JOURNAL OF VIROLOGY, Mar. 1985, p. 1012–1015
0022-538X/85/031012-04$02.00/0
Copyright © 1985, American Society for Microbiology
Vol. 53, No. 3

The Circular Intracellular Form of Epstein-Barr Virus DNA Is Amplified by the Virus-Associated DNA Polymerase

JAMES E. SHAW

Department of Medical Microbiology and Immunology, College of Medicine and Comprehensive Cancer Center, Ohio State University, Columbus, Ohio 43210

Received 9 August 1984/Accepted 28 November 1984

Selective DNA extraction and hybridization procedures were used to estimate the relative number of covalently closed circular viral genomes in cultures of Epstein-Barr virus (EBV)-transformed cells. In virus-producing P3HR-1 cultures that were exposed for 11 days to phosphonoacetic acid or to acyclovir, the content of covalently closed circular EBV DNA was reduced ca. 70% relative to a control culture without drug. The EBV plasmid content of Raji, a virus nonproducer cell line, was not reduced by exposure to these compounds. When P3HR-1 cultures were exposed to 12-*O*-tetradecanoylphorbol-13-acetate, the number of circular genomes per cell increased. These findings indicate that two enzyme activities synthesize circular EBV DNA and that the virus-associated DNA polymerase synthesizes most of the circular EBV DNA in a virus producer culture. It is suggested that the circular genomes synthesized by the viral enzyme are intermediates in the syntheses of linear virus DNA.

The DNA of Epstein-Barr virus (EBV) is a linear molecule (13) with ca. 180 kilobase pairs. It is synthesized in cultures of transformed human (9) or marmoset (12) B-cells that carry multiple latent copies of the viral DNA in a covalently closed circular form (4, 8; see reference 11). Linear EBV DNA is synthesized when cells with latent circular genomes enter the virus replicative cycle and express the EBV-associated DNA polymerase (6). Phosphonoacetic acid (PAA) and acyclovir (ACV) inhibit the activity of the viral enzyme in vitro (5), and they block virus replication in EBV producer cultures (3, 4, 18, 19). These compounds do not inhibit the synthesis of cellular DNA or the synthesis of EBV DNA in virus nonproducer cultures like Raji (14), in which all of the viral genomes reside in the latent circular form (11). Moreover, the synthesis of a small percentage of the viral DNA in EBV producer cultures like B95-8 (12) and P3HR-1 (9) is resistant to inhibition by PAA and ACV (3, 4, 18). The resistant viral DNA, like that in Raji, is synthesized in virus nonproducer cells and consists of covalently closed circular molecules (4, 8).

Synthesis of most of the viral DNA in EBV producer cultures occurs in virus-producing cells and is inhibited by PAA and ACV (3, 4, 18). The viral DNA that is synthesized in virus-producing cells is packaged in virions in a linear form (13). However, it has not been established how the linear genomes arise in cells with circular plasmids. One possibility is that the EBV DNA polymerase synthesizes linear DNA directly by using the resident plasmids as templates. Another possibility is that the viral enzyme amplifies the number of resident plasmids, and the nascent circular genomes are converted to a linear form. The second possibility is examined here.

In this study, evidence that the EBV DNA polymerase synthesizes covalently closed circular DNA is presented. To establish this point, the quantities of covalently closed circular EBV DNA in cultures exposed to viral enzyme inhibitors and in cultures without drug are compared. The data indicate that most of the circular EBV DNA in a virus producer culture is synthesized by the EBV-associated DNA polymerase, not by cellular enzymes.

Covalently closed circular EBV DNA is obtained directly from cells by an alkaline extraction procedure that removes linear and nicked circular forms of DNA. This procedure has been used to obtain highly purified circular EBV DNA of intracellular origin from virus producer and nonproducer cell lines (7). The procedure does not require lengthy incubations which favor the introduction of single-strand nicks in large DNA circles. Furthermore, loss of circular EBV DNA by trapping in viscous cellular lysates is not a serious problem with this procedure. Trapping can occur when cells are lysed under nondenaturing conditions, and it can lead to inconsistencies in the values obtained for the number of plasmids in an EBV-transformed cell line.

