Comparative Studies of Herpesvirus Papio (Baboon Herpesvirus) DNA and Epstein-Barr Virus DNA

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SUMMARY

An Epstein-Barr virus (EBV)-like herpesvirus has been isolated from a baboon cell line (594S/F9) by induction with the tumour promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA). The herpesvirus papio (HVP) DNA was mixed with B95 EBV DNA and was characterized by sedimentation in neutral glycerol gradient as 55S DNA, with a buoyant density of approx. 1.718 g/ml after equilibrium centrifugation in caesium chloride. DNA-DNA reassociation kinetics between B95 EBV and HVP DNA showed that HVP DNA shares about 40% homology with B95 EBV DNA. Blot hybridization of EcoRI fragments of HVP DNA with a 32P-B95 EBV DNA probe showed that most of the EcoRI fragments of HVP DNA were hybridized to B95 EBV DNA, suggesting that the homologous sequences were dispersed throughout the virus DNA.

INTRODUCTION

Several primate cell lines which contain the genomes of Epstein-Barr virus (EBV)-related herpesviruses have been established in the past several years (Agrba et al. 1975; Gerber et al. 1976, 1977; Rasheed et al. 1977; Deinhardt et al. 1978; Falk et al. 1978; Rabin et al. 1978a; Neubauer et al. 1979). The origin of these cell lines includes the baboon, gorilla, chimpanzee and orangutan. These primate herpesviruses, in general, are able to immortalize B lymphocytes in vitro. In addition, they share some antigenic features related to human EBV. The primate viruses are related to each other, but can be distinguished by anti-complement immunofluorescence assay (Rabin et al. 1978b). The primate cell line used in this study was 594S/F9, which was established from a baboon with lymphoid disease (Rabin et al. 1977). These cells were found to release EBV-like virus with in vitro transforming activity. They also contain antigens that can cross-react with EBV early antigen (EA), viral capsid antigen (VCA) and membrane antigen (MA), but not EBV nuclear antigen (EBNA) (Rabin et al. 1977, 1978a, b).

DNA-DNA reassociation kinetics between EBV DNA and virus DNA in the primate cell lines indicate that they share 30 to 40% homology of DNA sequences (Djatchenko et al. 1976; Falk et al. 1976, 1978; Rabin et al. 1978a; Neubauer et al. 1979). Some of these cell lines can produce a small amount of virus in culture, but it is very difficult to obtain a large amount of pure virus DNA. In this report we describe the isolation of HVP DNA induced by the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), that has been used

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to increase virus production in some EBV producer cell lines (zur Hausen et al. 1978, 1979). This virus DNA was characterized with regard to CsCl density, restriction enzyme patterns and homology to B95 EBV DNA.

METHODS

Cells. 594S/F9 cells were propagated at 37 °C in RPMI 1640 medium containing 8% calf serum. The cell culture was split once a week to $3 \times 10^5$ viable cells/ml. TPA (20 ng/ml) was added when the culture was diluted to a final vol. at 5 l. Following the addition of TPA, cells were sampled for virus antigens by immunofluorescence assay (FA) every 3 days. Five mCi $^3$H-thymidine was added to the 5 l culture on the third day. The cells were harvested when about 20% of the cells were positive for FA. B95-8 cells were similarly treated with TPA to produce B95 EBV.

