Proc. Natl. Acad. Sci. USA
Vol. 77, No. 1, pp. 433–436, January 1980
Cell Biology

Expression of Epstein–Barr virus genes in different cell types after microinjection of viral DNA

(early antigen/viral capsid antigen/thymidine kinase)

A. GRAESSMANN*, H. WOLF†, AND G. W. BORNKAMM‡

*Institut für Biochemie und Molekularbiologie der Freien Universität Berlin, Arnimallee 22, 1 Berlin 33, Germany; Max-von-Pettenkofer-Institut der Universität München, Pettenkoferstr. 9a, 8 München, Federal Republic of Germany; and ‡Institut für Virologie, Zentrum für Hygiene, Universität Freiburg, Hermann-Herderstr. 11, 78 Freiburg, Federal Republic of Germany

Communicated by Werner Henle, October 22, 1979

ABSTRACT **Gene expression of Epstein–Barr virus (EBV) was studied after microinjection of viral DNA into different types of cells. Raji TK⁻ cells, known to express viral gene functions after superinfection with the EBV-P3HR-1 virus strain, were attached to plastic dishes by using anti-lymphocyte IgG, phytohemagglutinin, or concanavalin A as a ligand. It was difficult to inject DNA into the small and fragile Raji cells. After formation of polykaryons by cell fusion, microinjection became more efficient. At 24 hr after injection of P3HR-1 virus DNA, 90–100% of the injected cells expressed the early antigen complex as observed by immunofluorescence staining; 70–80% of the cells simultaneously incorporated [³H]thymidine, indicating that thymidine kinase is expressed after injection of viral DNA. Additionally, synthesis of the virus capsid antigen was demonstrated in 20–30% of the recipient Raji cells. Human diploid fibroblasts, African green monkey kidney cells, and rat fibroblasts, which do not represent natural target cells for EBV, could also be induced to synthesis of early antigen complex by injection of P3HR-1 virus DNA.**

Epstein-Barr virus (EBV) has been found regularly associated with two human malignancies, Burkitt's lymphoma and nasopharyngeal carcinoma (for review, see refs. 1 and 2). The virus transforms human lymphocytes into permanently growing lymphoblasts (3–6) and induces a malignant lymphoproliferative disease upon inoculation into New World primates (7–10). It is of particular interest to study the transforming capacity of EBV because of a possible causative role of the virus in the development of malignancies in man.

One approach to the study of the EBV genome has been comparison of the DNA of viral strains with different biological properties (11–13). The majority of EBV isolates, regardless of whether derived from patients with infectious mononucleosis, Burkitt's lymphoma, or other diseases, are transforming viruses and do not induce the early antigen complex (EA) upon superinfection of Raji cells (a nonproducer cell line of Burkitt origin). In contrast, the strain P3HR-1 (originating from a Burkitt tumor) is a nontransforming virus with the unique capacity to induce the EA in Raji cells (14, 15).

Descriptive comparative studies have been published which identified sequences deleted from or inserted into different viral strains by partial denaturation mapping (16) and by analysis of the restriction enzyme cleavage patterns (17, 18). Although substantial progress has been made in the physical characterization of the EBV genome by Given and Kieff (18), attempts to map biological functions on the genome have not been reported. Considerable effort was made in different laboratories to adapt transfection techniques that appeared to be useful in the study of other herpes viruses (19–21) to EBV; however, so far they have been without success.

Here we report that biological functions of EBV can be studied by microinjection of EBV DNA into different types of cells. Raji cells were selected for microinjection of EBV DNA because they can be abortively infected with P3HR-1 virus, resulting even in virus particle production. In addition, viral gene expression was studied after injection of P3HR-1 virus DNA into human and rat fibroblasts and into African green monkey kidney cells, which are not naturally susceptible to viral infection.

MATERIALS AND METHODS

Cells and Virus. Human embryonic fibroblasts (strain WI-38 and our own isolates), secondary green monkey kidney cells (TC 7 and our own isolates), and rat fibroblasts (rat 1) were grown on glass slides in a minimal essential medium supplemented with 10% fetal calf serum. P3HR-1 cells were obtained from W. Henle; Raji TK⁻ cells were from M. Nonoyama. These cells were grown in RPMI-1640 (GIBCO) with 10% fetal calf serum. Spontaneous reversion of Raji TK⁻ cells to the TK⁺ phenotype was not observed, even though the cells were kept in nonselective medium for many months.

