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Reassociation Kinetics for Epstein-Barr Virus DNA: Nonhomology to Mammalian DNA and Homology of Viral DNA in Various Diseases

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The number of Epstein-Barr virus (EBV) genomes per cell in established leukocytic lines and tissue specimens has been evaluated by measuring DNA-DNA reassociation kinetics with hydroxyapatite chromatography. Under the proper conditions, this method is sufficiently sensitive to detect EBV DNA in the amount of 0.1 genome per cell. All the samples tested that have been suspected to be without EBV DNA by cRNA hybridization proved negative by this more sensitive specific analysis. These included Hela and Hep2 cells, a negative case of Burkitt's lymphoma, two negative cases of nasopharyngeal carcinoma, and two established human leukocytic lines. Homology tests conducted with single-strand-specific nuclease S1 indicated that the viral DNA from a nasopharyngeal carcinoma and infectious mononucleosis were more than 90% homologous to EBV DNA.

ZurHausen and his collaborators originally detected Epstein-Barr virus (EBV) DNA in various cells (7) and tumor tissues (8) by DNA-DNA membrane hybridization. We confirmed and extended their results by the more sensitive cRNA-DNA hybridization method (M. Nonoyama, C.-H. Huang, J. S. Pagano, G. Klein, and S. Singh, Proc. Nat. Acad Sci. U.S.A., in press; reference 12). In those papers Hela or Hep2 cells were used as EBV DNA-negative cell controls, but the negativity of these human cells had not actually been proved. In addition, the cRNA hybridization method cannot detect more than two genome equivalents of EBV DNA per cell against the background of results with Hela or Hep2 cells, and important questions about their negativity for the EBV genome have remained unanswered. Fresh tissue from one case of Burkitt's lymphoma and five cases of nasopharyngeal carcinoma obtained at biopsy, for example, have not exhibited detectable levels of EBV DNA by cRNA hybridization, in contrast to 22 Burkitt's lymphomas and 18 nasopharyngeal carcinomas in which the viral DNA was detectable (M. Nonoyama et al., in press). To ascertain the association of EBV DNA with these diseases, it is important to determine whether these biopsies are really negative for EBV DNA or contain a small amount of the EBV genome which could not be detected by cRNA hybridization.

With the development of methodology for DNA-DNA reassociation kinetics for EBV DNA, which involved in our method labeling nicked viral DNA in vitro with ^{3}H-thymidine triphosphate (TTP) and DNA polymerase I to make highly reactive EBV DNA (13), we are now able to answer questions of this kind. By using the same technique with single-strand-specific nuclease S1, we could further test the homology of EBV DNA with viral DNA associated with nasopharyngeal carcinoma and infectious mononucleosis.

MATERIALS AND METHODS

Preparation of EBV DNA. The HR1K cell line was used to produce EBV as described previously (12). Approximately 15% of the cells were positive for viral capsid antigen when tested by the indirect immunofluorescence method. The partially purified virus (12) was treated with Pronase (1 mg/ml) and sodium dodecyl sulfate (SDS) (1%) for 10 h at 37 C in 0.05 M Tris and 0.001 M EDTA buffer, pH 7.4. The extracted DNA was centrifuged at 21,500 rpm in an SW27 rotor for 15 h at 18 C. The viral DNA sedimented in the region of 55*S*. This DNA was purified by two cycles of isopycnic centrifugation in CsCl in an SW50 rotor at 35,000 rpm for 60 h at 1.718 g/cm^3 (12).

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Preparation of cellular DNA and DNA from tissue specimens. DNA was extracted from cells and specimens obtained at biopsy by treatment with Pronase (1 mg/ml) and 1% SDS in 0.05 M Tris buffer, pH 9.0, at 37 C overnight followed by two extractions with phenol. The DNA was precipitated with two volumes of ethanol and dissolved in 0.05 M Tris, pH 7.4, EDTA 0.01 M, and then treated with 20 μg of RNase per ml at 37 C for 1 h. After sonic disruption (13) the DNA was treated with phenol two times and precipitated by alcohol. The final DNA preparations were dissolved in 0.5 to 1 ml of 0.0025 M EDTA. The size of the DNA was about 4×10^6 daltons, determined by sedimentation through a neutral sucrose gradient (15) with SV40 component I and II DNA as markers.

