JOURNAL OF VIROLOGY, July 1976, p. 187-194
Vol. 19, No. 1

Printed in U.S.A.

Mechanisms of Infection with Epstein-Barr Virus

I. Viral DNA Replication and Formation of Noninfectious Virus Particles in Superinfected Raji Cells

YOSHIHIRO YAJIMA AND MEIHAN NONOYAMA*

Departments of Microbiology, Rush-Presbyterian-St. Luke's and University of Illinois Medical Centers, Chicago, Illinois 60612*

Received for publication 3 February 1976

Human lymphoblastoid Raji cells, which do not produce virus, supported replication of Epstein-Barr virus (EBV) upon superinfection. Early antigen, viral capsid antigen, and virions were produced in Raji cells superinfected with EBV. Viral DNA replicated under complete inhibition of host cell DNA synthesis to the extent that a few micrograms of EBV DNA were recovered from 10^7 superinfected Raji cells, corresponding to 5,000 viral genomes/cell. Homology of the synthesized viral DNA to parental EBV DNA was more than 90%. Virions produced by the Raji cells contained a 55S DNA but failed to induce early antigen, viral capsid antigen, and viral DNA synthesis after a second superinfection of Raji cells.

The mechanism of replication of Epstein-Barr virus (EBV) DNA, expression of EBV genomes, and host cell control over EBV replication must be studied to understand oncogenicity of EBV, which has been suspected to induce human cancers such as Burkitt's lymphoma and nasopharyngeal carcinoma (2, 4, 5).

As previously reported (12), Raji cells derived from a Burkitt lymphoma patient retained 50 to 60 latent EBV genomes per cell after propagation in vitro for many passages but produced neither early antigen (EA) nor viral capsid antigen (VCA). Raji cells superinfected by EBV prepared from virus-producing P3 HRI cells caused induction of membrane antigens and EA (1, 6, 9, 10, 13). Only few, if any, cells, however, formed VCA, and no infectious virus was produced. DNA synthesis of host cells was suppressed, whereas EBV DNA replicated to some extent and EBV genome equivalents increased to 1,000/cell (12, 13). These results indicated that some late step(s) of virus replication must be blocked either by a host cell mechanism or by defective parental EBV genomes used for superinfection.

In this report, we have studied further details of superinfection of Raji cells with EBV.

MATERIALS AND METHODS

Cells and medium. P3 HRI cells producing EBV (HRI EBV) were propagated at 35 C in 800-ml glass bottles with 200 ml of RPMI-1640 medium containing 5% calf serum. EBV nonproducer Raji cells and Simpson cells, free of EBV genomes, which were derived from a human myeloblastic leukemia patient (7), were also grown in RPMI 1640 medium supplemented with 5% calf serum. Superinfection was carried out in phosphate-free minimum essential medium (MEM) containing 2% calf serum.

Superinfection of Raji cells with EBV. A total of 5×10^6 Raji cells were infected with 1 ml of EBV concentrate, which was prepared by pelleting viruses from 200 ml of supernatant fluid of 4×10^8 HRI cells at 30,000 $\times$ *g* at 4 C for 1 h. After 1 h of adsorption at 35 C, the superinfected cells were collected by centrifugation at 300 $\times$ *g* for 10 min, resuspended in 5 ml of fresh phosphate-free MEM, and incubated at 35 C. The multiplicity of infection was 2 to 3, determined by EA-inducing units as described previously (13). The superinfected cells were labeled with [^{3}H]thymidine (1 to 100 μCi/ml) or ^{32}P (0.01 to 1 mCi/ml) between 10 and 60 h after infection. DNA was extracted from the superinfected Raji cells as follows. Cells were collected and suspended in 2 ml of 0.05 M Tris-hydrochloride (pH 9.0), 0.01 M EDTA, and 0.4 M NaCl, and then proteinase K and Sarkosyl were added to final concentrations of 100 μg/ml, and 1%, respectively. After incubation at 37 C for 3 to 15 h, DNA was extracted with phenol and precipitated by addition of 2 volumes of ethanol.

Preparation of ^{32}P-labeled EBV DNA from HRI cells. HRI cells were labeled with 100 μCi of carrier-free phosphate per ml in phosphate-free MEM for 3 days, and the cells were harvested and suspended in 0.05 M Tris, pH 9.0, 0.01 M EDTA, and 0.4 M NaCl. Cells were lysed with proteinase (100 μg/ml) and sodium dodecyl sulfate (1%), and 55S DNA was isolated from a glycerol gradient (10 to 30%) after centrifugation at 21,500 rpm at 18 C for 15 h in an SW27 rotor. 55S viral DNA was further purified through two cycles of CsCl equilibrium centrifugation at 38,000 rpm at 18 C for 48 h in a 50Ti angle rotor. The prepared [^{32}P]viral DNA had a specific radioac-

tivity of 10^5 counts/min per μg and did not reassociate at an increased rate together with excess of Simpson cell DNA.