The relative quantity of circular EBV DNA recovered from the transformed Raji line with the alkaline extraction procedure is shown in Fig. 1. Cells from 1, 10, 25, and 50 ml of a Raji culture were mixed with EBV DNA-negative BJAB cells such that each sample contained the same number of cells before extraction. The DNA of each extract was attached to nitrocellulose filters and hybridized to ^{32}P-labeled virion DNA. The data show that the quantity of radiolabeled EBV DNA that hybridized was directly proportional to the number of Raji cells in the sample before extraction. The linearity of the data indicates that these are reliable methods for quantitating the relative number of EBV plasmids in transformed cells. In the following studies, these procedures are used to determine the relative number of covalently closed circular genomes in cultures exposed to viral DNA polymerase inhibitors.

P3HR-1 and Raji cells were exposed to PAA or to ACV. After continuous exposure to these compounds for 11 days, covalently closed circular DNA was isolated. The EBV DNA obtained by the alkaline extraction procedure bands at the superhelical and relaxed closed circular positions of cesium chloride-ethidium bromide gradients (Fig. 2). On some gradients with P3HR-1 DNA, more than two peaks were detected by hybridization (unpublished data). The extra peaks of DNA were located in the lower region of the gradient and were composed of single-stranded molecules as determined by their sensitivity to S_1 endonuclease. Contamination of circular DNA extracts with single-stranded DNA

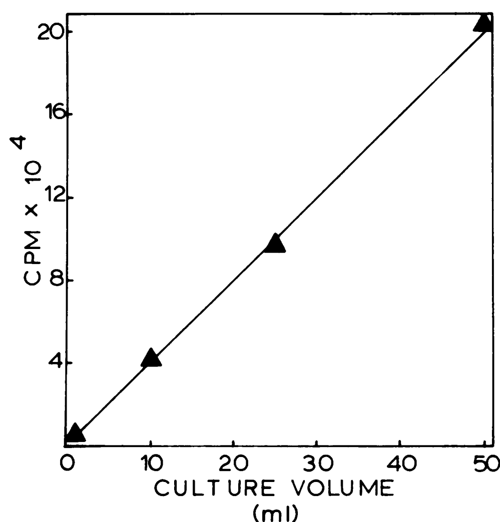

FIG. 1. Duplicate samples of 1, 10, 25, and 50 ml of a Raji (14) culture (10^6 cells per ml) were mixed with EBV DNA-negative BJAB cells (10) such that each sample contained a total of 5×10^7 cells. Covalently closed circular DNA was extracted from each sample under alkaline conditions and precipitated with ethanol as previously described (7). The DNA was collected by centrifugation and dissolved in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate). One fourth of each sample was mixed with an equal volume of 0.5 N NaOH. After heating for 10 min at 80°C and cooling to 0°C, the pH was adjusted between 7 and 8 by adding an equal volume of 0.6 M Tris-hydrochloride (pH 8), water, and concentrated HCl (100:100:3). The final volume was adjusted to 4 ml by adding 1.2 ml of 20× SSC. Each sample was passed through a nitrocellulose filter (0.45 μm; Schleicher & Schuell Inc.) by suction under low vacuum, and the attached DNA was washed once by passing 6 ml of 6× SSC through the filter. Filters were prepared for use by briefly soaking in water and then in 6× SSC. Just before adding the DNA, 6 ml of 6× SSC was passed through the filters by suction. All reagents were prefiltered through nitrocellulose before use. Filters with DNA were dried at room temperature and then heated under vacuum for 3 h at 80°C. For hybridizations, 1 μg of EBV DNA was labeled in vitro with [^{32}P]dCTP as previously described (15). The labeled DNA was filtered through G-50 Sephadex (10 mM Tris-hydrochloride [pH 8], 50 mM NaCl, 0.2 mM EDTA) and had a specific activity of 5×10^7 to 1×10^8 cpm/μg. Filters with immobilized DNA were soaked briefly in 6× SSC and then stacked in a cylindrical glass vessel with a diameter slightly greater than that of the filters (27 mm). Prehybridization solution (50% formamide, 6× SSC, 0.5% sodium dodecyl sulfate, 0.2% polyvinylpyrrolidone [molecular weight, 40,000], 0.2% bovine serum albumin, and 0.2% Ficoll [molecular weight, 400,000]) was added to cover the filters. After incubation at 45°C overnight, the filters were washed twice with 6× SSC at room temperature and then incubated at 45°C for 1 to 2 days in hybridization solution (50% formamide, 6× SSC, 0.5% sodium dodecyl sulfate, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll, 100 μg of sonicated salmon sperm DNA per ml, and 1 μg of ^{32}P-labeled EBV DNA). The hybridization solution was heated in a boiling water bath for 10 min and then quickly cooled to room temperature before adding to the filters. After hybridization, the filters were washed twice with 2× SSC for 5 min at room temperature and twice with 2× SSC containing 1% sodium dodecyl sulfate for 30 min at 60°C. The filters were cooled to room temperature and washed twice with 0.2× SSC for 30 min at room temperature. The filters were dried, and the radioactivity was measured by scintillation spectrometry. The radioactivity that associated with filters containing only BJAB DNA was subtracted from the filters with BJAB and Raji DNA. Each point is the mean of duplicate determinations.

