JOURNAL OF VIROLOGY, June 1981, p. 880-885
0022-538X/81/060880-06$02.00/0

Vol. 38, No. 3

Sedimentation Characteristics of Newly Synthesized Epstein-Barr Viral DNA in Superinfected Cells

PAMELA J. SIEGEL,[1,2] WENDY CLOUGH,[1,2,3]* AND JACK L. STROMINGER[1]

Sidney Farber Cancer Institute, Boston, Massachusetts 02115,[1] Harvard Medical School, Department of Microbiology and Molecular Genetics, Boston, Massachusetts 02115,[2] and Molecular Biology Division, University of Southern California, Los Angeles, California 90007[3]

Received 8 January 1981/Accepted 26 February 1981

Replicating Epstein-Barr virus (EBV) DNA molecules isolated from superinfected Raji cells were shown to consist of 80S to 65S and 58S (mature) molecules. Pulse-chase experiments showed that radioactive label of DNA molecules with the larger sedimentation coefficients was partially chased into 58S labeled forms. Formation of large concatemers of viral DNA could not be detected at any time after superinfection. The continuous presence of the 65S viral DNA intermediate throughout the replicative cycle combined with the observed inhibition of EBV DNA synthesis by addition of nontoxic levels of ethidium bromide to the superinfected cell culture led us to propose that EBV replication proceeds via a relaxed circular DNA intermediate.

Epstein-Barr virus (EBV)-transformed B lymphocytes in culture, as well as fresh lymphoid cells obtained either from the peripheral blood of patients with infectious mononucleosis or from Burkitt lymphoma tissue, contain the EBV genome in the form of a circular plasmid; however, the DNA in the EB virion has been shown to be linear (1, 9, 10, 13, 20). Since EBV has no known lytic cycle and since in virus-producing B-cell lines only a few percent of the cells at any one time are making virus, it has not yet been determined how the EBV DNA is replicated in productively infected cells, or what the relationships are between circular and linear viral DNA forms.

A system useful for studying replicating EBV DNA has been developed (12, 19, 21): nonproducer Raji cells, which have been shown to carry EBV DNA in circular plasmid form (10), after superinfection with the P3HR-1 strain of EBV undergo a large burst of viral DNA synthesis. This resembles an abortive lytic infection, since few virions are produced.

In the present report, the size classes of replicating EBV DNA present in superinfected cells at various times after infection were examined. The principal replicative intermediate was a 65S viral DNA form. Addition of ethidium bromide, an inhibitor of the replication of circular DNAs (6, 11, 17), to superinfected cells inhibited EBV DNA replication at concentrations noninhibitory to cellular DNA replication. Formation of large concatemers of viral DNA was not detected. The latter observation sets this abortive lytic viral system apart from cells infected with the lytic herpesviruses herpes simplex virus (HSV) and pseudorabies virus, for which formation of large concatemeric structures has been reported (2, 8).

MATERIALS AND METHODS

Cells and viruses. Raji cells, human B lymphocytes derived from a Burkitt's lymphoma patient, contain 50 to 60 EBV genomes per cell. P3HR-1 cell cultures (obtained from M. Nonoyama; viral capsid antigen 10% or greater) served as the source of virus. They were maintained at 35°C by diluting 1:1 once a week with RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum. Raji cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum.

Crude virus preparation. Cells and large debris were removed from the culture by centrifuging 500-ml portions in a Sorvall RC5-B centrifuge, using a Sorvall GS-3 rotor, at 5,000 rpm for 10 min at 4°C. This procedure was repeated two or three times. The virus was pelleted from the clarified supernatant by sedimentation at 9,000 rpm for 2 h at 4°C. The crude viral pellet was suspended in 1/100 the original volume of RPMI 1640 containing 1% heat-inactivated fetal calf serum. One-milliliter portions were quick-frozen and stored at −70°C. Before use, the virus was thawed at 37°C and sonicated for 60 s in an Ultramet III (Buehler, Ltd.) water bath sonicator. Pilot experiments were performed to determine quality of the virus preparations.

