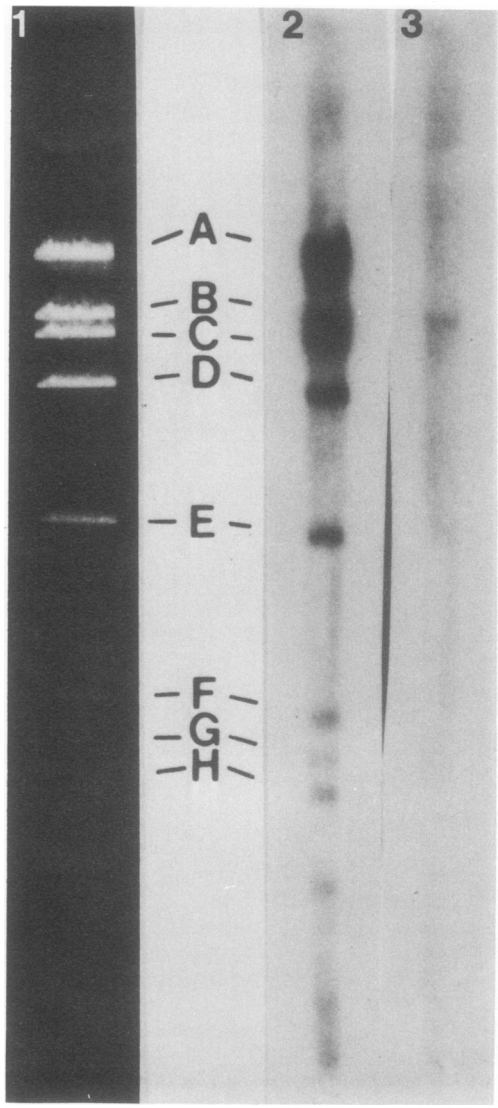

FIG. 10. *Identification of the Hsu I fragments of EBV B95-8 DNA which contain DNA homologous to EBV W91 DNA sequences not present in the DNA of the HR-1 strain. EBV B95-8 DNA Hsu I fragments (slot 1) were transferred onto a nitrocellulose filter. The filter was incubated with 165,000 cpm of ^{32}P-labeled EBV W91 DNA from which sequences homologous to EBV HR-1 DNA had been removed and exposed to X-ray film with a Cronex screen for 3 days (slot 3). The filter was then incubated with 400,000 cpm of ^{32}P-labeled EBV W91 DNA and exposed with X-ray film with a Cronex screen for 12 h (slot 2).*

not be necessary for in vitro transformation of lymphocytes to lymphoblasts or for partially permissive replication of EBV in lymphoblast cultures in vitro because the B95-8 strain possesses these two phenotypic properties (16–20). (ii) Labeled EBV B95-8 or W91 DNA, from which sequences homologous to the HR-1 strain had been removed, consistently identified the *Eco*RI fragment A and *Hsu* I fragment B of the B95-8 and W91 strains as fragments which contain DNA missing from the HR-1 strain. The maps of B95-8 and W91 DNAs are colinear in this region. The additional DNA which these strains contain is therefore in the region of overlap between *Eco*RI fragment A and *Hsu* I fragment B of EBV B95-8 and W91 DNAs, i.e., between 22 and 37 megadaltons from the left end of their maps.

Although we have no direct evidence that the DNA deleted in the HR-1 strain accounts for the inability of this strain of virus to transform lymphocytes to lymphoblasts with indefinite growth in vitro, several indirect lines of evidence favor this hypothesis. (i) This DNA is present in both the B95-8 and W91 transformation-positive strains of virus. (ii) Radiation of HR-1 virus with UV light or X-rays does not uncover latent transforming potential, suggesting that the failure of this strain of virus to transform is related to the lack of DNA necessary for transformation (7). (iii) Virus-specific RNA which is processed in lymphocytes restringently infected with EBV (5, 23) is encoded largely by DNA contained in the *Eco*RI-A fragment of B95-8 DNA (W. King, A. Powell, and E. Kieff, manuscript in preparation).