Labeling of EBV DNA with ^{3}H-TTP in vitro. DNA polymerase I (Kornberg enzyme) (9) was used to prepare highly radioactive EBV DNA (13). One microgram of purified EBV DNA was incubated with 0.05 μg of DNase I at 37 C for 20 min in 0.45 ml of the polymerase buffer (potassium phosphate, 70 mM (pH 7.4); $MgCl_2$, 7 mM; and 2-mercaptoethanol, 1 mM); the enzyme was inactivated by heating at 70 C for 10 min. This solution was mixed with 0.025 ml of twenty times concentrated reaction mixture (2 mM each of dCTP, dGTP, and dATP in polymerase buffer), 250 μCi of ^{3}H-TTP (16 Ci/mM), and 0.03 ml of DNA polymerase I (3 units). The mixture was incubated at 18 C for 5 to 10 h until the incorporation reached a plateau. The reaction was stopped by addition of Sarkosyl-97 to 1% and cooled in ice for 1 min. The specific activity, determined by measurement of the radioactivity of trichloroacetic acid precipitates of a portion of the reaction mixture at the end of the reaction, was 2.2×10^6 counts per min per μg of EBV DNA. The mixture was passed through Sephadex G50 equilibrated with Tris buffer, 0.01 M (pH 7.4); EDTA, 0.001 M; and 0.1% Sarkosyl-97. The DNA fractions were pooled, treated with water-saturated phenol, and precipitated in two volumes of ethanol at -20 C overnight. The average size of ^{3}H-EBV DNA thus obtained was about 2×10^5 daltons in a single-stranded form as determined by Fore (13, 15). The even labeling of EBV DNA with ^{3}H-TTP has also been discussed in the previous paper (13).

The DNA polymerase I was purified according to Richardson et al. (14) and had a specific activity of 13,000 units/mg of protein determined with deoxyadenylate-deoxythymidylate polymer as a primer.

DNA-DNA reassociation kinetics. Tritium-labeled EBV DNA fragments of 2.5×10^{-3} to 1.5×10^{-2} μg (6×10^3 to 3.6×10^4 counts/min) were added to 0.75 ml of a reaction mixture in 0.0025 M EDTA containing sheared cellular DNA or tissue DNA (0.5 to 5 mg) in a test tube. The mixture was denatured by heating at 100 C for 10 min and then chilling in ice. Six molar NaCl (0.25 ml) was added to the mixture to make a final concentration of 1.5 M. One milliliter of the reaction mixture in Tris, 0.01 M (pH 7.0), and NaCl, 1.5 M, was divided into 0.1 ml in each glass tube, and the tubes were sealed. The samples were incubated at 66 C for appropriate periods.

Hydroxyapatite (BioRad) in columns (3 by 1 cm) was equilibrated with 0.1 M phosphate buffer, pH 6.8, and 0.1% SDS in 5-ml syringes in a water bath maintained at 60 C. The capacity of the hydroxyapatite varied between 10 and 40 optical density units of DNA per centimeter of column bed. The samples were applied to the hydroxyapatite columns, and single-stranded DNA was collected in three eluates of 2.5 ml each of 0.14 M PO_4 buffer containing 0.1% SDS. Double-stranded DNA was recovered by elution three times at 60 C with 2.5-ml portions of 0.4 M phosphate buffer containing 0.5% SDS. Five percent of single-stranded DNA was eluted in the double-stranded DNA fraction, and no appreciable double-stranded DNA came out in the single-stranded DNA fraction. We have corrected for the 5% value for unreassociated DNA. The effluents were allowed to cool at 4 C for 20 min, precipitated with trichloroacetic acid (20% final), and filtered on membrane filters (Millipore Corp.). The filters were then removed and counted in a liquid scintillation spectrometer. The kinetics of reassociation follow the equation (2): $C/Co = 1/(1 + K\,Cot)$. Therefore, $Co/C = 1 + K\,Cot$, where C is the concentration of unreassociated ^{3}H-EBV DNA, Co is the total concentration of ^{3}H-EBV DNA, t is time, and K is the reassociation constant. The plots of Co/C values at various times can be expressed as single straight line (3).

Nuclease S1 assay (A. oryzae). Recently, single-stranded DNA-specific nuclease (nuclease S1) (1) has been used for DNA reassociation experiments (10). We obtained nuclease S1 from T. Ando, Institute of Physical and Chemical Research, Tokyo, Japan. The enzyme was suspended in water to 1 mg/ml and stored at -20 C. One-tenth milliliter of the reassociation mixture was diluted in 0.9 ml of the nuclease buffer containing 0.03 M sodium acetate buffer, pH 4.5, and 5×10^{-4} M zinc acetate. The mixture was divided in two samples. After the addition of 10 μg of nuclease S1 to one of the samples, the reaction was continued for 4 h at 37 C and stopped by adding 5% trichloroacetic acid containing 0.01 M sodium pyrophosphate. The reaction mixtures were chilled in ice for 5 min. The precipitates were collected on membrane filters (0.45 μm, Millipore Corp.), washed twice with 4 ml of cold trichloroacetic acid, and processed for liquid scintillation counting.