Purification of virus DNA. Seven days after TPA treatment, the 594S-F9 cells were removed by centrifugation at 8000 rev/min for 5 min. The virus in the supernatant was concentrated by centrifugation at 12000 rev/min at 4 °C for 90 min in a Beckman JA-14 rotor. The pellet of virus material was resuspended in TE buffer pH 8 (0.01 M-tris pH 8, 0.001 M-EDTA). Solutions of 10% NP40 and Triton X-100 were added to achieve final concentrations of 0.5% of each. Following Dounce homogenization in ice (25 strokes), the virus was freed of cellular debris by centrifuging at 9000 rev/min at 4 °C for 10 min in a JA-21 rotor. The resulting pellet was re-extracted twice as described. The pooled supernatant fractions were layered on top of a 10 ml 35% (w/w) sucrose cushion in TE pH 8. After centrifugation at 22000 rev/min at 4 °C for 60 min in an SW27 rotor, the virus pellet was resuspended in 2 ml TE pH 8. NP40 and Triton X-100 solutions were added to 0.5% of each. After Dounce homogenizing (50 strokes) in ice, the virus suspension was layered on to a 15 to 30% (w/v) linear sucrose gradient in TE pH 7.4. Following centrifugation at 22000 rev/min at 4 °C for 30 min in an SW27 rotor, 3 ml fractions were collected by puncturing the bottom of the cellulose nitrate tube with an 18G needle. The pellet was re-extracted and subjected to a 15 to 30% sucrose gradient centrifugation as described. The fractions containing the virus were isolated from the middle of the gradient and subsequently recovered by centrifugation at 22000 rev/min at 4 °C for 2 h in an SW27 rotor. To release the virus DNA from the partially purified virus, the pellet was resuspended in a small volume (0.4 ml) of TNE pH 9 (0.05 M-tris, 0.4 M-NaCl, 0.001 M-EDTA) and treated with proteinase K (200 µg/ml) and SDS (1%). After incubation overnight at 37 °C, virus DNA was isolated by centrifugation in a 10 to 30% (w/v) glycerol gradient in TNE (pH 8) at 38000 rev/min at 18 °C for 4 h in an SW41 rotor. Virus DNA was further purified, if necessary, by equilibrium density centrifugation in a cesium chloride gradient (10-2 g CsCl was dissolved in 8 ml TE pH 8, containing 0.1% Sarkosyl). Centrifugation was carried out at 38000 rev/min in a Ti50 rotor for 48 h at 20 °C.

DNA-DNA reassociation kinetics. Ten ng $^3$H-B95 EBV DNA (1.8 \times 10^4 \text{ ct/min}), labelled in vitro, were mixed with 0.5 µg cold HVP or B95 EBV DNA, heat denatured and reassociated in 0.5 M-NaCl at 66 °C. Samples were taken at intervals for S-1 nuclease assay to determine the degree of reassociation.

Restriction enzyme digestion and Southern blotting hybridization. The procedures for restriction enzyme digestion and agarose-gel electrophoresis were described elsewhere (Lee et al. 1977, 1979). Briefly, approx. 2 µg of HVP and B95 EBV DNA were individually digested with EcoRI and the digested DNA products were separated by 0.4% agarose-gel electrophoresis. The gel was then stained with ethidium bromide and photographed under u.v. light. λ-DNA EcoRI fragments and intact λ-DNA were used as mol. wt. markers. The DNA fragments in the gel were transferred to a nitrocellulose filter according to Southern's method (1975). $^{32}$P-labelled B95 EBV DNA, 10² ct/min (1 \times 10^8 ct/min/µg) prepared by
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Table I. Induction of EA and VCA in 594S/F9 cells by TPA and the yield of virus DNA from purified virions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days in incubation</th>
<th>% Positive cells</th>
<th>Virus DNA yield (μg/l)</th>
</tr>
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<tbody>
<tr>
<td>+ TPA*</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>25</td>
<td>21</td>
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<tr>
<td>- TPA</td>
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<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3</td>
<td>1</td>
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* TPA concentration 20 ng/ml.

in vitro nick-translation (Nonoyama & Pagano, 1973; Mackey et al. 1977; Rigby et al. 1977), was used as the probe to hybridize the DNA on the filter. Hybridization was performed in a small plastic bag at 41 °C for 40 h. The conditions were similar to those described by Dawid (1977). Briefly, the solution contained 0.6 M-NaCl, 0.2 M-tris pH 8, 0.02 M-EDTA, 50% formamide and 0.5% SDS. After hybridization, the filters were rinsed in a shaking water bath (41 °C) with 50% formamide containing 2 × SSC (0.15 M-NaCl, 0.015 M-sodium citrate) for 30 min. The filters were then rinsed with 2 × SSC for 5 h. Finally, the filters were washed with 2 × SSC containing 0.005 M-sodium pyrophosphate. The filters were air dried and autoradiographed with Kodak X-ray (RP/R2) film and high intensity screens (Du Pont, Lighting Plus X G).