Superinfection of Raji with EBV strain P3HR-1. Raji cells were then used for superinfection 24 h after feeding. The titer at the time of use was about 10^6 cells/ml. For each superinfection, 3×10^6 to 5×10^6 cells were used. For each 10^6 pelleted Raji cells, an equivalent of 30 ml of P3HR-1 supernatant was added (100 ml was concentrated to 1 ml). The virus was thawed quickly and sonicated for 60 s in a water bath sonicator before addition to the cells. The virus was

allowed to adsorb for 1 h at 37°C. RPMI 1640 with 1% heat-inactivated fetal calf serum was then added to bring the cell concentration to between 5×10^5 and 1×10^6 cells/ml. The infection was allowed to proceed at 35°C for an additional 4 h, after which the cells were pelleted, washed twice in phosphate-free minimal essential medium, and then suspended in phosphate-free minimal essential medium containing 1% heat-inactivated (dialyzed) fetal calf serum to a concentration of 5×10^5 to 1×10^6 cells/ml. At this time [*methyl*-^{3}H]thymidine (Schwarz/Mann; 18 Ci/mmol) was added as indicated (see figure legends). Infection was allowed to continue for a total of 24 h or as indicated in the text, at which time the DNA was extracted.

Preparation of DNA from superinfected cells. Essentially the method of Shaw et al. (15) was followed. The infected cells were pelleted and washed twice with phosphate-buffered saline and resuspended in 1 ml of the same. After the cells were well dispersed with a Pasteur pipette, 0.5 ml of lysis buffer (15) was added; cells were thoroughly lysed by rolling the tube on a flat surface for 10 min. Then 20 μl of freshly made pronase solution was added (Sigma pronase was dissolved to 10 mg/ml in 50 mM Tris–10 mM NaCl and heat treated at 80°C for 10 min to inactivate DNases and then at 37°C for 1 h just before use). Pronase treatment was carried out at 50°C for 1 h and then repeated in the same manner for an additional hour. After the pronase treatment, the DNA was usually centrifuged to equilibrium in CsCl density gradients or occasionally sedimented directly on a glycerol sizing gradient. The peak fractions of the density of viral DNA from CsCl density gradients were pooled and then diluted with buffer and recentrifuged either in another density gradient or in a glycerol gradient. In the latter case, samples were diluted 1:20 in a buffer containing 5 mM EDTA, 50 mM NaCl, and 1 mM Tris, pH 8.0.

Equilibrium centrifugation. CsCl and phosphate-buffered saline were added to samples, and the refractive index was adjusted to 1.4000 (25°C). Centrifugation was in a Beckman vertical rotor for 16 h at 32,000 rpm or a Beckman 50 titanium rotor for 70 h at 23,000 rpm, both at 20°C. Gradients were dripped through a 16-gauge needle hole punched into the polyallomer tube; where size of the molecules was critical, the hole was enlarged by a no. 11 scalpel blade. The refractive index was recorded for the first and every fifth fraction. Ten microliters of all fractions was precipitated with trichloroacetic acid and counted in a Beckman scintillation counter.

HSV marker DNA. HSV used as 55S marker DNA was obtained from P. Schaffer and grown in Vero cells (obtained from the American Type Culture Collection). Preparation of 55S virion marker DNA was as previously described (16).

HSV used as control in the experiments to examine concatemer formation was type 1 KOS strain from E. Wagner. H-11 HeLa cells (adapted to monolayer growth) in a 25-cm^2 T flask were infected when near confluency at a multiplicity of infection of 15 in the presence of phosphate-buffered saline (with calcium and magnesium). Adsorption was allowed to proceed for 1 h at 37°C with gentle shaking. Unadsorbed virus was then removed, and 3 to 4 ml of medium (medium 199 or Dulbecco minimal essential medium, with 2% dialyzed heat-inactivated fetal calf serum) was added to the flask. After 3 h at 37°C, [*methyl*-^{14}C]thymidine (Schwarz/Mann; 57 mCi/mmol) was added at a concentration of 0.5 μCi/ml, and the infection was allowed to proceed for an additional 3 h. Cells were then removed from the flask with gentle trypsinization and washed in phosphate-buffered saline. Cell lysis and centrifugation of DNA in CsCl density gradients were exactly as described for EBV-superinfected cells.