The same concentration of cellular DNA alone was reassociated as above at the same time and mixed with single- or double-stranded ^{3}H-EBV DNA in amounts equivalent to those used in the experiment above. The mixture was digested with S1 enzyme as above; there was digestion of 97 to 99% of single-stranded DNA and 10% of double-stranded DNA throughout the experiment. A correction was made for the under evaluation of double-stranded DNA by dividing by a factor of 0.9.

RESULTS

Demonstration of human DNA negative for EBV DNA. To test whether the reassociation kinetics are sensitive enough to detect as little as 0.1 genome equivalent per cell of EBV DNA, and to verify whether Hela and Hep2 DNA which have been used as negative controls

for the hybridization studies of EBV (12, 13) were truly negative, the reassociation kinetics were compared between calf thymus DNA and 0.1 or 0.05 genome equivalent of cold EBV DNA mixed with calf thymus DNA and Hela or Hep2 DNA. In each experiment 0.1 genome equivalent per cell of ^{3}H-EBV DNA was allowed to reassociate in the presence of 0.1 or 0.05 genome per cell of cold EBV DNA mixed with 5 mg of calf thymus DNA per ml. It is reasonable to assume that calf thymus DNA does not contain EBV DNA since the distribution of EBV is limited to man and to higher species of primates.

As in Fig. 1, the slopes for the mixture of ^{3}H-EBV DNA and Hep2 DNA or Hela DNA were identical to the slope for the mixture of ^{3}H-EBV DNA and calf thymus DNA, whereas the reaction for the reconstruction experiment containing 0.1 or 0.05 genome of cold EBV DNA, 0.1 genome of ^{3}H-EBV DNA, and 5 mg of calf thymus DNA per ml went 2.1 or 1.6 times as fast as it did for the control experiment without cold EBV DNA. These experiments show that Hep2 and Hela DNA contain as little homology to EBV DNA as does calf thymus DNA and that 0.05 genome per cell of EBV DNA can be detected accurately. From the figure it is expected that the limit of the sensitivity should be 0.02 to 0.03 genome per cell under these conditions; we can distinguish a slope of 1.2 to 1.3 times faster than that of the mixture of ^{3}H-EBV DNA and calf thymus DNA.

Table 1 summarizes the viscosity effects on the kinetics. The half Cot values increase with the addition of increased amounts of calf thymus DNA. The increased rate for Cot ½ or the decreased rate for the reassociation kinetics was about the same as in the case of SV40 DNA versus salmon sperm DNA (4) and suggests that significant contamination of calf thymus DNA with EBV DNA is not present. If a significant amount of EBV DNA were present in the calf thymus DNA, the rate for the reassociation should be accelerated with the increased amount of calf thymus DNA.

In our previous cRNA hybridization studies (M. Nonoyama et al., in press; reference 12), there were tissue specimens and established leukocytic lines in which EBV DNA could not be detected, although it was expected to be present. Since the DNA of some of these specimens and cells was still available, we conducted reassociation kinetics with different concentrations of DNA. In the first series of experiments, 5 mg of DNA and 0.1 genome per cell of ^{3}H-EBV DNA were used. In the second and third series,