occurred during initial studies when the interphase material was disturbed during quantitative removal of the aqueous phase after phenol extraction. To eliminate possible contamination with single-stranded DNA in subsequent experiments which required quantitative recovery of the aqueous phase, all samples were routinely digested with S_1 endonucl-

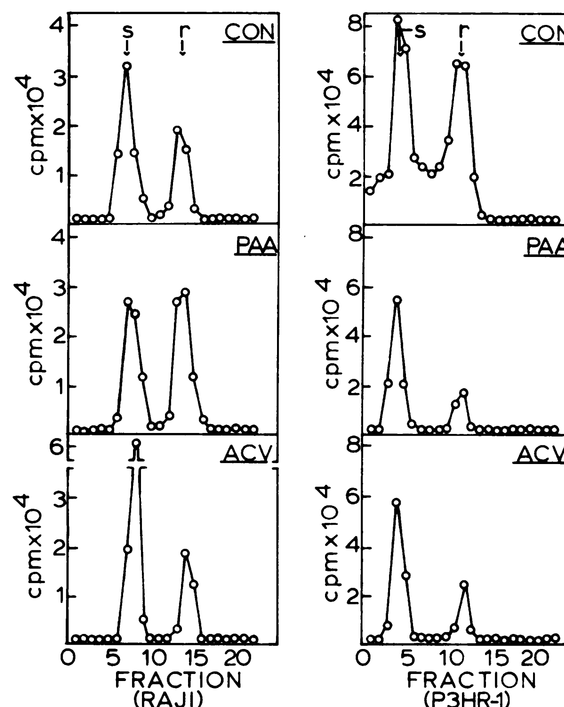

FIG. 2. Isopyknic centrifugation of EBV DNA extracted from Raji and P3HR-1 cells under alkaline conditions. Raji and P3HR-1 cultures were exposed for 11 days to 100 μM ACV or to 400 μM PAA or were incubated without drug (CON). Covalently closed circular DNA was extracted from 5×10^7 cells and precipitated with ethanol as described in the legend to Fig. 1. The ethanol precipitates were dissolved in 4.6 ml of a CsCl solution prepared by dissolving 1 g of CsCl with 1 ml of 5 mM Tris-hydrochloride (pH 7.6)–0.2 mM EDTA. To this solution, 61 μl of 1% ethidium bromide was added. The refractive index of the final mixture was 1.3875. The DNA was centrifuged overnight at 45,000 rpm in a 65 Ti vertical Beckman rotor at 20°C. Fractions (0.2 ml) were collected from the bottom of the tube, and the refractive index of every fifth fraction was determined. The density profiles of each set of gradients were identical. The banding positions of superhelical (s) and relaxed (r) closed circular forms of DNA are indicated. Ethidium bromide was removed from each fraction by a single extraction with 0.5 ml of 90% isopropanol. To each fraction, 0.5 ml of water containing 20 μg of sonicated salmon sperm DNA was added, and the DNA was precipitated overnight at −20°C with 1.5 ml of ethanol. Precipitates were collected by centrifugation at 2,500 rpm for 20 min at 4°C. Under these conditions, ^{3}H-labeled herpes simplex virus DNA was recovered quantitatively. The precipitates were dissolved in 0.2 ml of S_1 digestion buffer (30 mM sodium acetate [pH 4.5], 50 mM sodium chloride, 1 mM zinc chloride, 5% glycerol), and then 0.1 ml of S_1 digestion buffer containing 100 U of S_1 endonuclease (Sigma Chemical Co.) was added. After 5 min at 37°C, the fractions were cooled to 0°C, and 1.1 ml of 0.32 M NaOH was added. After incubation for 10 min at 80°C and cooling to 0°C, 1.4 ml of neutralization buffer and 1.2 ml of 20× SSC were added, and the DNA was immobilized on nitrocellulose filters and hybridized to ^{32}P-labeled EBV DNA (Fig. 1). To determine background radioactivity, filters without DNA were included during hybridization. The counts per minute above background is shown.