Immunofluorescence procedures. Anti-early antigen sera were gifts of G. Klein and L. Hutt. Anti-viral capsid antigen (VCA) serum was a gift of C. Edson. Indirect immunofluorescence was performed as described by Nyormoi et al. (14).

RESULTS

Glycerol gradient sedimentation of viral DNA. Pulse-labeling experiments were performed in which DNA of superinfected cells was labeled for 4-h periods over 24 h, starting 4 h after superinfection. In each case, DNA was extracted and centrifuged in CsCl density gradients, and the viral DNA was further sedimented in glycerol gradients. DNA extracted at any time period showed a similar size distribution upon glycerol gradient sedimentation. Figure 1 shows the 12- to 16-h and the 20- to 24-h time periods. The total amount of isotope incorporated into EBV DNA declined with advancing time after infection, suggesting that the rate of viral DNA synthesis was decreasing starting at a relatively early time during the infectious process. In addition to DNA molecules of 58S (mature length), 65S and 80S species were present (Fig. 1). At the earlier time period, small amounts of labeled DNA also sedimented as 100S and larger forms. There was proportionately more 65S to 80S material than 55S to 58S material at the earlier time period, as determined by measuring the areas under the curves on the graphs; the ratio 55S to 58S/65S to 80S labeled DNA forms increased threefold from the 12- to 16-h period to the 20- to 24-h period.

The 65S viral DNA has previously been found in Raji cells and has been characterized as an open circular form of viral DNA (10). The 80S form may be either a circular or linear replicative intermediate; a 100S species seen only in small amounts in these experiments has previously been characterized as a covalently closed supercoiled circle which is reduced to a 65S form after X-irradiation (10). In addition, no radioactivity was found in the pellet, preliminarily excluding the presence of much larger concatemeric replicative forms.

To examine directly the question of concatemer formation in EBV-superinfected cells, experiments were performed in which the DNA from 5-h [^{3}H]thymidine-labeled superinfected