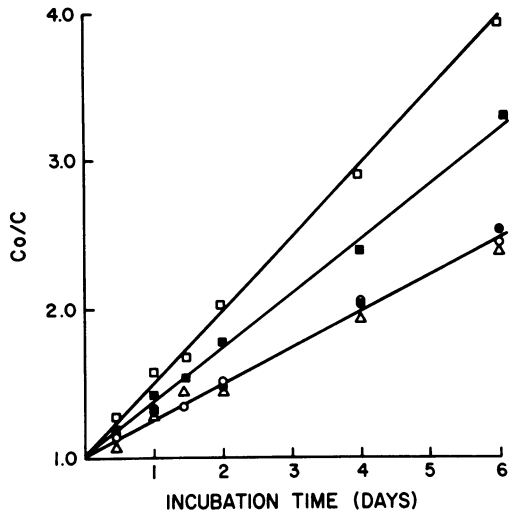

FIG. 1. *DNA-DNA reassociation kinetics for cold EBV DNA, Hela, Hep2, and calf thymus DNA with ^{3}H-EBV DNA. Each reaction mixture containing 5 mg of sheared and heat-denatured DNA and 2.4 × 10^4 counts/min (0.01 μg, 0.1 genome/cell) of ^{3}H-EBV DNA in 0.001 M EDTA, 1.5 M NaCl, pH 7.2, was allowed to reassociate at 66 C. Samples were removed at intervals, and single- and double-stranded DNA were separated on hydroxyapatite columns with 0.14 M phosphate buffer containing 0.1% SDS and 0.4 M phosphate buffer containing 0.1% SDS at 60 C. The results are plotted as a function of ^{3}H-EBV DNA Co/C value. Symbols: □, cold EBV DNA (0.1 genome), calf thymus, and ^{3}H-EBV DNA; ■, cold EBV DNA (0.05 genome), calf thymus, and ^{3}H-EBV DNA; ○, Hep2 DNA and ^{3}H-EBV DNA; ●, Hela DNA and ^{3}H-EBV DNA; △, calf thymus DNA and ^{3}H-EBV DNA. Co is the initial concentration of DNA, and C is the concentration of unreassociated DNA. The number of genome equivalents was calculated as previously (12, 13).*

TABLE 1. *Viscosity effect for the reassociation kinetics*

Calf thymus DNA (mg/ml)	^{3}H-EBV DNA (μg/ml)	Cot 1/2 (mol·s/liter)
0	0.01	0.61×10^{-2}
0.5	0.01	0.63×10^{-2}
1.0	0.01	0.75×10^{-2}
2.0	0.01	0.80×10^{-2}
5.0	0.01	1.03×10^{-2}

1 mg of DNA and 0.25 genome per cell of ^{3}H-EBV DNA or 0.5 mg of DNA and 0.25 genome per cell of ^{3}H-EBV DNA were used. In each series the negative controls for EBV DNA were a mixture of calf thymus DNA and ^{3}H-

EBV DNA. In the first series we tested DNA from the only biopsy ("Fausta") of Burkitt's lymphoma so far found without detectable EBV DNA by cRNA hybridization (M. Nonoyama et al., in press) and "Molt" DNA, which was extracted from established leukocytes derived from a patient with acute lymphatic leukemia (from J. Minowada, Roswell Park) and also negative for EBV DNA by cRNA hybridization.

As shown in Fig. 2, there were no significant differences in the reassociation rates between these two DNAs and calf thymus DNA, which indicates that Fausta DNA and Molt DNA contain less than one EBV genome in every 30 to 50 cells.

Two samples of DNA of nasopharyngeal carcinoma negative for EBV DNA by cRNA hybridization (M. Nonoyama et al., in press) and DNA from another established leukocytic line, "Simpson," derived from a patient with multiple myeloma (Minowada) and negative for EBV DNA by cRNA hybridization (M. Nonoyama et al., in press), were tested in the second and third series of experiments (Fig. 3, 4). None of the test DNAs showed any significant difference in the reassociation kinetics from calf thymus DNA. Simpson DNA and the specimens of nasopharyngeal carcinoma DNA contained less than one EBV genome in every 10 to 20 cells.

Homology between EBV DNA and hybridizable DNA in EBV-containing cells and tissues. We have demonstrated the homology between EBV DNA and hybridizable DNA in Burkitt's lymphoma and nasopharyngeal carcinoma by DNA reassociation kinetics with hydroxyapatite chromatography (13). However, this technique only showed that the DNA had sequences hybridizable to EBV DNA in every 10^6 daltons, as we used DNA of that size and did not prove complete homology.

Recently, nuclease S1, a single-strand-specific nuclease, has been used in reassociation kinetics instead of hydroxyapatite column chromatography. When hydroxyapatite is used, DNA recovered in 0.4 M phosphate buffer is a mixture of true double-stranded DNA and partially double-stranded DNA which might give a misleading test of homology. When nuclease S1 is used for such experiments more definite conclusions about the homology should be possible since any single-stranded tail attached to reassociated DNA should be removed.

We first compared the kinetics of ^{3}H-EBV DNA reassociation by the nuclease S1 method and the hydroxyapatite method. As in Fig. 5, the reassociation rate with the nuclease S1 method was half as fast as with the hydroxyapatite method, which indicated the removal of tails attached to reassociated DNA by the nuclease; it therefore appeared reasonable to evaluate homology by the nuclease S1 method.