EBV was purified from P3HR-1 cells. Virus was induced by addition of 12-*O*-tetradecanoylphorbol-13-acetate (20 ng/ml; Sigma) 1 day after the cells were split (22). Virus was purified from cells and supernatants as described (23), starting from 1.2 liters of culture fluid, except that dextran T-10 (Pharmacia) was used for gradient centrifugation instead of sucrose (24). The visible virus bands were collected, and the virus was pelleted at 20,000 rpm for 1 hr at 4°C in a SW 27 rotor (Beckman), resuspended in virus suspension buffer (VSB; 0.01 M Tris·HCl/0.01 M KCl/0.005 M EDTA, pH 7.4), and stored at −70°C. One of five virus preparations was labeled with [³H]-thymidine (1 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) 1 day after addition of the phorbol.

Purification of P3HR-1 Virus DNA. Virus isolated from a total of 12 liters of P3HR-1 cell cultures was lysed with 1% sarcosyl/0.025 M Tris·HCl/0.01 M EDTA, pH 8.5, and incubated with proteinase K (100 μg/ml; Merck) at 37°C for 2 hr. Then, 0.01 M Tris·HCl/1 mM EDTA, pH 7.5, and solid CsCl were added to a final volume of 10 ml and a final density of 1.705 g/ml. The lysate was centrifuged for 72 hr at 30,000 rpm in a 50 Ti anglehead rotor (Beckman) at 20°C. Fractions (four drops) were collected from the bottom of the tube through a syringe needle (1.25-mm inner diameter) at a restricted flow rate. Aliquots (3 μl) were assayed for radioactivity, and the DNA concentration was determined in the fractions neighboring the peak fraction by measuring the optical density at 260 nm. The DNA concentration in the peak fraction estimated from the specific activity was 140 μg/ml. A portion (100 μl) of the peak fraction was loaded onto a Sephadex G-50 column (5.0

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: EBV, Epstein–Barr virus; EA, early antigen complex; VCA, virus capsid antigen.

× 0.35 cm) and eluted with 0.01 M Tris·HCl/1 mM EDTA, pH 7.5, in 25-μl fractions. The two peak fractions of the Sephadex column with DNA concentrations of 100 μg/ml were used for microinjection experiments.

Attachment of Raji Cells to Plastic Petri Dishes. Plastic 60-mm petri dishes containing 2.5 ml of 2.5% glutaraldehyde were incubated for 2 hr at room temperature. The plates were washed four times with sterile water and covered with 200 μl of anti-lymphocyte IgG (1.3 mg/ml) (kindly provided by H. Rodt). Alternatively, other ligands known to bind to cell surfaces, such as phytohemagglutinin P (Serva), concanavalin A (Serva), pokeweed mitogen (Serva), and poly(*l*-lysine) (Sigma) were used in a concentration of 100 μg/ml for coating the plates. After incubation for 1–2 hr at 37°C in a humidified atmosphere, the fluid was once more homogeneously distributed on the surface of the plates and allowed to dry. After drying, the plates were washed twice with phosphate-buffered saline without Mg^{2+} and Ca^{2+} and filled with 3 ml of RPMI-1640 medium without serum. Raji cells were washed four times in serum-free RPMI-1640 medium. About 10^7 cells were seeded per 60-mm plate and were allowed to settle for 2–3 hr at 37°C in an atmosphere of 5% CO_2 in air.

Fusion of Raji Cells. Cell fusion was induced by polyethylene glycol 1000 (Sigma) as described by Norwood *et al.* (25). Plates with attached Raji cells were washed three times with phosphate-buffered saline (free of Mg^{2+} and Ca^{2+}) in order to remove loosely bound cells. The saline was aspirated and 1.5–2.5 ml of 45–47% (wt/wt) polyethylene glycol in serum-free RPMI-1640 medium, preincubated at 37°C, was added to the cell layer. The glycol solution was left on the cells for 60–70 sec; then it was removed by three successive washes with phosphate-buffered saline. The cells were incubated in serum-free RPMI-1640 medium at 37°C in a humidified CO_2 incubator. Cell fusion started immediately after polyethylene glycol treatment and proceeded for several hours.