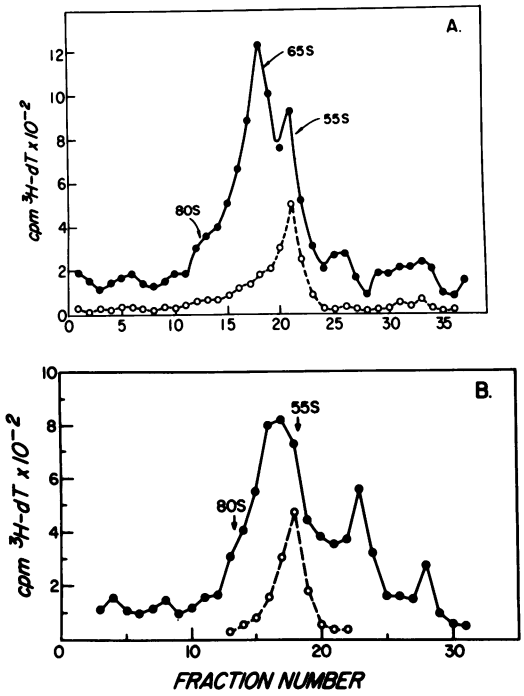

FIG. 1. *Glycerol gradient sedimentation of pulse-labeled EBV DNA isolated from a CsCl density gradient. Log-phase Raji cells were superinfected with the P3HR-1 strain of EBV (15, 19). [^{3}H]thymidine (18 Ci/mmol) was added (17 μCi/ml) for 4-h periods at (A) 12 to 16 h postinfection and (B) 20 to 24 h postinfection. In both cases, DNA was extracted and sedimented in CsCl density gradients as previously described (see Materials and Methods). Those fractions representing the peak of viral DNA were pooled and diluted 20-fold with a buffer of 20 mM Tris-hydrochloride (pH 8.0) and 1 mM EDTA (pH 8.0). Samples were layered onto 10 to 30% glycerol gradients containing 1 M NaCl, 20 mM Tris-hydrochloride (pH 8.0), and 1 mM EDTA (pH 8.0). These gradients were sedimented in a Beckman SW27 rotor, using 36-ml gradients in cellulose nitrate tubes, in the presence of ^{32}P-labeled HSV 55S DNA as marker. Sedimentation was at 25,000 rpm for 220 min at 4°C (12). Symbols: ●, EBV [^{3}H]DNA; ○, HSV ^{32}P-labeled 55S marker DNA.*

cells was extracted, centrifuged in CsCl density gradients, and sedimented in sucrose gradients in the presence of [^{14}C]thymidine-labeled HSV DNA extracted from HSV-infected cells at a comparably early time. Jacob and Roizman (8) had previously shown that concatemers of replicating HSV DNA could be isolated from near the bottom of such sucrose gradients. Our data from similar experiments showed that although significant amounts of ^{14}C-labeled replicating HSV DNA could be isolated from the lower region of such sucrose gradients, detectable amounts of ^{3}H-labeled EBV DNA were not present in the lower portion of the gradients (data not shown).

Pulse-chase experiments were carried out during early as well as late times after infection. First, EBV DNA was pulse-labeled with [^{3}H]-thymidine for 30 min at either 4 h (Fig. 2A) or 24 h (Fig. 2B) postinfection, followed by immediate extraction of DNA and subsequent centrifugation in CsCl and glycerol gradient sedimentation. At both times, we observed material similar in size to mature size DNA (58S) and 65S DNA, as well as a shoulder of 80S DNA. To determine which forms were the precursors for mature DNA, at 4 h (Fig. 2C) and 24 h (Fig. 2D) postinfection, the 30-min [^{3}H]thymidine pulse was followed by a chase of cold thymidine (20 μg/ml for 60 min or 10 μg/ml for 120 min). At both times, the chase of cold thymidine, although incomplete, caused a shift in the relative amount of labeled DNA from the 65S to 80S to the 55S to 58S (mature) category. At the 4-h time period, the shift caused by the chase was slight; the ratio of labeled 55S to 58S/65S to 80S forms as calculated by determining the areas under the curves was only increased by 15% after the chase. However, the same ratio was increased almost fourfold after the chase in the case of the 24-h pulse-chase experiment, indicating that a major shift of the labeled 65S to 80S to the 55S to 58S forms had occurred. As stated above, the rate of [^{3}H]thymidine incorporation into viral DNA was greater at earlier times postinfection in our superinfection system. If the 65S to 80S DNA was truly the precursor to the 58S mature DNA, then the higher rate of continuing synthesis of the precursor DNA forms at earlier times than at later times would in fact make those forms more difficult to chase at the earlier time.

Effect of ethidium bromide on EBV DNA synthesis. In a second approach to the question of whether a relaxed circular DNA replicative intermediate was indeed present, experiments using addition of ethidium bromide to the cells were performed. The replication of circular DNA molecules, e.g., mitochondrial and plasmid DNAs and proviral DNA of retroviruses, has been shown to be very sensitive to addition of the intercalating dye ethidium bromide to the culture (6, 11, 17). Correspondingly, we found EBV DNA synthesis in superinfected cells to be more sensitive than lymphocyte DNA synthesis to addition of ethidium bromide (Table 1). Raji cellular DNA synthesis in nonsuperinfected cells, as measured by labeling with [^{3}H]thymidine over a period of hours, was not affected by 500 ng of ethidium bromide per ml of medium, whereas 500 ng of ethidium bromide per ml