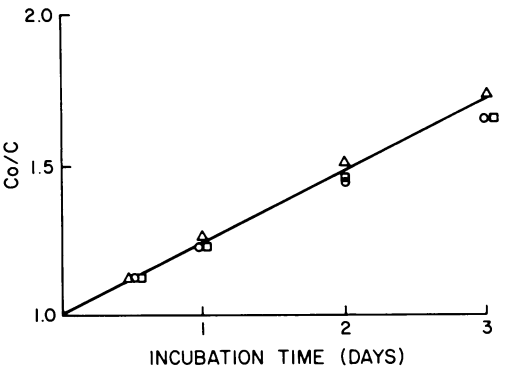

FIG. 2. *DNA-DNA reassociation kinetics for DNA from tissues obtained at biopsy from Burkitt's lymphoma and a human lymphocytic line for EBV DNA. Each reaction mixture contained 5 mg of sheared and heat-denatured DNA and 2.4 × 10^4 counts/min (0.01 μg, 0.1 genome/cell) of ^{3}H-EBV DNA in 1 ml of 0.001 M EDTA, 1.5 M NaCl solution, pH 7.2, and was allowed to reassociate at 66 C. The fractionation was done as in Fig. 1. Symbols: O, human lymphocyte DNA (Molt cell line) and ^{3}H-EBV DNA; □, Burkitt's lymphoma (Fausta) DNA and ^{3}H-EBV DNA; Δ, calf thymus DNA and ^{3}H-EBV DNA.*

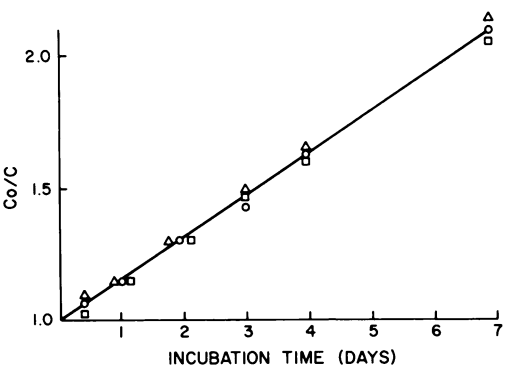

FIG. 3. *DNA-DNA reassociation kinetics for DNA from a nasopharyngeal carcinoma and from a human lymphocytic line negative for EBV DNA. Each reaction mixture contained 1 mg of sheared and heat-denatured DNA and 1.2 × 10^4 counts/min (0.005 μg, 0.25 genome/cell) of ^{3}H-EBV DNA in 1 ml of 0.001 M EDTA, 1.5 M NaCl solution, pH 7.2, and was allowed to reassociate at 66 C. The fractionation was done as in Fig. 1. Symbols: O, DNA from nasopharyngeal carcinoma and ^{3}H-EBV DNA; □, lymphocyte (Simpson cell line) DNA and ^{3}H-EBV DNA; Δ, calf thymus DNA and ^{3}H-EBV DNA.*

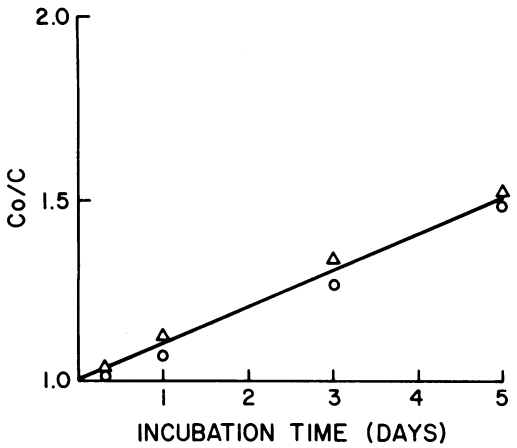

FIG. 4. *DNA-DNA reassociation kinetics for DNA from a nasopharyngeal carcinoma and ^{3}H-EBV DNA. Each reaction mixture contained 500 μg of sheared and heat-denatured DNA and 6 × 10^3 counts/min (0.0025 μg, 0.25 genome/cell) of ^{3}H-EBV DNA in 1 ml of 0.001 M EDTA, 1.5 M NaCl solution, pH 7.2, and was allowed to reanneal at 66 C. The fractionation was done as in Fig. 1. Symbols: O, DNA from nasopharyngeal carcinoma and ^{3}H-EBV DNA; Δ, calf thymus DNA and ^{3}H-EBV DNA.*

The kinetic complexity of the in vitro-labeled EBV DNA was determined from the kinetics by the nuclease S1 method of Fig. 5 by the equation of Wetmur and Davidson (16). The value k2 corrected for the standard conditions described by these authors was 69.4 liters per mol per s, and the kinetic complexity was 9.56 × 10^7. The value did not change significantly in the absence of 0.5 mg of calf thymus DNA.