Immunofluorescence Staining. Fluorescence staining for EPV EA and virus capsid antigen (VCA) was by standard procedures using indirect immunofluorescence techniques (14, 26).

Microinjection. Microinjection has been described in detail (27).

Autoradiography. Kodak nuclear track emulsion (NTB) was used at a dilution of 1:10 (in H_2O). After exposure for 3 days at 4°C, autoradiograms were developed with Kodak D-19 developer for 10 min at room temperature. After washing for 5 min in distilled water, autoradiograms were fixed with Kodak Unifix for 5 min, washed again for 10 min in distilled water, and air dried.

RESULTS

Expression of EBV DNA in Raji Cells After Microinjection. Large cells firmly adherent to a solid support are well suited for microinjection experiments. The lymphoid Raji cells, however, grow in suspension and are significantly smaller than most mononuclear cells (e.g., human fibroblasts). Raji cells can be attached efficiently to the surface of coated plastic dishes (Fig. 1*b*). Coating the dishes with anti-lymphocyte IgG, phytohemagglutinin, or concanavalin A as ligands gave results about equally good. In the case of concanavalin A, serum-containing medium could be used. Plates coated with pokeweed mitogen or poly(*l*-lysine) had less binding capacity or appeared

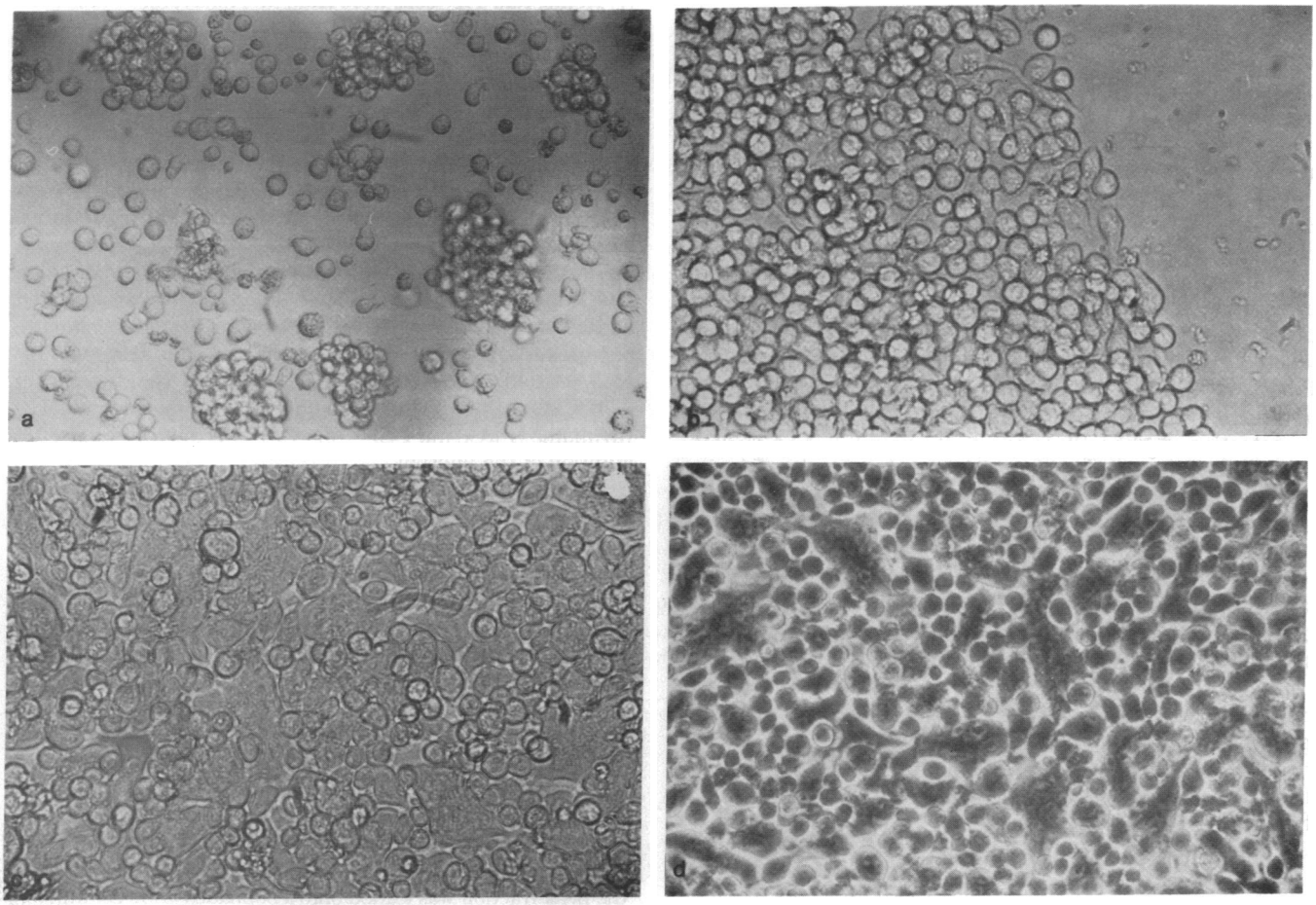

FIG. 1. (*a*) Suspension culture of Raji cells. (*b*) Adherent and spread Raji cells, 5 hr after plating onto an anti-lymphocyte IgG-coated plastic dish; uncoated regions are free of cells. (*c*) Fused Raji cells, 10 hr after addition of polyethylene glycol. (*d*) Phase-contrast picture of fused cells. (×120.)

to be more toxic to the cells. Pretreatment of the cells with glutaraldehyde prior coating was not essential but seemed to give somewhat better reproducible attachment.