Indirect immunofluorescence. Staining for early antigen (EA) and viral capsid antigen (VCA) was done by the procedures of Henle & Henle (1966). Two ml of cells were washed with phosphate-buffered saline (PBS) and fixed on a slide with cold acetone. Human EBV-positive serum (EA plus VCA, or VCA) was dropped on the slide and incubated for 45 min at 37 °C. The slide was washed again with PBS and incubated with fluorescein-conjugated caprine anti-human IgG (Hyland Laboratories, Costa Mesa, Calif., U.S.A.).

RESULTS

Induction of virus antigen by TPA

TPA has been shown to increase EBV antigens and genomes in the productive cell lines, P3 HR-1 and B95-8 (zur Hausen et al. 1978, 1979). It was tested for its effect on the 594S/F9 cell line. As shown in Table I, 25% and 21% of the cells were positive for EA and VCA, respectively, as the result of TPA induction (Table 1).

Comparison of the pattern of restriction enzyme-digested HVP DNA obtained with and without TPA treatment

HVP was isolated from either TPA-treated or non-TPA-treated cells. The virus DNA was extracted from purified virions and digested with restriction enzymes. The products were separated by agarose-gel electrophoresis and stained with ethidium bromide. Fig. 1 indicates no apparent differences between TPA-treated and non-treated HVP DNA. The yield of the virus DNA was increased approx. 20-fold by TPA induction (Table 1) and, therefore, HVP DNA for the following experiments was obtained by TPA treatment of cell cultures.

Comparison of size and density between HVP and B95 EBV DNA

The size of HVP DNA was compared to that of 55S B95 EBV DNA (Pritchett et al. 1975) by centrifuging the mixture of 3H-HVP DNA and 32P-B95 EBV DNA through a glycerol gradient. As shown in Fig. 2, 3H-HVP DNA and 32P-EBV DNA sedimented at about the
Fig. 1. Restriction enzyme patterns of HVP DNA from baboon lymphoblastoid cells isolated with and without TPA treatment. Two μg HVP DNA were cleaved with EcoRI, BamHI or SalI, individually, and then separated by 0.4% agarose-gel electrophoresis. The gel was stained with ethidium bromide and photographed under u.v. light. HVP virus DNA treatment: (a, b) cleaved with EcoRI, (a) with and (b) without TPA; (c, d) cleaved with BamHI, (c) with and (d) without TPA; (e, f) cleaved with SalI, (e) with and (f) without TPA.

same rate, indicating that the size of HVP DNA was not distinguished from that of 55S B95 EBV DNA. The density of HVP DNA in CsCl was also compared with that of B95 EBV DNA. As seen in Fig. 3, 3H-HVP DNA banded at the same position as 32P-B95 EBV DNA (1.718 g/ml), as previously determined by Pritchett et al. (1975). Thus, the density of HVP DNA in CsCl was not distinguishable from that of B95 EBV DNA.
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Fig. 2. Sedimentation of HVP DNA. The method for obtaining HVP particles is described in the text. The mixture of H-HVP and 32P-B95 EBV was lysed with 200 μg proteinase K and 1% Sarkosyl and layered on a 10 to 30% glycerol gradient containing 50 mM-tris-HCl pH 8, 1 mM-NaCl, 1 mM-EDTA, 0.1% Sarkosyl. Centrifugation was carried out at 38000 rev/min and 18°C in an SW41 rotor for 4 h. Fractions were collected from the top to the bottom by an ISCO fractionator Model 183. ■—■, 3H-HVP DNA; ○—○, 32P-B95 EBV DNA.