DNA was extracted from specimens of Burkitt's lymphoma and of nasopharyngeal carcinoma. DNA was also extracted from established cell lines transformed from human cord blood leukocytes by throat washings from patients with infectious mononucleosis. Such washings apparently contain an EBV-like agent that can transform cord blood cells (5, 11). One and one-half genomes per cell of ^{3}H-EBV DNA were added to 0.5 mg of these DNAs per ml, and the reassociation experiments were conducted. Figure 6 shows the results, which indicate that reassociation in all the DNA preparations proceeded linearly without a break in the curves as far as Co/C values of 10 to 20. This means that DNA hybridizable to EBV DNA in the tissues of Burkitt's lymphoma and nasopharyngeal carcinoma and in cells transformed by throat washings of patients with infectious mononucleosis was more than 90% homologous or probably identical to EBV DNA. The average number of genomes per cell in these experiments was 8.2 for the Burkitt's lymphoma, 13.5 for the nasopharyngeal carcinoma, and 7.5, 6.3, and 5.5 for the transformed cells if it is assumed that the reaction should proceed in proportion to the amount of cold EBV DNA, as in the hydroxyapatite method.

DISCUSSION

The use of calf thymus DNA as a negative control is reasonable because EBV infects only

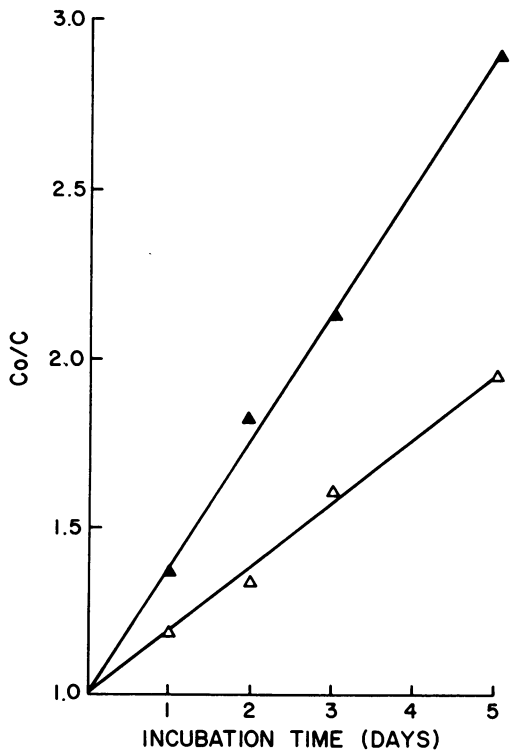

FIG. 5. *Comparison of hydroxyapatite and nuclease S1 for the reassociation rate of ^{3}H-EBV DNA. Each reaction mixture contained 500 μg of sheared and heat-denatured DNA and 3.6 × 10^4 counts/min (0.015 μg, 1.5 genome/cell) of ^{3}H-EBV DNA in 1 ml of 0.001 M EDTA, 1.5 M NaCl, pH 7.2, and was allowed to reanneal at 66 C. Samples were removed at intervals, and single- and double-stranded DNA were separated on hydroxyapatite with 0.15 M phosphate buffer containing 0.1% SDS and 0.4 M phosphate buffer containing 0.1% SDS at 66 C, respectively. For the nuclease S1 assay, single-stranded DNA was digested with 10 μg of nuclease S1 as described in Materials and Methods. Symbols: ▲, calf thymus DNA and ^{3}H-EBV DNA on hydroxyapatite; Δ, calf thymus DNA and ^{3}H-EBV DNA with nuclease S1.*

