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Homology Between Burkitt Herpes Viral DNA and DNA in Continuous Lymphoblastoid Cells from Patients with Infectious Mononucleosis

(DNA·DNA reassociation/Epstein–Barr virus/DNA/matching base pairs)

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ABSTRACT **At least 90% of the sequences of purified, *in vitro* labeled, DNA from Epstein–Barr virus (prepared from HR-1, Burkitt's lymphoblastoid cells) are homologous to the DNA of the herpes virus contained in cell lines derived from patients with infectious mononucleosis. The thermal stability of the homologous and heterologous hybrid DNA molecules could not be differentiated, indicating at least 97% matching of base pairs between DNA of Epstein–Barr virus and the herpes viral DNA contained in the lymphoblasts from patients with infectious mononucleosis.**

In 1964, a herpes virus, subsequently designated Epstein–Barr virus (EBV), was identified in continuous lymphoblastoid cell cultures established from biopsy tissue obtained from patients with Burkitt's lymphoma (BL) (1). EBV antigens have been demonstrated reproducibly in biopsy tissues (3, 4) and in continuous cultures of lymphoblastoid cells derived from BL tumor biopsies (2), suggesting that the virus is etiologically related to Burkitt's tumor. An antigenically indistinguishable herpes virus has been found in lymphoblastoid cell cultures established from patients with infectious mononucleosis (IM) (5). Epidemiologic studies have demonstrated that this herpes virus is the causative agent of classical, heterophile positive, IM (6). Serologically, EBV cannot be differentiated from the agent associated with IM. Nevertheless, the unity of these two agents remains an issue particularly in light of the fact that: (*i*) differences in the surface properties of lymphoblastoid cell cultures established from patients with BL and IM have been reported (7), and (*ii*) herpes simplex viruses differing in epidemiology and pathogenicity can be segregated into two distinct groups using more discriminatory immunologic analyses and by studies of DNA·-DNA homology (8). The purpose of the studies described in this report is to determine: (*i*) the percentage of the EBV genome complementary to the genome of the herpes virus present in lymphoblastoid cells derived from patients with IM, and (*ii*) the extent of homology between EBV DNA and the sequences complementary to this DNA present in continuous lymphoblastoid cultures from patients with IM.

MATERIALS AND METHODS

Cells. The Kaplan and Ditzel continuous lymphoblastoid cell cultures established from patients with IM (9) were maintained in RPMI-1640 medium supplemented with 10% fetal-calf serum. The HR-1 lymphoblastoid cell line established from a Burkitt tumor biopsy (10) was maintained in McCoy's medium supplemented with 8% fetal-calf serum. Cultures were incubated at 37°.

Purification of Cell DNAs. DNA was extracted and purified from Kaplan and Ditzel and HR-1 continuous lymphoblastoid cell lines and from adult human liver and neonatal thymic tissue as described (11). Highly polymerized calf-thymus DNA was obtained from Worthington Biochemical Corp.

Purification of EBV DNA. Cultures of HR-1 cells were maintained in spent medium at 32° for 10 days. The extracellular fluid was adjusted to 5% (w/v) polyethylene glycol 6000 and 0.5 M sodium chloride. After 24 hr at 4° the resultant sediment was separated by centrifugation at 6000 × *g* for 10 min at 4°. The pellet was resuspended in 0.05 M sodium phosphate buffer, pH 6.8 (phosphate buffer), and homogenized with 30 strokes of a tight-fitting Dounce homogenizer. The conditions used for purification of herpes viral nucleocapsids on sucrose velocity gradients and for extraction and purification of herpes viral DNA on neutral sucrose velocity gradients have been described (12). Nucleic acid banding at 52–62 S on neutral sucrose velocity gradients was subjected to equilibrium centrifugation in neutral cesium chloride, and DNA banding at a density of 1.716–1.720 $g \cdot cm^{-3}$ was obtained. A preparation yielding a single homogeneous band at 1.718 $g \cdot cm^{-3}$ on centrifugation in the analytical centrifuge (model E, Spinco Corp.) served as a substrate for *in vitro* labeling.

In Vitro Labeling of EBV DNA. Purified EBV DNA was labeled with [^{3}H]thymidine triphosphate, 46 Ci/mmol (New England Nuclear Corp.), using polymerase I of *Escherichia coli* (13, 14). The resultant EBV DNA had a specific activity of 5×10^6 $cpm \cdot \mu g^{-1}$. The DNA was stored at 4° until use. All experiments were performed within 2 weeks of preparation of the labeled DNA.