DNA-DNA reassociation kinetics. DNA-DNA reassociation kinetics were performed as previously reported (7). Small amounts of ^{32}P-labeled EBV DNA, prepared from superinfected Raji cells or directly from HRI cells, were mixed with excess amounts of Raji, Simpson, salmon sperm DNA, or [^{3}H]EBV DNA from superinfected Raji cells in 0.01 M Tris-hydrochloride (pH 7.5)-0.001 M EDTA. After boiling for 15 min, the NaCl concentration of the mixtures was adjusted to 0.5 or 1.5 M in a final volume of 1 ml. DNA-DNA reassociation kinetics were conducted at 66°C, and the amount of reassociated DNA was assayed by S_1 nuclease digestion (200 U/0.5 ml) in 0.03 M acetate buffer, pH 4.5, 7.2 $\times$ 10^{-3} M zinc acetate, and 0.2 M NaCl at 37 C for 3 h.

Isolation of virus particles. Raji cells superinfected with EBV were harvested, suspended in RSB buffer (Tris-hydrochloride, pH 7.1, 0.1 M; KCl, 0.01 M; $MgCl_2$, 0.01 M), and incubated for 30 min at room temperature, followed by a 10-min incubation with 1% Nonidet P-40. The cells were treated by adding 1/10 volume of 5 M NaCl, and the lysate was layered on top of a 30 to 55% (wt/wt) sucrose gradient in TNM buffer (Tris-hydrochloride, pH 7.5, 0.01 M; NaCl, 0.15 M; $MgCl_2$, 0.01 M). After centrifugation at 25,000 rpm at 4 C for 2 h in an SW27 rotor, the gradient was fractionated from the bottom of the tube, and radioactivity in a portion of each fraction was counted in 3A70B scintillation liquid (Research Products International Corp.) in a Packard Tri-Carb scintillation counter.

Determination of sedimentation value for the isolated virion DNA. Virions prepared from superinfected Raji cells were lysed with 100 μg of proteinase K per ml and 1% Sarkosyl. DNA was loaded onto a 10 to 30% (vol/vol) glycerol gradient in TNE buffer (Tris, 0.01 M, pH 7.4; NaCl, 1.0 M; EDTA, 0.001 M) and centrifuged at 30,000 rpm at 10 C for 6 h in an SW41 rotor in a Beckman ultracentrifuge. Herpes simplex virus type 2 DNA served as a marker for the sedimentation value of 55*S* (8).

RESULTS

Replication of EBV DNA in superinfected Raji cells. Superinfection of Raji cells with EBV in phosphate-free MEM caused complete inhibition of cellular DNA synthesis. Almost all DNA replicated in the superinfected cells after 10 h of infection had a density of 1.718 g/cm^3 in CsCl, specific for EBV DNA (Fig. 1), and showed a sedimentation value of 55*S* (Fig. 2). The yield of EBV DNA was about 2 to 3 μg from 5 $\times$ 10^6 superinfected Raji cells (determined after alcohol precipitation of CsCl peak fractions), and the specific radioactivity of EBV DNA, 2 $\times$ 10^6 to 5 $\times$ 10^6 counts/min per μg, was obtained when 100 μCi of [^{3}H]thymidine or 1 mCi of ^{32}P per ml was added to the medium of superinfected Raji cells. The DNA thus prepared