Single mononucleated Raji cells tolerated injection of only about 5×10^{-12} ml. This volume could be increased about 10-fold when polyethylene glycol-fused Raji cells containing 5–10 nuclei were used as recipient cells for microinjection (Fig. 1 *c* and *d*). No spontaneous EBV antigen synthesis was observed in cells made adherent and fused.

After injection of EBV DNA (100 μg/ml) into fused Raji cells, the following virus induced functions were tested: (*i*) synthesis of EA; (*ii*) expression of thymidine kinase; and (*iii*) synthesis of VCA. EA and VCA were detected by indirect immunofluorescence; thymidine kinase was studied by uptake of ^{3}H-labeled thymidine into Raji TK$^-$ cells. To study EA and thymidine kinase expression, microinjected cells were incubated for 24 hr in serum-free medium with [^{3}H]thymidine (0.1 μCi/ml), fixed with methanol, stained for EA, and subjected to autoradiography. Of the injected cells, 90–100% stained for EA and 70–80% of these incorporated [^{3}H]thymidine (Fig. 2 *a* and *b*). Grains were observed over EA-positive cells but not over mock-injected or uninjected cells. Also autoradiography of cells immediately after DNA injection did not reveal any grains.

In order to test, whether thymidine incorporation was a consequence of EA induction, EA synthesis was induced in Raji TK$^-$ cells by the diterpene ester 12-*O*-tetradecanoylphorbol-13-acetate TPA. About 10% of the Raji cells expressed EA after 4 days but did not reveal any [^{3}H]thymidine uptake.

VCA expression was studied 48 hr after injection of viral DNA into Raji cells; 20–30% of the recipient cells stained for VCA. The VCA-positive cells were always found to incorporate [^{3}H]thymidine (Fig. 2 *c* and *d*).

The induction of EA and thymidine kinase was specific for intact viral DNA; *Eco*RI digestion of the DNA abolished both effects.