Reassociation Kinetics. Less than 2×10^{-3} μg of labeled DNA was mixed with 500 μg of cell DNA, sonicated for 1 min at 4° and 30-W output with an Artek sonifier with microprobe, denatured in 0.15 N NaON, neutralized with 2 M phosphate buffer and incubated for various times at 64° in 0.5 M phosphate buffer. Single-stranded DNAs were separated from double-stranded DNAs by chromatography on hydroxyapatite gel (Biogel HTP) at room temperature. DNAs were adsorbed to hydroxyapatite (HAP) in 0.04 M phosphate buffer. Single-stranded DNA was eluted with 0.18 M phosphate buffer and double-stranded DNA with 0.4 M phosphate

Abbreviations: Burkitt lymphoma, BL; HSV, herpes simplex virus; infectious mononucleosis, IM; Epstein–Barr virus, EBV; hydroxyapatite gel, HAP; C_0t, product of concentration of nucleotide sequences of DNA and time of incubation.

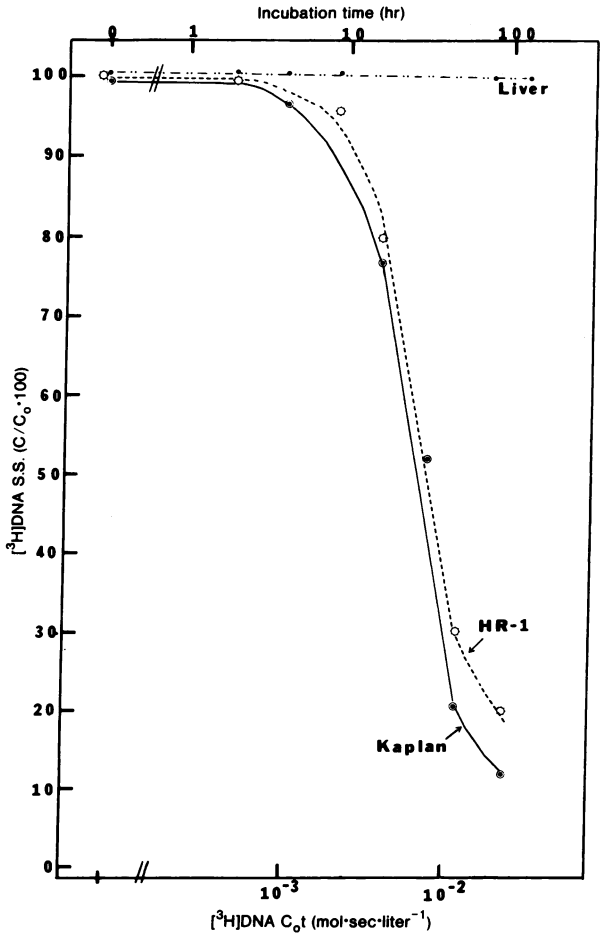

FIG. 1. Renaturation of EBV [^{3}H]DNA in the presence of 2.5×10^5-fold excess of unlabeled human-liver DNA (●); HR-1 BL lymphoblastoid-cell DNA (○); or Kaplan IM lymphoblastoid-cell DNA (⊙). Conditions of incubation and for separation of single- and double-stranded nucleic acid are described in *Methods*. Duration of incubation is indicated in the *upper abscissa*. The corresponding C_0t for input labeled EBV DNA is indicated in the *lower abscissa*. The concentration of EBV homologous sequences in the various cell DNAs was determined from the equations $C_0 = C_0t_{50}$ (14)$/t_{50}$ observed, after correction for Na^+. The number of genome equivalents was calculated by dividing the number of EBV homologous DNA molecules by the number of molecules of cell DNA in the reaction mixture. The values of 1×10^8 for the molecular weight of EBV DNA and 6×10^{12} for the molecular weight of lymphoblastoid cell DNA (17) were used in these calculations. *S.S.*, single-stranded.

buffer. All studies were done with a single lot of HAP, which was extensively pretested under the conditions used.

Thermal Chromatography. The procedures for chromatography and thermal elution from HAP have been described (15). Columns containing 1 g of HAP(2 ml of scrbent) were used. Fractions consisting of 6 column volumes were collected every 3 min.