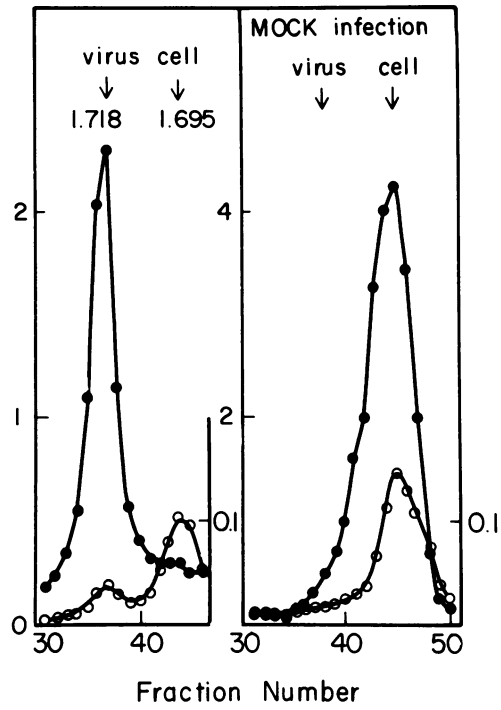

FIG. 1. *CsCl equilibrium centrifugation of DNA prepared from Raji cells superinfected with EBV. A 5-ml portion of Raji cells (10^6 cells/ml) was infected with EBV concentrated from 200 ml of P3 HRI supernatant fluid. A 10-μCi/ml amount of [^{3}H]thymidine was added to the culture from 10 to 60 h after infection, and DNA was extracted from the superinfected Raji cells by proteinase K-sodium dodecyl sulfate treatment, followed by phenol extraction and ethanol precipitation of DNA. DNA was dissolved in 0.01 M Tris-hydrochloride, pH 7.5, 0.001 M EDTA (TE), centrifuged in CsCl at 38,000 rpm at 18 C for 48 h in a type 50Ti angle rotor. Fractions were collected in 80 tubes by puncturing the bottoms of the tubes. A 0.5-ml portion of TE was added to each fraction, and the optical density at 260 nm was measured. A portion of each fraction was counted for radioactivity. Mock infection was carried out in the same manner without addition of virus concentrate. Symbols: ●, ^{3}H counts per minute (10^{-4}); ○, optical density at 260 nm.*

showed a single symmetrical peak of 1.718 g/cm^3 in CsCl by analytical centrifugation in a Beckman model E centrifuge (Miyagi, personal communication). The number of EBV genomes in superinfected Raji cells was estimated at approximately 5,000 genomes/cell, calculated from the yield of viral DNA and from the ratio between the molecular weights of virus DNA (10^8) and cellular DNA (4 $\times$ 10^{12}). When RPMI 1640 was used for superinfection, results were not as satisfactory as in phosphate-free MEM (Fig. 3). Therefore, phosphate-free MEM was used throughout the experiment, which gave us

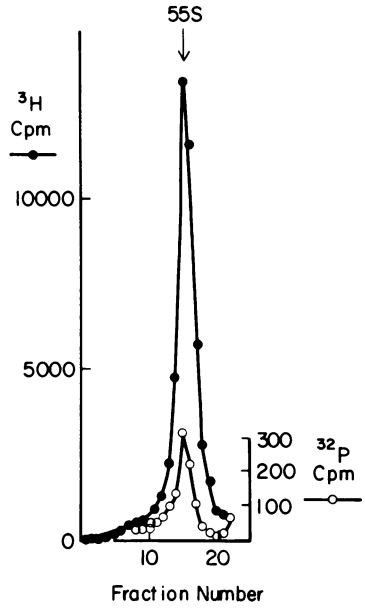

FIG. 2. *Sedimentation of viral DNA from Raji cells superinfected with EBV. [^{3}H]viral DNA from Fig. 1, obtained from superinfected Raji cells, was centrifuged through a 10 to 30% glycerol gradient with ^{32}P-labeled herpes simplex virus type 2 DNA in an SW41 rotor for 6 h at 30,000 rpm at 18 C. Fractions were collected from the top of the tube. Sedimentation was from left to right. Symbols: ●, [^{3}H]viral DNA from Fig. 1, obtained by superinfection of Raji cells; ○, ^{32}P-labeled herpes simplex virus type 2 DNA.*

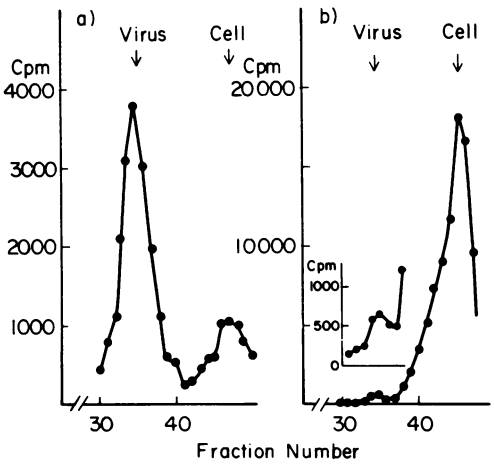

FIG. 3. *Comparison of media for efficiency of the superinfection. The conditions were described in Materials and Methods and in Fig. 1. (a) Superinfection in phosphate-free MEM; (b) superinfection in RPMI 1640.*

an additional advantage for ^{32}P labeling of viral DNA.