Fig. 3. CsCl equilibrium centrifugation of HVP DNA. 3H-HVP DNA was mixed with 32P-labelled B95 DNA and unlabelled λ-DNA and centrifuged to equilibrium in CsCl at 38000 rev/min and 18°C for 40 h; 110 fractions were collected from the bottom of the tube. Absorbance at 260 nm was measured for each fraction. Then a sample of each fraction was precipitated by TCA and counted in a Beckman scintillation counter. CsCl density was measured by a refractometer. The arrow indicates the position of λ-DNA density. ■—■, 3H-HVP DNA; ○—○, 32P-B95 EBV DNA; ●—●, density.

DNA–DNA reassociation kinetics between B95 EBV DNA and HVP DNA

Previous studies indicated that DNA from baboon lymphoblastoid cell lines (8CB-1, 13CB-1 and 16CB-1) hybridized to approx. 40% of an EBV DNA probe (Djatchenko et al. 1976; Falk et al. 1976, 1978). Since an adequate amount of purified HVP DNA could now be obtained by TPA induction, the homology between EBV and this baboon virus DNA was re-examined by reassociation kinetics using purified B95 EBV and HVP DNA. The result is shown in Fig. 4. As expected, the hybridization reaction between unlabelled and 3H-labelled B95 EBV DNA was linearly accelerated, while the hybridization rate between unlabelled HVP DNA and 3H-B95 EBV DNA increased sharply during the initial 2 h, then levelled off. Since the self-hybridization of 3H-B95 EBV DNA (closed circles in Fig. 4) was insignificant at the break point region of the kinetics between unlabelled HVP and 3H-EBV DNA probe, it is estimated that HVP DNA has approx. 40% homologous sequences with B95 EBV DNA as previously reported (Falk et al. 1976).

Blotting hybridization between HVP DNA and B95 EBV DNA

In general, the patterns of the EcoRI fragments of HVP DNA and B95 EBV DNA are quite different, as shown in Fig. 5(a), although the reassociation kinetics between these two DNAs show 40% homology (Fig. 4). The following experiments were designed to identify
Fig. 4. DNA–DNA reassociation kinetics between 3H-labelled B95 DNA and HVP virus DNA. Ten ng 3H-labelled B95 DNA (1.8 x 10⁴ ct/min) were mixed with 0.5 µg virus DNA. The mixture was heat-denatured and reassociated in 0.5 M-NaCl at 66 °C. Samples were taken at intervals up to 20 h and reassociated DNA was assayed by S1 nuclease. The data were analysed using the equation (C₀/C)²⁻² = 1 + Kₐₜ (Morrow, 1974; Smith et al. 1975), where C₀ is the initial concentration of 3H-labelled B95 DNA, C is the amount of S1 nuclease-sensitive DNA at time t and K is the kinetics constant. □—□, 3H-labelled B95 DNA + B95 DNA; △—△, 3H-labelled B95 DNA + HVP virus DNA; ●—●, 3H-labelled B95 DNA alone.

HVP DNA fragments which share sequence homology with B95 EBV DNA. 32P-B95 EBV DNA, labelled in vitro, was used as a probe to hybridize B95 and HVP DNA fragments (Fig. 5b) which had been blot transferred to nitrocellulose filters. All of the B95 EBV DNA fragments were hybridized as expected (Fig. 5b, lane 1). Most of the HVP DNA fragments were also hybridized (Fig. 5b, lanes 2 and 3), although the intensity of each band was weaker than those in B95 EBV DNA (Fig. 5b, lane 2). It was noted, however, that the intensity of band C of HVP DNA was somehow stronger than others. These results indicated that most of the EcoRI fragments of HVP DNA share a certain degree of homology with B95 EBV DNA. The reciprocal experiment was conducted by using 32P-labelled HVP DNA as a probe to hybridize to HindIII-digested fragments of HVP and B95 EBV DNA. A similar result was obtained showing that most of B95 EBV DNA fragments hybridized to the HVP DNA probe, whereas all the HVP DNA fragments hybridized to the probe (data not shown).