RESULTS

The extent to which EBV DNA sequences are represented by complementary DNA sequences in lymphoblastoid cultures established from patients with IM was determined by comparison of the kinetics of reassociation of labeled EBV DNA in the preseence of excess HR-1 or Kaplan-cell DNA. In parallel experiments, labeled EBV DNA was reassociated in the presence of adult human-liver, calf-thymus, or neonatal human-thymus DNA, to control the effect of cell DNA on the viscosity of the reaction mixture.

The results shown in Fig. 1 are typical of several series of experiments which indicated the following:

(*i*) Under the conditions of low (less than 2×10^{-3} μg) input EBV [^{3}H]DNA used in these experiments, there was no detectable reassociation of the labeled DNA in the presence of human liver-cell (Fig. 1), neonatal human-thymus, or calf-thymus DNAs.

(*ii*) The HR-1 Burkitt's tumor lymphoblastoid cultures maintained under nonpermissive conditions (37° with frequent feeding) contain sequences complementary to greater than 85% of the EBV genome (Fig. 1). The reassociation of DNA molecules follows the equation $C/C_0 = 1/1 + kC_0t$, where C_0 is the initial concentration of denatured DNA, C is the concentration of denatured DNA remaining at each time t, and k is the reassociation constant characteristic of each DNA molecule. At 50% reassociation, this equation can be reduced to $C_0t_{50} = 1/k$. The C_0t_{50} for sonicated EBV DNA on HAP has been found to be 0.14 mol·sec·liter^{-1} under the conditions used here (14), in good agreement with the value for herpes simplex viral DNA (16). From the known C_0t_{50} and the experimentally determined time at which 50% of denatured DNA reassociates, the initial EBV homologous DNA concentration, C_0, can be calculated. After correction to standard salt concentration (0.15 M Na^+), the time necessary to permit 50% of the labeled EBV DNA to reanneal in the presence of HR-1 cell DNA was determined to be 140 hr. From this value we estimate that the HR-1 cells that do not produce virus contain about 12 genome equivalents per cell. Our determinations of genome equivalents is somewhat inexact since we did not possess a sufficient quantity of EBV [^{3}H]DNA to observe the reassociation of EBV [^{3}H]DNA alone.

(*iii*) DNA extracted from the Kaplan lymphoblastoid-cell line was fully complementary to EBV DNA to the extent that the reassociation could be followed in these experiments (90%) (Fig. 1). After correction to standard salt concentration, the time necessary to permit 50% of the labeled EBV DNA to reanneal in the presence of Kaplan-cell DNA was 120 hr. We estimate from this that the Kaplan-cell DNA contains approximately 14 genome equivalents of EBV DNA per cell. In two experiments in which EBV [^{3}H]DNA was reassociated in the presence of Ditzel IM cell DNA, renaturation was followed to 60% and was found to parallel the reassociation of EBV [^{3}H]DNA with HR-1 DNA.

The extent of heterogeneity between the nucleotide sequences of EBV DNA and the complementary nucleotide sequences in IM lymphoblastoid-cell DNA was evaluated by determination of the thermal stability of reassociated heteroduplex DNA. The conditions for reassociation of EBV [^{3}H]-DNA with Kaplan or HR-1 cell DNAs were as described in *Methods*. The reassociation reaction was continued for sufficient time to permit the annealing of 85% of the input EBV [^{3}H]DNA with complementary cell DNAs. The results shown in Fig. 2 indicate that (*i*) heteroduplex DNA (EBV [^{3}H]DNA·Kaplan DNA, Fig. 2*A* and *C*) eluted as a single sharp peak at 80° with a TE_{50} (temperature at which 50% of [^{3}H]DNA eluted) of 79°; (*ii*) the major fraction of homoduplex DNA EBV [^{3}H]DNA·HR-1 DNA, Fig. 2*B* and *C*) eluted at 79° with a TE_{50} of 78–79°. The amount of duplex DNA eluting between 60 and 70° varied, but did not exceed 20% of the total duplex DNA.