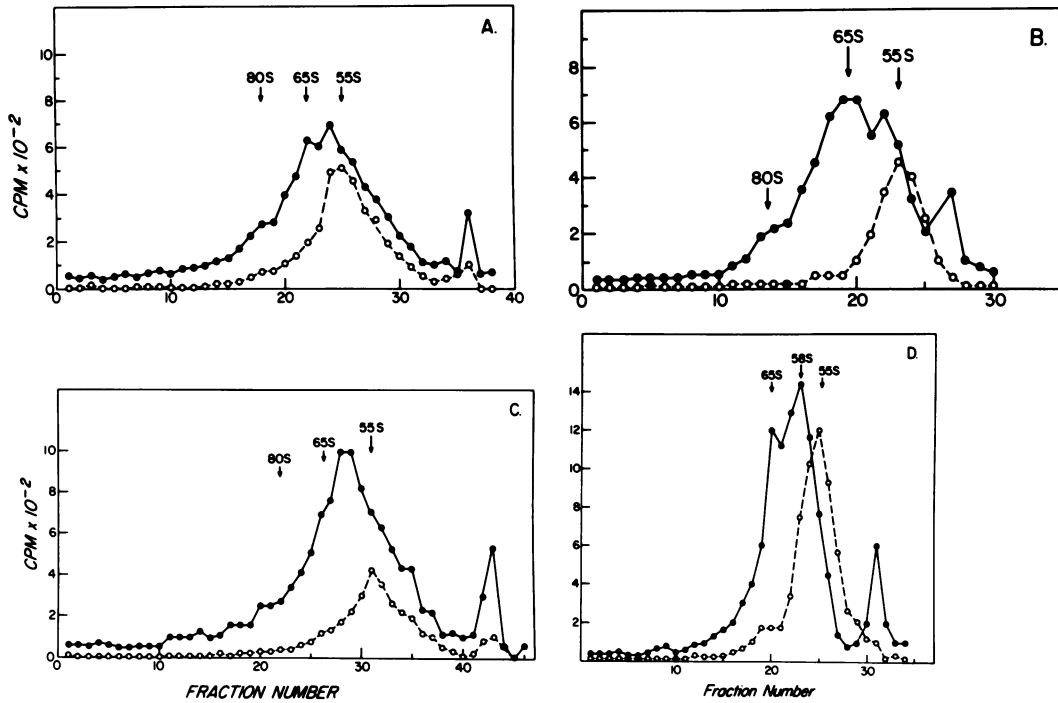

FIG. 2. *Glycerol gradient sedimentation of viral DNA from pulse- and pulse-chase-labeling experiments performed at early and late times after superinfection. Raji cell superinfection with P3HR-1 virus was as described (15, 19). A 30-min pulse of [^{3}H]thymidine (50 μCi/ml) was added at the indicated times. Chases were performed by subsequent addition of unlabeled thymidine at either 20 μg/ml for 60 min or 10 μg/ml for 120 min. (A) 30-min pulse at 4 h postinfection, DNA extracted immediately; (B) 30-min pulse at 24 h postinfection, DNA extracted immediately; (C) 30-min pulse followed by chase at 4 h postinfection; (D) 30-min pulse followed by chase at 24 h postinfection. In all experiments, the extracted DNA was sedimented in CsCl density gradients, and the material of viral density was resedimented in glycerol gradients (^{3}H; ●) in the presence of HSV marker DNA (^{32}P; ○) (see legend to Fig. 1).*

decreased viral DNA synthesis to 50% of its normal value in superinfected cells. Concentrations of 20 and 100 ng/ml (sufficient to inhibit replication of covalently closed supercoiled plasmids [6, 11, 17]) inhibited EBV DNA synthesis at the 10 to 17% level. It has previously been shown that inhibition of EBV DNA synthesis leads to a decline in VCA production (4, 5, 7, 14, 18). The percentage of cells in the P3HR-1 producer cell line expressing VCA declined with increasing concentration of ethidium bromide proportionately to the observed inhibition of DNA synthesis; i.e., 500 ng of ethidium bromide per ml yielded a 50% inhibition of both DNA and VCA production. On the other hand, the percentage of early antigen-positive cells did not decline, but increased with increasing drug concentration.