ease just before denaturation and attachment of the DNA to nitrocellulose filters. The activity of the S_1 enzyme was monitored by measuring the extent of hydrolysis of ^{3}H-labeled single- and double-stranded herpes simplex virus DNA. With conditions for S_1 digestion identical to those for the EBV samples, 95% of the native herpes simplex virus DNA was resistant to hydrolysis, whereas greater than 95% of the denatured herpes simplex virus DNA was reduced to acid-soluble material which was too small to bind to nitrocellulose filters.

To compare the data of drug-treated and control cultures, the quantity of ^{32}P-labeled EBV DNA that hybridized to the circular DNA was divided by the total quantity of DNA in the cells before extraction. Total cellular DNA was determined by the diphenylamine reaction (2) on samples of cells removed from a culture at the same time cells were removed for DNA extraction. The data (Table 1) show that the content of covalently closed circular EBV DNA in P3HR-1 cultures that were exposed to PAA or to ACV was reduced to 32 and 30%, respectively, of the P3HR-1 control culture without drug (100%). The plasmid content of Raji cells was not reduced by exposure to these compounds. When these experiments were repeated with the same or different cultures of Raji and P3HR-1 cells, results similar to those in Table 1 were obtained for Raji. The values for P3HR-1 cells, however, ranged from 5 to 40% of control levels. A range of values would be expected for circular DNA that is synthesized by the EBV-associated DNA polymerase because the enzyme is expressed in virus-producing cells, and virus-producing cells vary in number in EBV producer cultures. This is consistent with the data in Table 3, which show that the quantity of circular EBV DNA in P3HR-1 cells that were exposed to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) exceeded by approximately twofold that of the control culture without promoter. There was also a twofold increase in the number of cells expressing virus capsid antigens. These data are consistent with previous findings which show that TPA increases the number of virus-producing cells in a culture (20), and after exposure to TPA significant increases in the virus-associated DNA polymerase activity can be detected in EBV producer cultures (5).

The relative quantity of circular EBV DNA banding as superhelical DNA on cesium chloride-ethidium bromide gradients is shown in Tables 2 and 3. The values obtained for P3HR-1 cells exposed to PAA and ACV were, respectively, 44 and 51% of the culture without drug, whereas the values for Raji were not reduced significantly from the control level. The value for P3HR-1 increased to 264% of the control level when cells were exposed to TPA (Table 3).

These studies show that the content of covalently closed circular EBV DNA in a virus producer culture is reduced by ca. 70% when the culture is exposed to compounds that block virus replication and inhibit the activity of the EBV-associated DNA polymerase. Furthermore, they show that the circular viral DNA increases in cells exposed to TPA. These findings indicate that the viral enzyme amplifies the circular DNA in latently infected cells that enter the virus-replicative cycle. It is suggested that the nascent circular genomes are intermediates in the synthesis of linear virus DNA. This is consistent with a report by Siegel et al. (17), who showed that 65S DNA is chased into 59S molecules in pulse-chase experiments with superinfected Raji cells. Relaxed circular and linear forms of EBV DNA have sedimentation coefficients of 65S and 59S, respectively (1). Superhelical DNA is also an intermediate in the synthesis of virus DNA (Tables 2 and 3).