Reassociation kinetics of EBV DNA obtained from superinfected Raji cells. Radioactive viral DNA thus obtained from superinfected Raji cells was able to reassociate at an increased rate to Raji cell DNA, which contained EBV DNA homologous to HRI-EBV DNA (14) but not to EBV genome-free Simpson cell DNA or to salmon sperm DNA (Fig. 4), indicating that radioactive DNA from superinfected Raji cells was mostly EBV DNA and contained little radioactive cellular DNA.

To test the complexity of viral DNA from superinfected Raji cells, small amounts of [^{32}P]EBV DNA prepared directly from HRI cells were reassociated with excess amounts of [^{3}H]viral DNA from superinfected Raji cells. [^{32}P]HRI-DNA was reassociated together with [^{3}H]viral DNA from superinfected Raji cells to the extent of more than 90% (Table 1). Therefore, there was no significant difference revealed by the reassociation kinetics between parental HRI-EBV DNA and viral DNA obtained from superinfected Raji cells.

Production of EBV particles in superinfected Raji cells. In previous reports, EA, but little if any VCA, was detected in superinfected Raji cells (3, 6). However, with superinfection

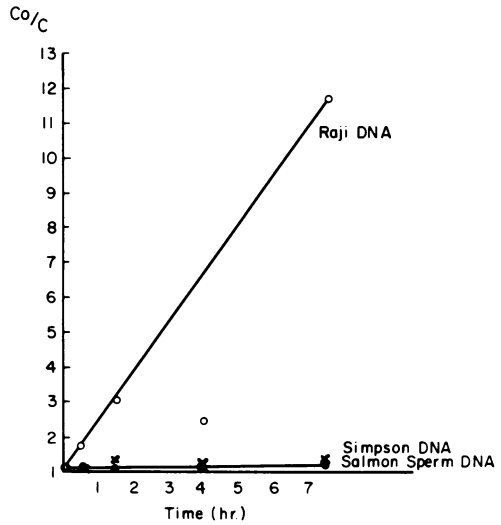

FIG. 4. *Specificity of reassociation kinetics of EBV DNA obtained from superinfected Raji cells. Heat-denatured ^{32}P-labeled EBV DNA (0.005 μg) from superinfected Raji cells was incubated with 1 mg of heat-denatured Raji, Simpson, or salmon sperm DNA and allowed to be reassociated at 66 C in 1.5 M NaCl. Amounts of reassociated DNA were assayed by S_1 nuclease. Symbols: ○, Raji DNA; ●, Simpson DNA; ×, salmon sperm DNA.*

TABLE 1. *Homology between viral DNA from superinfected Raji cells and EBV DNA from HRI cells*

DNA[a]	Label	% Reassociation		
		0	1 h	5 h
[^{32}P]HRI virus DNA	^{32}P	6.2	8.3	20.2
[^{32}P]HRI virus DNA + ^{3}H-labeled superinfected Raji virus DNA	^{32}P	5.5	64.8	93.1
	^{3}H	5.9	65.3	94.8

[a] [^{32}P]HRI virus DNA was directly prepared from HRI cells by isolation of 55S DNA in a glycerol gradient and repeated centrifugation in CsCl, [^{3}H]viral DNA from superinfected cells (2 μg, 10^4 counts/min) and [^{32}P]HRI-EBV DNA (0.1 μg, 10^4 counts/min) were mixed, boiled, and reassociated at 66 C in 0.5 M NaCl. Reassociated DNA was measured by S_1 nuclease.

in phosphate-free MEM, not only EA but also VCA were efficiently produced, as demonstrated by immunofluorescent tests. Figure 5 indicates a linear relationship between the appearance of VCA and the concentration of EBV used for superinfection (showing 30% of cells positive for VCA). The efficient induction of VCA in superinfected Raji cells suggested possible formation of virus particles, which was confirmed by electron microscopy. Both intracellular (Fig. 6) and extracellular (Fig. 7) virions were detected in superinfected Raji cells. Virions were isolated from superinfected Raji cells by Nonidet P-40 extraction and subsequent centrifugation in 30 to 60% (wt/wt) sucrose gradients. Figure 8 shows that the EBV peak appeared at 48% sucrose, the expected density of EBV nucleocapsids. 55S DNA of complete unit length was also obtained from isolated virions by proteinase K-Sarkosyl treatment (Fig. 9). Virion preparations, one obtained from supernatant fluid of superinfected Raji cells and the other prepared by freeze-thawing of superinfected Raji cells, were assayed for infectivity by superinfection of Raji cells. Neither EA nor VCA was observed, and no radioactive viral DNA was detected by CsCl centrifugation of DNA extracted from the superinfected Raji cells, indicating that the virions produced were not infectious for Raji cells.