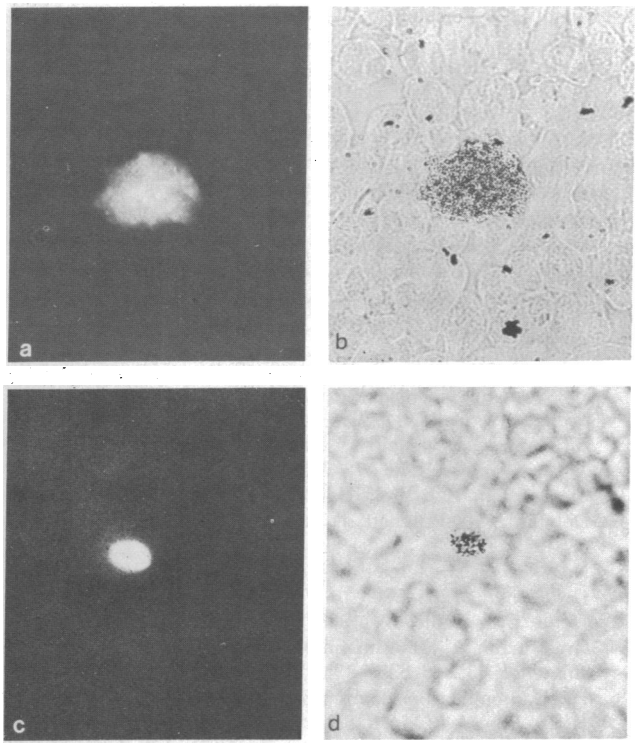

FIG. 2. Expression of viral antigens and thymidine kinase in Raji TK$^-$ cells injected with EBV DNA. (*a*) EA-positive multinucleated Raji cell, 24 hr after injection. (*b*) Autoradiogram of section in *a*. (*c*) VCA-positive mononucleated Raji cell, fixed and stained 48 hr after injection. (*d*) Autoradiogram of area in *c*. (×450.)

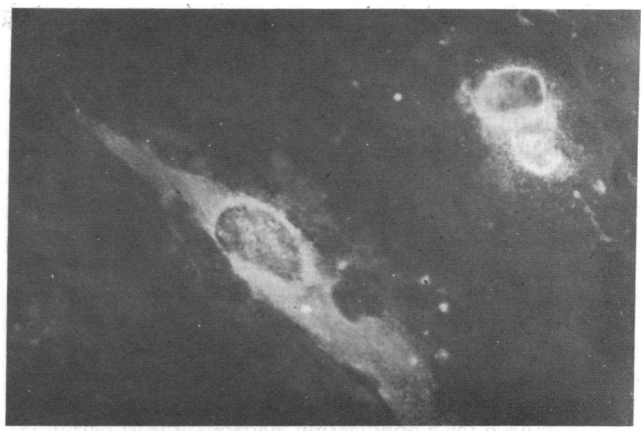

FIG. 3. EA-positive rat fibroblasts, fixed and stained 48 hr after injection of EBV DNA. (×450.)

The presence of a vast majority of uninjected cells surrounding a defined number of injected cells within a defined area of the plate served as an internal control for antigen expression and thymidine kinase activity.

Expression of EBV DNA in Human Fibroblasts, Monkey Kidney Cells, and Rat Fibroblasts. Expression of EA and VCA was also studied in human fibroblasts, secondary African green monkey kidney cells, and rat fibroblasts after microinjection of EBV DNA. These cells do not represent natural target cells for EBV and cannot be infected by the virus.

At 48 hr after injection, about one-fourth human fibroblasts, monkey kidney cells, and rat fibroblasts revealed EA staining (Fig. 3). Fig. 4 shows the time course of EA appearance in WI-38 cells injected with viral DNA. EA-positive cells were first observed at 48 hr after injection. At this time, 25–30% of the cells exhibited EA fluorescence. With prolonged incubation the number of EA-positive cells decreased slightly. After 8 days, 15% of the injected cells were still EA positive. No VCA synthesis could be observed in cells studied 48, 72, or 96 hr after injection of viral DNA.

Digestion of P3HR-1 virus DNA with *Eco*RI destroyed the EA-inducing activity.

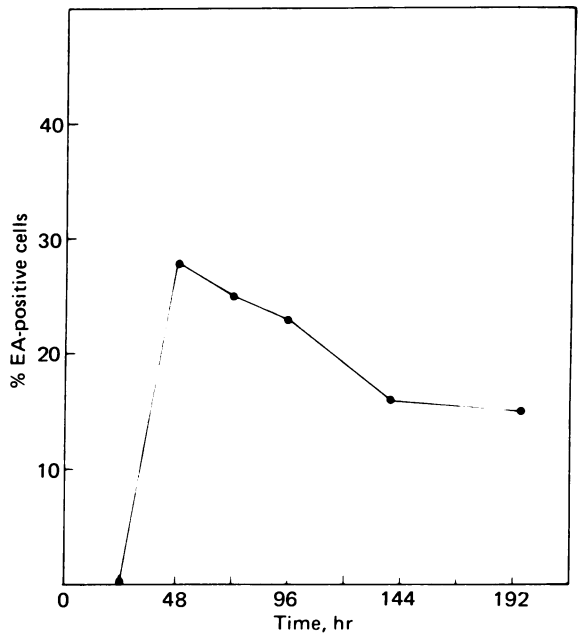

FIG. 4. Time course of EA synthesis in human fibroblasts. Each time point is based on 100 injected cells.