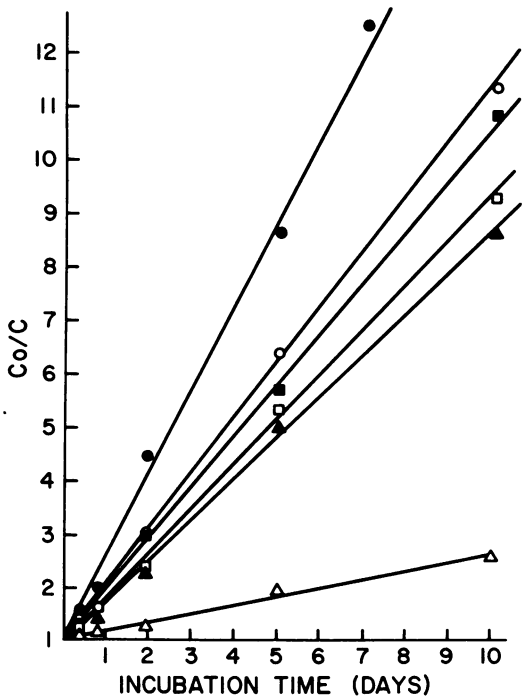

FIG. 6. *Homology between EBV DNA and viral DNA from Burkitt's lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis determined by DNA-DNA reassociation. Each reaction mixture contained 500 μg of sheared and heat-denatured DNA and 3.6 × 10⁴ counts/min (0.015 μg, 1.5 genome/cell) of ³H-EBV DNA in 1 ml of 0.001 M EDTA, 1.5 M NaCl, pH 7.2, and was allowed to reassociate at 66 C. Samples were removed at intervals, and single-stranded DNA was digested with 10 μg of nuclease S1 at 37 C for 4 h. Symbols: ●, DNA from a nasopharyngeal carcinoma and ³H-EBV DNA; ○, DNA from a Burkitt's lymphoma and ³H-EBV DNA; ■, DNA from infectious mononucleosis no. 1 cell line and ³H-EBV DNA; □, DNA from infectious mononucleosis no. 2 cell line and ³H-EBV DNA; ▲, DNA from infectious mononucleosis no. 3 cell line and ³H-EBV DNA; △, calf thymus DNA.*

man and higher species of primates. In addition, the absence of EBV DNA or any related sequences in calf thymus DNA was confirmed in the following way. When ³H-EBV DNA was mixed with 5 mg or 0.5 mg of calf thymus DNA or without calf thymus DNA, the reaction rates with 0.5 mg and without calf thymus DNA were not appreciably different. However, the rate with 5 mg of calf thymus DNA was 60% slower due to the higher viscosity, and there was no acceleration of the reaction, which should have been expected if any hybridizable DNA had been present in calf thymus DNA.

Under the conditions used in these experiments, it is now possible to detect less than one EBV genome in every 30 to 50 cells (kinetics of 1.2 to 1.3 times faster than the reaction for the mixture of calf thymus DNA and ³H-EBV DNA of 0.1 genome per cell). This slight difference in kinetics can be more easily compared by Co/C versus t than by conventional plots of C/Co versus Cot.

Hela cells and Hep2 cells did not contain an appreciable amount of EBV DNA, as expected, and can now serve as a good negative control both for cRNA hybridization and DNA-DNA reassociation kinetics. One specimen of Burkitt's lymphoma, Fausta, in which EBV DNA had not been detected by cRNA hybridization (M. Nònoyama et al., in press), proved to be unassociated with EBV DNA (less than one genome of EBV DNA in every 30 to 50 cells). Since this specimen, which was histopathologically and clinically Burkitt's lymphoma, was from a large surgical biopsy of the ovary that should contain more than one tumor cell in every 30 to 50 normal cells, EBV apparently did not have a direct relation with this case of Burkitt's lymphoma. All of the other 22 tissue specimens of the disease were clearly positive for EBV DNA in genome equivalent amounts ranging from 5 to 100 as determined by cRNA-DNA hybridization (M. Nonoyama et al., in press).

Two cases of nasopharyngeal carcinoma which had been negative for EBV DNA by cRNA hybridization (M. Nonoyama et al., in press) also did not contain EBV DNA or carried less than one genome of EBV in every 10 to 20 cells. We have three more negative cases of nasopharyngeal carcinoma by cRNA hybridization, which have not been tested by DNA-DNA reassociation kinetics. Eighteen out of 23 specimens of nasopharyngeal carcinoma were positive for EBV DNA (M. Nonoyama et al., in press).

Most established human leukocytic cell lines were associated with EBV DNA (J. S. Pagano, M. Nonoyama, and C.-H. Huang, in press; reference 6), but there were two exceptional cases. One, Molt, derived from a patient with acute lymphatic leukemia, and the other, Simpson, derived from multiple myeloma, did not contain appreciable amounts of EBV DNA: less than one genome in every 30 to 50 cells for the former, and less than one genome in every 10 to 20 cells for the latter.

We cannot exlude the possibility in the negative cases that these cells or tissues might contain deleted EBV genome of less than 10% of

whole DNA. Since EBV DNA is large enough to code two to three hundred cistrons, 10% of the genome could be functionally significant.