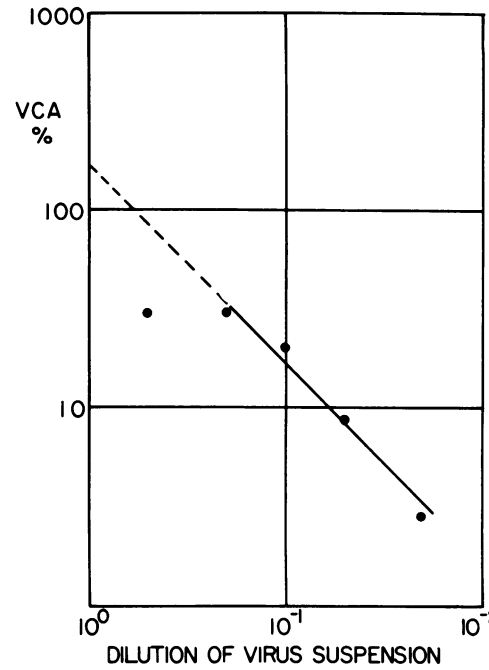

FIG. 5. *Appearance of VCA in Raji cells superinfected with various concentrations of EBV. Raji cells were infected with EBV at an appropriate dilution, and cell smears were prepared at 48 h after infection by acetone fixation. VCA was determined, using anti-VCA human serum, by indirect immunofluorescent test.*

DISCUSSION

The present results indicate that superinfection of Raji cells with EBV in phosphate-free MEM is a useful system for studying EBV replication and host-virus interaction. Superinfection in RPMI 1640 or in MEM with phosphate occasionally resulted in a good infection, as examined by the extent of viral DNA replication, but results were not consistent. It seems that phosphate depletion from the medium is not an absolute requirement for EBV replication, but cells in phosphate-free MEM tend to become physiologically favorable for the viral infection. Although no infectious virus was recovered from the superinfected cells in phosphate-free MEM, EBV DNA was synthesized extensively with formation of EBV particles under complete inhibition of host cell DNA synthesis. The synthesized EBV DNA showed DNA homology of more than 90% to parental HRI-EBV DNA.

Practically, it is now possible to prepare a few micrograms of EBV DNA of complete size from 10^7 superinfected Raji cells, whereas we needed at least 10 to 20 liters of HRI culture for preparation of the equivalent amount of EBV DNA. Furthermore, highly radioactive EBV DNA was easily prepared by this method to perform DNA-DNA reassociation kinetics. Complementary RNA was also prepared from this DNA with the same extent of background