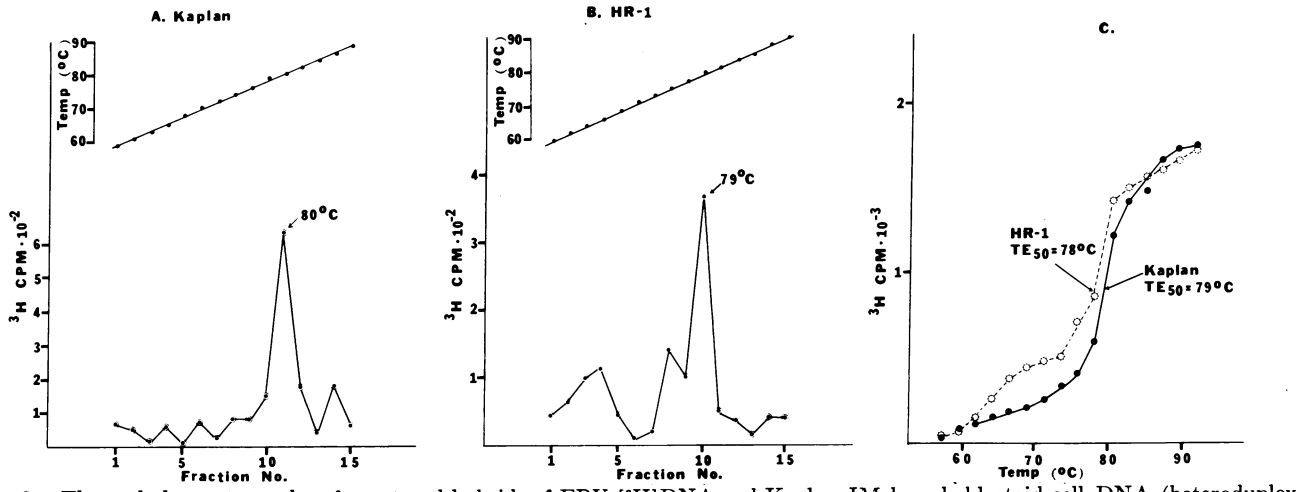

FIG. 2. Thermal chromatography of renatured hybrids of EBV [^{3}H]DNA and Kaplan IM lymphoblastoid-cell DNA (heteroduplex DNA; *A*); and of hybrids of EBV [^{3}H]DNA and HR-1 BL lymphoblastoid-cell DNA (homoduplex DNA; *B*). Fractions eluted from the column were precipitated onto Millipore HAWP filters by addition of 100% trichloroacetic acid to a final concentration of 5% and assayed in a Beckman LS230 liquid scintillation counter. The cumulative data for *A* and *B* are plotted in *C*.

DISCUSSION

Previous studies of EBV purified from "high-producing" lymphoblastoid cultures established from biopsies of Burkitt's tumor demonstrated that (*i*) EBV DNA is a double-stranded molecule of about 10^8 daltons (17); (*ii*) EBV DNA banded at 1.718 g·cm^{-3} in neutral CsCl, corresponding to 57 guanine plus cytosine moles percent (18); (*iii*) the C_0t_{50} of EBV DNA was found to be 0.14 mol·liter·sec^{-1} (14). Multiple copies of the EBV genome have been found in producing as well as non-virus-producing Burkitt's lymphoblastoid cultures (14, 19) in biopsy material of Burkitt's tumor (20), and in biopsies of patients with nasopharyngeal carcinoma (14, 20). The immunologically closely related herpes virus of infectious mononucleosis shares with EBV the important biological property of capacity to transform normal human lymphocytes *in vitro* (21). Attempts to purify the herpes virus from IM lymphoblasts have been unrewarding.

Our studies evaluate the extent of genetic relatedness between EBV and the closely related IM herpes virus using whole-cell DNA and purified EBV DNA labeled *in vitro* as test reagents. The reassociation reaction of IM lymphoblast DNA with EBV [^{3}H]DNA was found to parallel in rate the homologous HR-1·EBV [^{3}H]DNA reassociation at the point at which 90% of the labeled EBV DNA had reannealed. We conclude from this that more than 90% of the genome of EBV is capable of base pairing with the DNA of the herpes virus identified in IM. Heteroduplex DNA(EBV·Kaplan cell DNA duplexes) eluted from HAP at 79°–80°, as expected for DNA of 57 guanine plus cytosine moles percent. Similar studies of the herpes simplex viruses (HSV) had demonstrated that 50% of the genome of HSV-1 was not capable of annealing to HSV-2 (15). Complementary sequences between HSV-1 and HSV-2 had a high degree (15%) of unmatched base pairs, as evidenced by lowered thermal stability (15). In contrast to the earlier studies of herpes simplex viruses (15), we have been unable to demonstrate any differnces between EBV DNA and the DNA of the herpes virus associated with IM.

We conclude from our data that the extent of homology between EBV DNA and the DNA of herpes virus present in IM lymphoblasts is at least 90% and that the extent of base pairing between the homologous sequences is at least 97%. We have limited the extent to which differences in viral genome could explain the disease states. One alternative hypothesis is that factors unrelated to the virus may determine the outcome of infection.

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