DISCUSSION

Microinjection allows the transfer of about 10^{-11} ml into each mononucleated tissue culture cell (27). For a DNA with a molecular weight of 100×10^6 the DNA concentration has to be at least 17 μg/ml in order to transfer statistically one viral genome per injected cell. Obviously, higher DNA concentrations should be used to obtain an efficient transfer of intact DNA into the cells, because some of the DNA might be sheared during the injection step. Induction of EBV synthesis in carrier cell lines by diterpene esters increases the yield of virus DNA considerably (22, 28). This enabled us to prepare viral DNA in a concentration of 100 μg/ml from CsCl density gradients without any other concentration procedure, which may lead to fragmentation of the high molecular weight DNA. Freezing of the DNA, required for lyophilization, destroys at least part of the biological activity of EBV DNA and was therefore avoided. (P3HR-1 virus DNA at a concentration of 25 μg/ml, which was used in the initial experiments, lost its EA-inducing activity after two cycles of freezing and thawing.)

It was not possible to use the small and not adherent lymphoid Raji cells directly for microinjection. But we were able to anchor these cells by using plates coated with anti-lymphocyte IgG, phytohemagglutinin, or concanavalin A, and the problem of the small cell size of the lymphoblasts was solved by cell fusion of the attached cells to polykaryons.

Because Raji cells were attached to plastic petri dishes, acetone could not be used for fixation, and the cells had to be fixed with methanol, which inactivates the R component of the EA (29). Thus, only the expression of the EA D component could be studied.

We have shown that P3HR-1 virus DNA is capable of inducing EA, VCA, and thymidine kinase after injection of viral DNA into Raji cells. From these experiments, however, no conclusion can be drawn as to whether the genes expressed after viral DNA injection are encoded by the input viral DNA or by endogenous EBV genomes. It even cannot be excluded that thymidine kinase expression is due to the activation of a previously silent cellular gene. It was our main objective to demonstrate that injected viral DNA shows the same biological activity as the infecting P3HR-1 virus.

The high rate of conversion of Raji cells to EA expression after injection of P3HR-1 virus DNA indicates that the injection process is highly efficient. This is not surprising if one considers that, in plaque-purified simian virus 40 preparations, about 100 particles are required to yield 1 plaque-forming unit in tissue culture infection, whereas 1 virus particle is infectious upon injection into the cell (30).

EA can also be induced in virus-nonsusceptible human fibroblasts, African green monkey kidney cells, and rat fibroblasts after injection of P3HR-1 virus DNA. This demonstrates that the natural barrier against an EBV infection of these cells must be located in one of the early events—adsorption, penetration, or uncoating. Virus adsorption studies indicate that these cells do not possess receptors for EBV (unpublished observation). A similar mode of host range restriction, which could be by-passed by injection of virus particles or viral DNA, has been demonstrated for simian virus 40 and polyoma viruses (for review, see ref. 30). The fact that human fibroblasts, monkey kidney cells, and rat fibroblasts did not synthesize viral capsid proteins may point to an additional regulation mechanism for the expression of late viral genes in these cells.

Until now, no EBV nuclear antigen expression could be detected after injection of viral DNA into human and rat fibroblasts. The reason for this may be technical and requires further investigation.

The expression of EBV genes after microinjection of viral DNA obviously provides an approach to mapping biological functions on the viral genome. We have been able to induce EA by injection of DNA fragments, which will be reported in detail elsewhere. It will be of special interest to study the transforming potential of EBV DNA and of EBV DNA fragments and to learn if transformation can be initiated by injection of viral DNA into human fibroblasts.