DISCUSSION

The data presented in Fig. 1 and 2 indicate that the EBV DNA forms of larger S value (65S to 80S) were precursors to those forms of smaller S value (55S to 58S) that were the size of mature viral DNA. The fact that a continuum of viral DNA sizes between 65S and 80S is always found and that all of this material is relatively decreased in amount after chase suggests that various sizes of replicating molecules up to twice the size of parental DNA exist.

Our data show that EBV DNA replication in this abortive lytic system differs in two aspects from replication of the lytic HSV and pseudorabies virus. First, the concatemer formation reported for HSV and pseudorabies virus (2, 8) was not detected at any time after EBV superinfection. Second, pseudorabies, unlike EBV, shows no mature-length DNA after comparable pulse-labeling with [^{3}H]thymidine (2), but does fragment into molecules of less than mature-size DNA (3). The absence of large concatemeric forms in the EBV superinfection system studied here suggests the possibility that one block in EBV's abortive lytic cycle is the synthesis of

TABLE 1. *Effect of ethidium bromide on DNA synthesis in superinfected Raji cells and P3HR-1 producer cells*

Ethidium bromide (ng/ml)	Raji				P3HR-1	
	Viral DNA[a]		Cell DNA		% of cells VCA positive[b]	% of cells EA positive[b]
	cpm × 10^2	%	cpm × 10^4	%		
0	5.32	100.0	6.33	100.0	8.7	4.45
20	4.40	82.7	6.65	105.0	ND[c]	ND
100	4.73	88.9	6.20	97.9	7.6	6.90
500	3.01	56.6	6.45	101.9	4.5	8.00
1,000	ND		7.50	118.5	3.0	9.05

[a] Raji cells were superinfected with P3HR-1 virus in the presence or absence of ethidium bromide; 10 μCi of [^{3}H]thymidine per ml was added. After 24 h, DNA was extracted and viral DNA was separated from host DNA in CsCl gradients. The total amount of [^{3}H]thymidine incorporation for each concentration was determined by calculating the area under the plotted curves of the gradients.

[b] Antigen production was determined in P3HR-1 producer cells. Immediately after feeding, ethidium bromide was added to the cells. After 1 week in the dark, antigen determinations were made by using indirect immunofluorescence techniques (14). EA, Early antigen.

[c] ND, Not done.

such large DNA forms. But it is not clear that EBV DNA replication in superinfected cells is of a defective type, since mature viral DNA lengths (58S) are produced from the 65S precursor molecules.

We also report here that viral DNA synthesis in superinfected cells and VCA production in producer cells are both inhibited to the 50% level by addition of ethidium bromide to the cells at 500 ng/ml, a concentration that is noninhibitory to cellular DNA synthesis. This degree of ethidium bromide sensitivity of the EBV DNA synthesis in association with our finding that the predominant viral replicative intermediate is a 65S DNA form leads us to propose that EBV DNA synthesis proceeds through a relaxed circular intermediate such as a rolling circle.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA-19235, CA-23070, and CA-21082 from the National Institutes of Health. P.J.S. was a fellow of the Helen Hay Whitney Foundation, and W.C. is the recipient of Public Health Service research career development award CA-00707 from the National Cancer Institute.

We thank Sarah Wright for her assistance in the preparation of this manuscript.