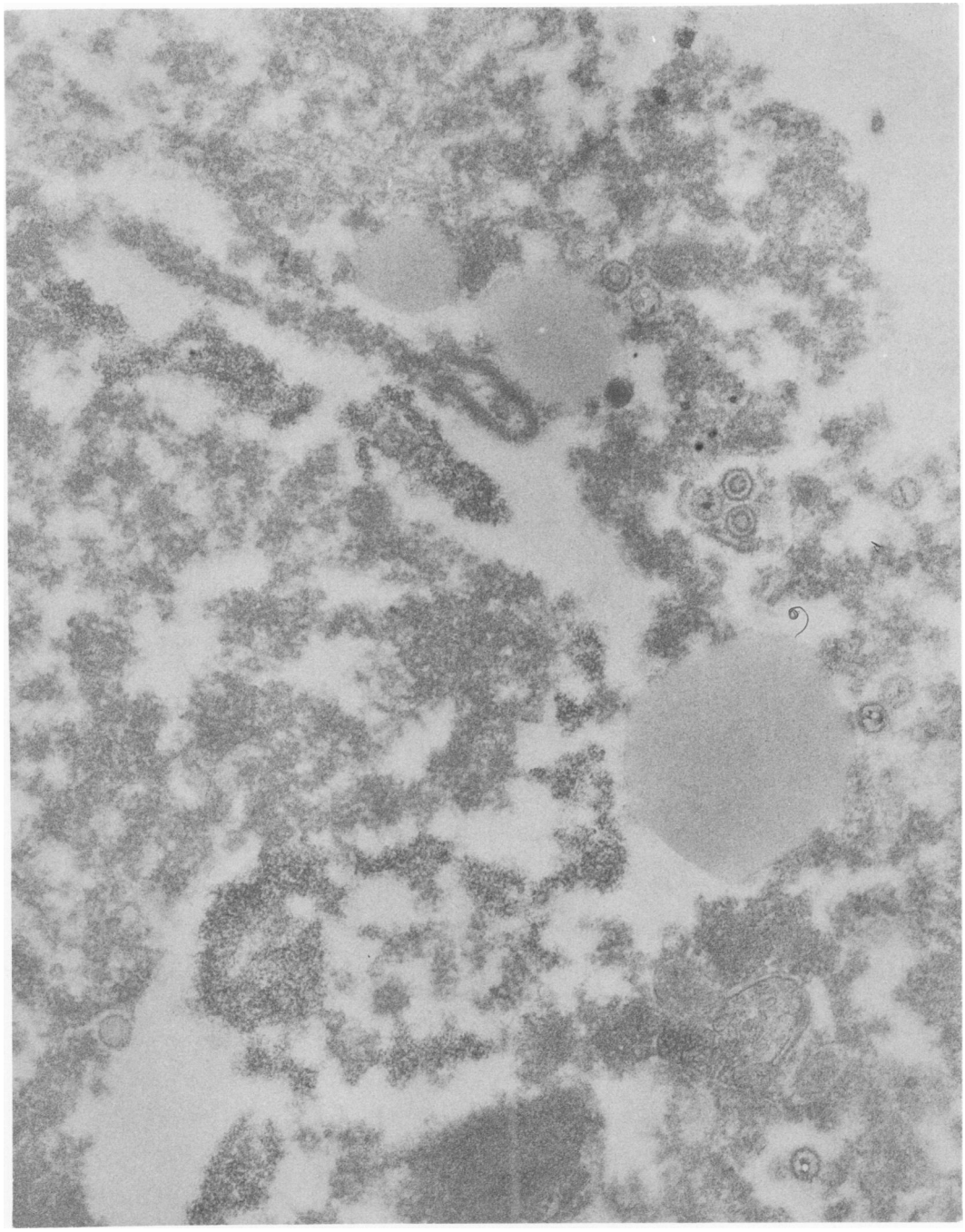

FIG. 6. *Observation of intracellular viruses in superinfected Raji cells. Superinfected Raji cells were fixed with glutaraldehyde in cacodylate buffer (pH 7.2) and with osmic acid.*

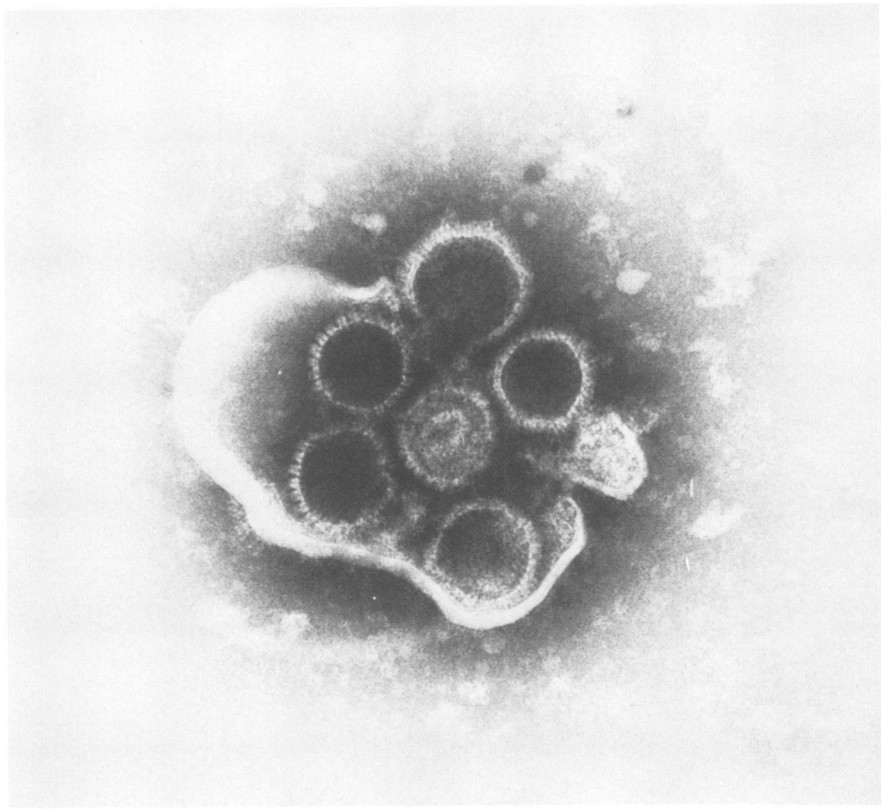

FIG. 7. *Extracellular viruses in supernatant fluid of superinfected Raji cells. Virus was negatively stained with 2% phosphotungstic acid, pH 7.0.*