DISCUSSION

The baboon cell line 594S/F9 produces a limited amount of herpesvirus which is biologically similar to EBV in that it transforms human B lymphocytes and produces cross-reacting virus antigens with EBV. TPA is an effective agent for inducing virus replication in EBV producer cells. Comparison of the restriction enzyme (EcoRI or BamHI) patterns of B95 EBV DNA revealed no significant differences between TPA-treated and untreated virus preparations (zur Hausen et al. 1979). This report demonstrates that TPA also increases virus production in this 594S/F9 primate cell culture. No significant difference between the restriction enzyme patterns of TPA-treated or untreated HVP DNA preparations was
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Fig. 5. Blot hybridization of EcoRI fragments of HVP and B95 EBV DNA with 32P-B95 EBV DNA probe. The procedures for DNA digestion and transfer are described in the text. The DNA filter was hybridized with 32P-labelled B95 DNA in a plastic bag. (a) Before blot hybridization, the gels were stained with ethidium bromide and photographed under u.v. light: 1, B95 virus DNA/EcoRI; 2, HVP virus DNA/EcoRI. (b) After hybridization and washing, the filter was exposed to autoradiography film (RP/R2): 1, B95 virus DNA/EcoRI; 2, HVP virus DNA/EcoRI; 3, HVP virus DNA/EcoRI having a longer exposure time to X-ray film.

observed. In addition, spontaneously induced and TPA-induced HVP both retain biological activity for transformation of human lymphocytes (Y. S. Lee et al., unpublished data). Thus, the difference, if any, between HVP DNA obtained by TPA induction and spontaneously produced virus DNA would be expected to be minimum. HVP DNA was shown to have a sedimentation coefficient similar to 55S EBV DNA (Pritchett et al. 1975) and the density of HVP DNA in CsCl was indistinguishable from EBV DNA density (Pritchett et al. 1975). Similar data were obtained in an earlier study by Djatchenko et al. (1976). EcoRI restriction enzyme patterns of HVP DNA were shown to be reasonably uniform with a total mol. wt. of $110 \times 10^6$, determined by the λ-DNA markers, both EcoRI-digested and undigested. HVP
DNA shares 40% homology with EBV DNA by DNA–DNA reassociation kinetics. Southern blotting hybridization permitted the identification of the fragments of the homologous sequences between B95 EBV DNA and HVP DNA. Using 32P-labelled B95 EBV DNA as a probe, most of the HVP DNA fragments were found to contain homologous sequences with B95 EBV DNA. When a reciprocal experiment was carried out using 32P-labelled HVP DNA as the probe, a similar result was obtained. Thus, the homology of B95 EBV DNA sequences with HVP DNA is dispersed throughout the virus DNA and is not restricted to a certain portion. The C fragment of HVP DNA hybridized significantly higher than other bands. Preliminary experiments indicated that the highly repeated Bam V fragment of B95 EBV DNA also hybridized to the C fragment of HVP DNA. This C fragment contains a highly repeated fragment of 2.0 x 10^6 mol. wt. revealed by Kpn enzyme which is also hybridizable to the Bam V fragment of EBV DNA (Y. S. Lee et al., unpublished data). Thus, the strong hybridization of the C fragment should reflect the presence of repeated sequences in that fragment. The degree of homology between the Bam V fragment and the 2 x 10^6 mol. wt. fragment of Kpn has not yet been determined. It is possible that the rest of the primate herpesviruses antigenically related to EBV, found in gorilla and orangutan, which contain 30 to 40% homology to EBV DNA (Rabin et al. 1978a; Neubauer et al. 1979), may have a similar relationship to EBV DNA. Identification of corresponding homologous fragments between HVP and B95 EBV DNA is now in progress.

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