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. **Ben-Porat, T., S. Kaplan, B. Stehn, and A. S. Rubenstein.** 1976. Concatameric forms of intracellular herpes virus DNA. Virology **69**:547-560.
3. **Ben-Porat, T., and S. Tokazewski.** 1977. Replication of herpes virus DNA. II. Sedimentation characteristics of newly synthesized DNA. Virology **79**:292-301.
4. **Benz, W., P. Siegel, and J. Baer.** 1978. Effects of adenine arabinoside on lymphocytes infected with Epstein-Barr virus. J. Virol. **27**:475-482.
5. **Gergely, L., G. Klein, and I. Ernberg.** 1971. The action of DNA antagonists on Epstein-Barr virus (EBV)-associated early antigen (EA) in Burkitt lymphoma lines. Int. J. Cancer **7**:293-302.
6. **Guntaka, R. V., B. W. J. Mahy, J. M. Bishop, and H. E. Varmus.** 1975. Ethidium bromide inhibits appearance of closed circular viral DNA and integration of virus-specific DNA in duck cells infected by avian sarcoma virus. Nature (London) **253**:507-511.
7. **Hampar, B., J. G. Derge, L. Martos, M. Tagamets, and M. Burroughs.** 1972. Sequence of spontaneous Epstein-Barr virus activation and selective DNA synthesis in activated cells in the presence of hydroxyurea. Proc. Natl. Acad. Sci. U.S.A. **69**:2589-2593.
8. **Jacob, R. J., and B. Roizman.** 1977. Anatomy of herpes simplex virus DNA. VIII. Properties of the replicating DNA. J. Virol. **23**:394-411.
9. **Kaschka-Dierich, C., A. Adams, T. Lindahl, G. Borkamm, G. Bjursell, G. Klein, B. Giovanella, and S. Singh.** 1976. Intracellular forms of Epstein-Barr virus DNA in human tumor cells *in vivo*. Nature (London) **260**:302-306.
10. **Lindahl, T., A. Adams, G. Bjursell, B. W. Bornkamm, C. Kaschka-Dierich, and U. Jehn.** 1976. Covalently closed circular duplex DNA of Epstein-Barr virus in human lymphoid cell lines. J. Mol. Biol. **102**:511-530.
11. **Nass, M. M. K.** 1970. Abnormal DNA patterns in animal mitochondria: ethidium bromide-induced breakdown of closed circular DNA and conditions leading to oligomer accumulation. Proc. Natl. Acad. Sci. U.S.A. **67**:1926-1933.
12. **Nonoyama, M., and J. S. Pagano.** 1972. Replication of viral DNA and breakdown of cellular DNA in Epstein-Barr virus infection. J. Virol. **9**:714-716.
13. **Nonoyama, M., and J. S. Pagano.** 1972. Separation of Epstein-Barr virus DNA from large chromosomal DNA in nonvirus-producing cells. Nature (London) New Biol. **238**:169-171.
14. **Nyormoi, O., D. A. Thorley-Lawson, J. Elkington, and J. L. Strominger.** 1976. Differential effects of phosphonoacetic acid on the expression of Epstein-Barr viral antigens and virus production. Proc. Natl. Acad. Sci. U.S.A. **73**:1745-1748.
15. **Shaw, J. E., T. Seebeck, J.-H. H. Li, and J. S. Pagano.** 1977. Epstein-Barr virus DNA synthesized in superinfected Raji cells. Virology **77**:762-771.
16. **Skare, J., and W. C. Summers.** 1977. Structure and function of herpesvirus genomes. II. EcoR1, XbaI and Hind*III* endonuclease cleavage sites on herpes simplex virus type 1 DNA. Virology **76**:581-595.

17. **Stanfield, S., and D. R. Helinski.** 1976. Small circular DNA in *Drosophila melanogaster*. Cell **9**:333–345.
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. **Tanaka, A., M. Miyagi, Y. Yajima, and M. Nonoyama.** 1976. Improved production of Epstein-Barr virus DNA for nucleic acid hybridization studies. Virology **74**:81–85.
20. **Tanaka, A., and M. Nonoyama.** 1974. Latent DNA of Epstein-Barr virus: separation from high molecular weight cell DNA in a neutral glycerol gradient. Proc. Natl. Acad. Sci. U.S.A. **71**:4658–4661.
21. **Yajima, Y., and M. Nonoyama.** 1976. Mechanisms of infection with Epstein-Barr virus. I. Viral DNA replication and formation of noninfectious viral particles in superinfected Raji cells. J. Virol. **19**:187–194.