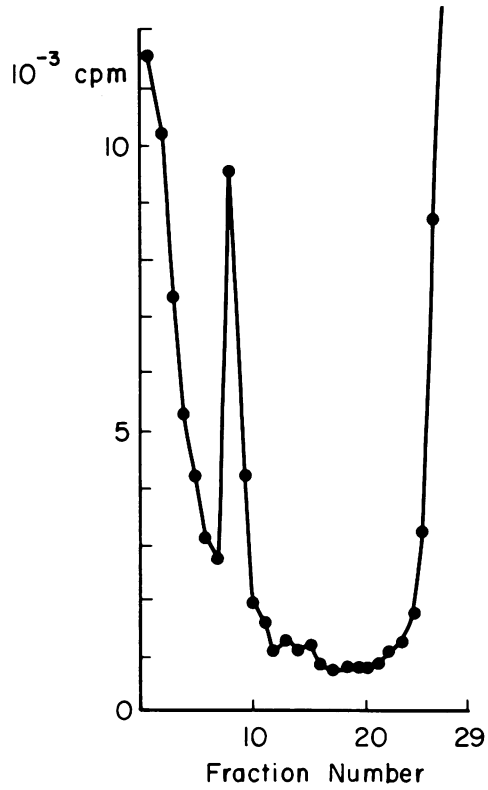

FIG. 8. *Centrifugation of virions isolated from superinfected Raji cells in a sucrose gradient. Raji cells (10^6 cells/ml) were infected with EBV concentrate as in Fig. 1. A 25-μCi portion of ^{32}P per ml was added 20 h after infection, and the cells were harvested after 5 days of incubation at 35 C. Virions were isolated and centrifuged in a 30 to 55% (wt/wt) sucrose gradient as described in the text. The bottom of 55% sucrose is on the left.*

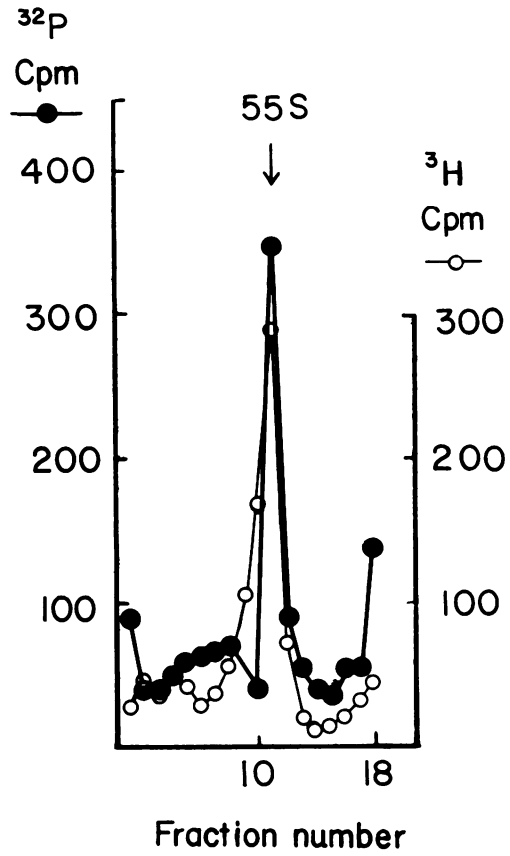

FIG. 9. *Sedimentation of EBV from virions recovered from superinfected Raji cells. EBV obtained as in Fig. 6 was lysed with 100 μg of proteinase K per ml and 1% sarkosyl and layered directly on a 10 to 30% (vol/vol) glycerol gradient and centrifuged in a Spinco SW41 rotor for 6 h at 30,000 rpm and 18 C. Fractions were collected from the top of the tube. Sedimentation was from left to right. Symbols: ●, [^{32}P]viral DNA; ○, ^{3}H-labeled herpes simplex virus type 2 DNA.*

hybridization to cellular DNA as complementary RNA prepared from HRI virus DNA.

Virus DNA replicated in superinfected Raji cells presumably originated from superinfecting virus DNA and not from latent virus DNA, based on the result of Klein et al. (10) that infection of EBV genome-negative BJA-B cells with EBV induced EA. We do not know, however, whether or not latent viral DNA was also induced to replicate by the superinfection.