The excellent technical assistance of E. Guhl, M. Haus, and U. Zimber is gratefully acknowledged. We thank Dr. H. zur Hausen and Dr. H. Delius for helpful discussions and critically reading the manuscript. This work was supported by Die Deutsche Forschungsgemeinschaft (Gr. 384/5, Ha 449/12, Wo 227/2, and SFB 51).

1. zur Hausen, H. (1975) *Biochim. Biophys. Acta* **417**, 25–53.
2. Henle, W. & Henle, G. (1979) in *The Epstein-Barr Virus*, eds. Epstein, M. A. & Achong, B. G. (Springer, Berlin), pp. 61–78.
3. Henle, W., Diehl, V., Kohn, G., zur Hausen, H. & Henle, G. (1967) *Science* **157**, 1064–1065.
4. Pope, J. H., Horne, M. K. & Scott, W. (1969) *Int. J. Cancer* **4**, 255–260.
5. Gerber, P., Whang-Peng, J. & Monroe, J. H. (1969) *Proc. Natl. Acad. Sci. USA* **63**, 740–747.
6. Miller, G., Lisco, H., Kohn, H. L. & Stitt, D. (1971) *Proc. Soc. Exp. Biol. Med.* **37**, 1459–1465.
7. Shope, T., Dechairo, D. & Miller, G. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2487–2491.
8. Epstein, M. A., Hunt, R. D. & Rabin, H. (1973) *Int. J. Cancer* **12**, 309–318.
9. Wolf, H., Werner, J. & zur Hausen, H. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39**, 791–796.
10. Miller, G., Shope, T., Coope, D., Waters, L., Pagano, J. S., Bornkamm, G. W. & Henle, W. (1977) *J. Exp. Med.* **145**, 948–967.
11. Sugden, B., Summers, W. C. & Klein, G. (1976) *J. Virol.* **18**, 765–775.
12. Hayward, S. D. & Kieff, E. (1977) *J. Virol.* **23**, 421–429.
13. Rymo, L. & Forsblom, S. (1978) *Nucleic Acids Res.* **5**, 1387–1402.
14. Henle, W., Henle, G., Zajac, B. A., Pearson, G., Waubke, R. & Scriba, M. (1970) *Science* **169**, 188–190.
15. Miller, G., Robinson, J., Heston, L. & Lipman, M. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 4006–4010.
16. Delius, H. & Bornkamm, G. W. (1978) *J. Virol.* **27**, 81–89.
17. Raab-Traub, N., Pritchett, R. & Kieff, E. (1978) *J. Virol.* **27**, 388–398.
18. Given, D. & Kieff, E. (1978) *J. Virol.* **28**, 524–542.
19. Sheldrick, P., Laithier, M., Lando, D. & Ryhiner, M. L. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3621–3625.
20. Fleckenstein, B., Bornkamm, G. W. & Ludwig, H. (1975) *J. Virol.* **15**, 398–406.
21. Miller, G., Wertheim, P., Wilson, G., Robinson, J., Geelen, J. L. M. C., van der Noordaa, J. & van der Eb, A. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 949–953.
22. zur Hausen, H., O'Neill, F., Freese, U. K. & Hecker, E. (1978) *Nature (London)* **272**, 373–375.
23. Fleckenstein, B. & Wolf, H. (1974) *Virology* **58**, 55–64.
24. Dolyniuk, M., Pritchett, R. & Kieff, E. (1976) *J. Virol.* **17**, 935–949.
25. Norwood, T. H., Zeigler, C. J. & Martin, G. M. (1976) *Somatic Cell Genet.* **2**, 263–270.
26. Henle, G. & Henle, W. (1966) *J. Bacteriol.* **91**, 1248–1256.
27. Graessmann, A., Graessmann, M. & Mueller, C. (1980) *Methods Enzymol.* **65**, 816–825.
28. zur Hausen, H., Bornkamm, G. W., Schmidt, R. & Hecker, E. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 782–785.
29. Henle, G., Henle, W. & Klein, G. (1971) *Int. J. Cancer* **8**, 272–282.
30. Graessmann, A., Graessmann, M. & Mueller, C. (1979) *Curr. Top. Microbiol. Immunol.* **87**, 1–22.