The growth rate and viability of EBV-transformed cells are not affected significantly when cultures are exposed to 100 μM ACV or to 400 μM PAA (3, 4, 20), the concentrations used in the present study. At these inhibitor concentrations, VCA expression decreases maximally within 2 to 5 days and remains at a low level until the drug is removed from the culture medium. Thus, viral DNA polymerase inhibitors decrease VCA expression as well as the circular viral DNA content of P3HR-1 cells.

Some of the circular EBV DNA of P3HR-1 cells was synthesized in the presence of viral enzyme inhibitors (Fig.

TABLE 1. Relative quantities of covalently closed circular EBV DNA extracted from drug-treated and control cultures[a]

Culture	Treatment	10^5 cpm[b]	10^2 μg of DNA[c]	10^2 cpm/μg of DNA	Ratio[d]
Raji	Control	1.14	4.60	2.48	1.00
	PAA	1.49	5.20	2.86	1.15
	ACV	1.28	4.60	2.78	1.12
P3HR-1	Control	5.06	4.80	1.05	1.00
	PAA	1.62	4.80	0.34	0.32
	ACV	1.58	5.00	0.32	0.30

[a] Summary of data in Fig. 2.
[b] Total gradient counts per minute minus background.
[c] Total DNA in cells from which the covalently closed circular DNA was obtained. Total DNA was determined by the diphenylamine reaction (2) with salmon sperm DNA as standard.
[d] Ratios were obtained by dividing each value in the column headed 10^2 cpm/μg of DNA by the Raji or P3HR-1 control value in that column.

TABLE 2. Relative quantities of circular EBV DNA banding at the superhelical density[a]

Culture	Treatment	10^5 cpm[b]	10^2 μg of DNA[c]	10^2 cpm/μg of DNA	Ratio[d]
Raji	Control	0.61	4.60	1.33	1.00
	PAA	0.63	5.20	1.21	0.91
	ACV	0.81	4.60	1.76	1.32
P3HR-1	Control	2.22	4.80	4.83	1.00
	PAA	1.01	4.80	2.10	0.44
	ACV	1.02	5.00	2.04	0.51

[a] Summary of data in Fig. 2.
[b] Total counts per minute banding at the superhelical density (fractions 6 to 9, Raji; fractions 3 to 6, P3HR-1 [Fig. 2]).
[c,d] As indicated in Table 1.

TABLE 3. Relative quantities of covalently closed circular EBV DNA in TPA-treated cells[a]

P3HR-1 culture treatment	Hybridized to:	10^5 cpm[b]	10^2 μg of DNA[c]	10^2 cpm/μg of DNA	Ratio[d]
Control	Gradient	1.49	7.46	2.00	1.00
TPA	Gradient	2.75	7.12	3.86	1.93
Control	Superhelical DNA	0.54	7.46	0.72	1.00
TPA	Superhelical DNA	1.35	7.12	1.90	2.64

[a] P3HR-1 cells were exposed to 40 ng of TPA per ml for 4 days. The total quantity of covalently closed circular EBV DNA and the quantity of EBV DNA banding at the superhelical density were determined as described in the legends to Fig. 1 and 2 and in Tables 1 and 2.
[b] Counts of ^{32}P per minute.
[c] As indicated in Table 1.

2). The synthesis of EBV DNA in virus producer cultures exposed to PAA and ACV was observed previously (3, 4, 8, 18). The resistant DNA is attributed to the synthesis of circular latent genomes in virus nonproducer cells. The viral DNA in nonproducer cells is nucleosomal in structure (16), and like cellular chromatin, it is synthesized by enzymes (presumably host enzymes) that are resistant to inhibitors of viral DNA polymerase activity.

In summary, these studies show that the circular EBV DNA isolated from a virus producer culture is a mixture of molecules synthesized by different enzymes. Most of the circular EBV DNA is synthesized by the EBV-associated DNA polymerase insofar as the synthesis of most of the DNA is blocked by compounds that inhibit viral enzyme activity. A provisional assignment based on these findings is that the circular genomes synthesized by the viral enzyme are intermediates in the synthesis of linear virus DNA.

This work was supported by Ohio State University Bremer funds and by grant IN16U from the American Cancer Society. Acyclovir was a gift from the Burroughs Wellcome Co.