Virus recovered from Raji cells superinfected with EBV were not infectious upon a second superinfection of Raji cells, for which several possibilities can be considered: (i) Raji cells do not permit production of infectious progeny EBV; (ii) EBV from HRI cells itself is a defective virus; (iii) virus DNA replicated in superinfected Raji cells is altered or modified; (iv) viral coat proteins are nonfunctional; (v) the choice of host cells for the second infection was wrong and the infection should be done by using cells free of EBV genomes such as BJA-B (10) or human cord blood cells.

In regard to the third possibility, it may be possible that the virus obtained by superinfection of Raji cells is a transforming virus rather than a lytically infectious virus. EBV from B95-8 cells (11), a transforming virus, did not induce any viral antigens in superinfected Raji cells (10) but did induce transformation of human cord blood cells (11). It has been reported that B95-8 virus DNA contained only 85% of the DNA sequences of HRI EBV (15), suggesting that the "extra region" of HRI EBV DNA may contain structural or regulatory genes for com-

plete infection of EBV. HRI EBV, therefore, may contain a potential ability for transformation, and slight modification or lack of the "extra region" of EBV DNA might result in production of transforming virus.

ACKNOWLEDGMENTS

We thank F. Deinhardt and L. Wolfe for their comments.

This study was supported by grants from the Public Health Service National Cancer Institute (CA-15281-01), the American Cancer Society (VC-185), and the Leukemia Research Foundation, Inc. and by Public Health Service contract N01-CP-33219, Virus Cancer Program, National Cancer Institute.

LITERATURE CITED

1. **Adams, A., and G. Klein.** 1973. Superinfection with Epstein-Barr virus of human lymphoid cell lines of different origin. Nature (London) New Biol. **242**:234–236.
2. **Epstein, M. A., B. G. Achong, and Y. M. Barr.** 1964. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet **1**:702–703.
3. **Gergely, L., G. Klein, and I. Einberg.** 1971. Appearance of Epstein-Barr virus associated antigens in infected Raji cells. Virology **45**:10–21.
4. **Henle, G., W. Henle, P. Clifford, V. Diehl, G. W. Kafuko, B. G. Kirya, G. Klein, R. H. Morrow, G. M. R. Munube, P. Pike, P. M. Tukei, and J. L. Ziegler.** 1969. Antibodies to Epstein-Barr virus in Burkitt's lymphoma and control groups. J. Natl. Cancer Inst. **43**:1147–1158.
5. **Henle, W., G. Henle, P. Burtin, Y. Cachin, P. Clifford, A. DeSchryver, G. de The, V. Diehl, C. H. Ho, and G. Klein.** 1970. Antibodies to Epstein-Barr virus in nasopharyngeal carcinoma, other head and neck neoplasmas and control groups. J. Natl. Cancer Inst. **44**:225–231.
6. **Henle, W., G. Henle, B. A. Zajad, G. Pearson, R. Waubke, and M. Scriba.** 1970. Differential reactivity of human serums with early antigens induced by Epstein-Barr virus. Science **169**:188–190.
7. **Kawai, Y., M. Nonoyama, and J. S. Pagano.** 1973. Reassociation kinetics for Epstein-Barr virus DNA: nonhomology to mammalian DNA and homology of viral DNA in various diseases. J. Virol. **12**:1006–1012.
8. **Kieff, E. D., S. L. Bachenheimer, and B. Roizman.** 1971. Size composition and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J. Virol. **8**:125–132.
9. **Klein, G., L. Dombos, and B. Gothoskar.** 1972. Sensitivity of Epstein-Barr virus (EBV) producer and nonproducer human lymphoblastoid cell lines to superinfection with EB-virus. Int. J. Cancer **10**:44–57.
10. **Klein, G., B. Sugden, W. Leibold, and J. Menezes.** 1974. Infection of EBV genome negative and positive human lymphoblastoid cell lines with biologically different preparations of EBV. Intervirology **3**:232–244.
11. **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.
12. **Nonoyama, M., and J. S. Pagano.** 1971. Detection of Epstein-Barr viral genome in nonproductive cells. Nature (London) New Biol. **233**:103–106.
13. **Nonoyama, M., and J. S. Pagano.** 1972. Replication of viral deoxyribonucleic acid in Epstein-Barr virus infection. J. Virol. **9**:714–716.
14. **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.
15. **Pritchett, R. F., S. D. Howard, and E. D. Kieff.** 1975. DNA of Epstein-Barr virus. I. Comparative studies of the DNA of Epstein-Barr virus from HRI and B95-8 cells: size, structure and relatedness. J. Virol: **15**:556–569.