The reassociation kinetics measured by nuclease S1 gave the kinetic complexity of 9.56×10^7, which is approximately the molecular weight of EBV DNA. This suggests that the S1 method is almost equivalent to the measurement by optical density and also that EBV DNA radioactivity labeled in vitro retains the integrity of original EBV DNA.

Because of the difference in the diseases, there has been speculation over whether the virus from Burkitt's lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis are identical or merely closely related to EBV. We have presented a partial answer previously with the use of hydroxyapatite chromatography (13). In this communication, we have shown more conclusively with the use of nuclease S1 which removes all or most of tails from partially double-stranded DNA that the viral DNA in nasopharyngeal carcinoma and infectious mononucleosis is more than 90% homologous to EBV DNA. Since we could not detect EBV DNA in peripheral leukocytes of patients with infectious mononucleosis, we defined the infectious mononucleosis virus as the transforming agent found in throat washings from patients (5, 11).

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LITERATURE CITED

1. Ando, T. 1966. A nuclease specific for heat-denatured DNA isolated from a product of *Aspergillus oryzae*. Biochim. Biophys. Acta **114**:158-168.
2. Britten, R. J., and D. E. Kohne. 1968. Nucleotide sequence repetition. Carnegie Inst. Year B. **65**:78-105.
3. Frenkel, N., B. Roizman, E. Cassai, and A. Nahmias. 1972. A DNA fragment of herpes simplex 2 and its transcription in human cervical cancer tissues. Proc. Nat. Acad. Sci. U.S.A. **69**:3784-3789.
4. Gelb, L. D., D. E. Kohne, and A. M. Martin. 1971. Quantitation of simian virus 40 sequences in African green monkey, mouse and virus-transformed cell genomes. J. Mol. Biol. **57**:129-145.
5. Gerber, P., M. Nonoyama, S. Lucas, E. Perlin, and B. E. Goldstein. 1973. Oral excretion of Epstein-Barr virus by healthy subjects and patients with infectious mononucleosis. Lancet **2**:988-989.
6. Hausen, H. zur, V. Diehl, H. Wolf, H. Schulte-Holthausen, and U. Schneider. 1972. Occurrence of Epstein-Barr virus genomes in human lymphoblastoid cell lines. Nature N. Biol. **237**:189-190.
7. Hausen, H. zur, and H. Schute-Holthausen. 1970. Presence of EB virus nucleic acid homology in a virus free line of Burkitt tumor cells. Nature (London) **227**:245-248.
8. Hausen, H. zur, H. Schulte-Holthausen, G. Klein, W. Henle, G. Henle, P. Clifford, and L. Santesson. 1970. EBV DNA in biopsies of Burkitt tumors and anaplastic carcinomas of the nasopharynx. Nature (London) **228**:1056-1058.
9. Kelly, B. R., N. R. Cozzarelli, M. P. Deutscher, I. R. Lehman, and A. Kornberg. 1970. Enzymatic synthesis of deoxyribonucleic acid. XXXII. Replication of duplex deoxyribonucleic acid by polymerase at a single strand break. J. Biol. Chem. **245**:39-45.
10. Leong, J. A., A. C. Garapin, N. Jackson, L. Fanshier, W. Levinson, and J. M. Bishop. 1972. Virus-specific ribonucleic acid in cells producing Rous Sarcoma virus: detection and characterization. J. Virol. **9**:891-902.
11. Miller, G., J. C. Niederman, and L. Andrews. 1973. Prolonged oraphageal excretion of Epstein-Barr virus after infectious mononucleosis. New Engl. J. Med. **288**:229-232.
12. Nonoyama, M., and J. S. Pagano. 1971. Detection of Epstein-Barr viral genome in nonproductive cells. Nature N. Biol. **233**:103-106.
13. Nonoyama, M., and J. S. Pagano. 1973. Homology between Epstein-Barr virus DNA and viral DNA from Burkitt's lymphoma and nasopharyngeal carcinoma determined by DNA-DNA reassociation kinetics. Nature (London) **242**:44-47.
14. Richardson, C., C. L. Schildkraut, H. V. Aposhian, and A. Kornberg. 1964. Enzymatic synthesis of deoxyribonucleic acid. XIV. Further purification and properties of deoxyribonucleic acid polymerase of *E. coli*. J. Biol. Chem. **239**:222-231.
15. Studier, F. W. 1965. Sedimentation studies of the size and shape of DNA. J. Mol. Biol. **11**:373-390.
16. Wetmur, J. G., and N. Davidson. 1968. Kinetics of renaturation of DNA. J. Mol. Biol. **31**:349-370.