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LITERATURE CITED

1. **Dolyniuk, M., R. Pritchett, and E. Kieff.** 1976. Proteins of Epstein-Barr virus. I. Analysis of the polypeptides of purified enveloped Epstein-Barr virus. J. Virol. **17**:935–949.
2. **Gerber, P.** 1973. Oral excretion of Epstein-Barr virus. Lancet **i**:1001.
3. **Gerber, P., F. Nkrumah, R. Pritchett, and E. Kieff.** 1976. Comparative studies of Epstein-Barr virus strains from Ghana and the United States. Int. J. Cancer **17**:71–81.
4. **Gerber, P., J. Whang-Peng, and J. H. Monroe.** 1969. Transformation and chromosome changes induced by Epstein-Barr virus in normal human leukocyte cultures. Proc. Natl. Acad. Sci. U.S.A. **63**:740–747.
5. **Hayward, S. D., and E. Kieff.** 1976. Epstein-Barr virus-specific RNA. I. Analysis of viral RNA in cellular extracts and in the polyribosomal fraction of permissive and nonpermissive lymphoblastoid cell lines. J. Virol. **18**:518–525.
6. **Hayward, S. D., and E. Kieff.** 1977. The DNA of Epstein-Barr virus. II. Comparison of the molecular weights of restriction endonuclease fragments of the DNA of strains of Epstein-Barr virus and identification of end fragments of the B95-8 strain. J. Virol. **23**:421–429.
7. **Henderson, E., L. Heston, E. Grogan, and G. Miller.** 1978. Radiobiological inactivation of Epstein-Barr virus. J. Virol. **25**:51–59.
8. **Henle, W., V. Diehl, G. Kohn, H. zur Hausen, and G. Henle.** 1967. Herpes-type virus and chromosome marker in normal leukocytes after growth with irradiated Burkitt cells. Science **157**:1064–1065.
9. **Henle, W., G. Henle, B. 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.
10. **Hinuma, Y., M. Konn, J. Yamaguchi, D. Wudarski, J. Blakeslee, and J. Grace.** 1967. Immunofluorescence and herpes type virus particles in the ^{32}P HR-1 Burkitt lymphoma clone. J. Virol. **1**:1045–1051.
11. **Jondal, M., and G. Klein.** 1973. Surface markers on human B and T lymphocytes. II. Presence of Epstein-Barr virus receptors on B lymphocytes. J. Exp. Med. **138**:1365–1378.
12. **Kelly, R. B., N. R. Cozzarelli, M. P. Deutscher, I. R. Lehman, and A. Kornberg.** 1970. Enzymatic synthesis of deoxyribonucleic acid. J. Biol. Chem. **245**:39–45.
13. **Kieff, E., and J. Levine.** 1974. Homology between Burkitt herpes viral DNA and DNA in continuous lymphoblastoid cells from patients with infectious mononucleosis. Proc. Natl. Acad. Sci. U.S.A. **71**:355–358.
14. **Klein, G.** 1971. Immunologic aspects of Burkitt's lymphoma. Adv. Immunol. **14**:187–250.
15. **Klein, G.** 1973. The Epstein-Barr virus, p. 521–555. *In* A. S. Kaplan (ed.), The herpesviruses. Academic Press, New York.
16. **Marmur, J.** A procedure for isolation of DNA from microorganisms. Methods Enzymol. **6**:726–738.
17. **Menezes, J., W. Leibold, and G. Klein.** 1975. Biological differences between different Epstein-Barr virus (EBV) strains with regard to lymphocyte transforming ability. Exp. Cell Res. **92**:478–484.
18. **Miller, G., D. Coope, J. Niederman, and J. Pagano.** 1976. Biological properties and viral surface antigens of Burkitt lymphoma and mononucleosis-derived strains of Epstein-Barr virus released from transformed marmoset cells. J. Virol. **18**:1071–1080.
19. **Miller, G., and M. Lipman.** 1973. Comparison of the yield of infectious virus from clones of human and simian lymphoblastoid lines transformed by EBV. J. Exp. Med. **138**:1398–1412.
20. **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. U.S.A. **71**:4006–4010.
21. **Miller, G., T. Shope, H. Lisco, D. Still, and M. Lipman.** 1972. Epstein-Barr virus: Transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leukocytes. Proc. Natl. Acad. Sci. U.S.A. **69**:383–387.
22. **Nilsson, K.** 1971. High frequency establishment of human immunoglobulin-producing lymphoblastoid lines from normal and malignant lymphoid tissue and peripheral blood. Int. J. Cancer **8**:432–442.
23. **Orellana, T., and E. Kieff.** 1977. Epstein-Barr virus-specific RNA. II. Analysis of polyadenylated viral RNA in restringent, abortive, and productive infections. J. Virol. **22**:321–330.
24. **Pattengale, P. K., R. W. Smith, and P. Gerber.** 1973.

Selective transformation of B lymphocytes by EB virus. Lancet ii:93–94.

25. **Pritchett, R. F., S. D. Hayward, and E. Kieff.** 1975. DNA of Epstein-Barr virus. I. Comparison of DNA of virus purified from HR-1 and B95-8 cells: size, structure, and relatedness. J. Virol. **15**:556–569.
26. **Rigby, P., M. Diechmann, C. Rhodes, and P. Berg.** 1977. Labeling DNA to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–252.
27. **Roizman, B., and E. Kieff.** 1975. Herpes simplex and Epstein-Barr viruses in human cells and tissues: a study in contrasts, p. 241–322. *In* F. F. Becker (ed.), Cancer: a comprehensive treatise, vol. 2. Plenum Publishing Corp., New York.
28. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
29. **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.