LITERATURE CITED

1. **Adams, A., and T. Lindahl.** 1975. Epstein-Barr virus genomes with properties of circular DNA molecules in carrier cells. Proc. Natl. Acad. Sci. U.S.A. **72**:1477–1481.
2. **Burton, K.** 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.
3. **Colby, B. M., J. E. Shaw, A. K. Datta, and J. S. Pagano.** 1982. Replication of Epstein-Barr virus DNA in lymphoblastoid cells treated for extended periods with acyclovir. Am. J. Med. **73**:77–81.
4. **Colby, B. M., J. E. Shaw, G. B. Elion, and J. S. Pagano.** 1980. Effect of acyclovir [9-(2-hydroxyethoxymethyl)guanine] on Epstein-Barr virus DNA replication. J. Virol. **34**:560–568.
5. **Datta, A. K., R. J. Feighny, and J. S. Pagano.** 1980. Induction of Epstein-Barr virus-associated DNA polymerase by 12-O-tetradecanoylphorbol-13-acetate. J. Biol. Chem. **225**:5120–5125.
6. **Goodman, S. R., C. Prezyna, and W. Clough-Benz.** 1978. Two Epstein-Barr virus-associated DNA polymerase activities. J. Biol. Chem. **253**:8617–8628.
7. **Griffin, B. E., E. Björck, G. Bjursell, and T. Lindahl.** 1981. Sequence complexity of circular Epstein-Barr virus DNA in transformed cells. J. Virol. **40**:11–19.
8. **Gussander, E., and A. Adams.** 1979. Intracellular state of Epstein-Barr virus DNA in producer cell lines. J. Gen. Virol. **45**:331–340.
9. **Hinuma, Y., M. Konn, J. Yamaguchi, D. J. Wudarski, J. R. Blakeslee, Jr., and J. T. Grace, Jr.** 1967. Immunofluorescence and herpes-type virus particles in the P3HR-1 Burkitt lymphoma cell line. J. Virol. **1**:1045–1051.
10. **Klein, G., T. Lindahl, M. Jondal, W. Leibold, J. Menezes, K. Nilsson, and C. Sundstrom.** 1974. Continuous lymphoid cell lines with characteristics of B cells (bone-marrow-derived) lacking the Epstein-Barr virus-genomes and derived from three human lymphomas. Proc. Natl. Acad. Sci. U.S.A. **71**:3283–3286.
11. **Lindahl, T., A. Adams, G. Bjursell, G. W. Bornkamm, C. Kaschka-Dierich, and U. Jehn.** 1976. Covalently-closed circular duplex DNA of Epstein-Barr virus in a human lymphoid cell line. J. Mol. Biol. **102**:511–530.
12. **Miller, G., and M. Lipman.** 1973. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. Proc. Natl. Acad. Sci. U.S.A. **70**:190–194.
13. **Pritchett, R., M. Pederson, and E. Kieff.** 1976. Complexity of EBV homologous DNA in continuous lymphoblastoid cell line. Virology **74**:227–231.
14. **Pulvertaft, R. J. V.** 1965. A study of malignant tumors in Nigeria by short term tissue culture. J. Clin. Pathol. **18**:261–273.
15. **Rigby, P. W., J. Dieckmann, M. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase 1. J. Mol. Biol. **113**:237–251.
16. **Shaw, J. E., L. F. Levinger, and C. W. Carter, Jr.** 1979. Nucleosomal structure of Epstein-Barr virus DNA in transformed cell lines. J. Virol. **29**:657–665.
17. **Siegel, P. J., W. Clough, and J. L. Strominger.** 1981. Sedimentation characteristics of newly synthesized Epstein-Barr viral DNA in superinfected cells. J. Virol. **38**:880–885.
18. **Summers, W. C., and G. Klein.** 1976. Inhibition of Epstein-Barr virus DNA synthesis and late gene expression by phosphonoacetic acid. J. Virol. **18**:151–155.
19. **Yajima, Y., A. Tanaka, and M. Nonoyama.** 1976. Inhibition of productive replication of Epstein-Barr virus DNA by phosphonoacetic acid. Virology **71**:352–354.
20. **Zur Hausen, H., F. O'Neill, U. Freese, and H. Hecher.** 1978. Persisting oncogenic herpesvirus induced by tumor promoter TPA. Nature (London) **272**:373–375.