JOURNAL OF VIROLOGY, Nov. 1984, p. 549–556
0022-538X/84/110549-08$02.00/0

Vol. 52, No. 2

Electron Microscopic Evidence for Replication of Circular Epstein-Barr Virus Genomes in Latently Infected Raji Cells

EVA GUSSANDER AND ALICE ADAMS†*
Department of Pathology, Uppsala University, S-751 85 Uppsala, Sweden

Received 6 February 1984/Accepted 30 July 1984

Raji cells, collected at various times from a synchronized culture, were gently lysed, and the high-molecular-weight DNA was enriched ca. 10-fold for latent Epstein-Barr virus (EBV) genomes by equilibrium density gradient centrifugation in neutral CsCl. The heavy-density DNA pool, which included more than 90% of the total intracellular EBV DNA sequences, was further fractionated by velocity sedimentation on neutral glycerol gradients, and material from fractions containing potential EBV DNA replicative forms was examined in the electron microscope. Early in the cellular S phase, when the EBV DNA content was found to be doubling in parallel with host chromosome replication, half of the 50- to 55-μm circular EBV genomes were observed to have two or more DNA branch points or forks. Most molecules were in a relaxed theta configuration, indicative of the Cairns mode of DNA replication. In the supercoiled state, the two daughter strands of the partially replicated molecules were seen to be wrapped around each other. Two theta structures had more than two DNA forks, indicating that DNA replication can initiate more than once on the same DNA molecule. Late in the S phase, the EBV DNA sedimenting at positions where theta structures were found with early S phase samples was composed of catenated dimers rather than partially replicated genomes. It is concluded that the circular EBV genomes, which are the major intracellular form in latently infected cells, are maintained as independent replicons and are not synthesized from an integrated template.

The Epstein-Barr virus (EBV) is a herpesvirus whose complete genome, in multiple copies, is maintained in a predominately nonexpressed form in the majority of human B lymphocytes growing in continuous cell culture (21, 27). The physical state of the EBV DNA present in such cells has been partially characterized. The Burkitt lymphoma-derived Raji cell line, which has served as the prototype for these investigations, harbors ca. 50 copies of the 110 × 10^6-dalton viral genome (19, 22). The intracellular state of the majority of these EBV genomes is a circular DNA plasmid (4, 18). Circular forms, which can be isolated either in the covalently closed, supercoiled structure or as a relaxed, open DNA circle (16), have subsequently been found in all cells with more than two to three copies of the EBV genome thus far examined (1).

In addition to the circular DNA species, some EBV DNA sequences are covalently bound to cellular DNA (3, 5), and the possibility of one or more integrated copies of the complete viral genome has not been excluded. Even in the case of a single cell line where no evidence of integrated EBV DNA sequences was detected by the density analysis method used (2), there could still be a complete EBV genome integrated into an unusually guanine-cytosine-rich region of the host chromosome. Thus, the possibility that the circular EBV DNA forms are not independent replicons, but are the products of one or more integrated genomes, has remained open.

Catenated dimers of two interlocking DNA circles are intermediates in the replication of simian virus 40 DNA (25). The finding of catenated dimers of EBV DNA circles (11) suggests, by analogy, that the circular forms might indeed be independent replicons. However, catenated circular DNA forms can arise by recombination (7) and thus, to our knowledge, it has not been conclusively demonstrated that the circular EBV genomes in latently infected cells are capable of self-replication.

The amount of EBV DNA in non-virus-producing cell lines has been reported to double early in the cellular S phase (13). In the present investigation we succeeded in isolating replicative intermediates of the circular EBV genomes during this period from a synchronized culture of Raji cells. We conclude from the electron micrographs presented that circular herpesvirus genomes can replicate via a Cairns-type intermediate in cells which are not productively infected.

MATERIALS AND METHODS

Cell culture and synchronization. Mycoplasma-free Raji cells were maintained in exponential growth by passage every other day in antibiotic-free RPMI 1640 medium supplemented with 8% fetal calf serum. Cell synchrony was induced by the double thymidine blocking method, as previously employed with this cell line (12). Briefly, 2 × 10^8 Raji cells, in which DNA synthesis had been blocked by a 24-h exposure at 37°C to medium containing 3 mM thymidine, were washed free of thymidine, suspended at a concentration of 4 × 10^5 cells per ml in medium containing 5 μg of deoxycytidine per ml, and incubated for 8 h at 37°C. The cells were then washed and exposed to a second 3 mM thymidine block for 16 h. Allowing for a 5 to 10% loss of cells during the collecting and washing steps, the total cell number doubled during the 8-h period when the culture was released from the first thymidine block.

Synchronized cells were collected after the second thymidine block, washed twice in cold Hanks balanced salt solution, and at zero time suspended at a concentration of 4 × 10^5 cells per ml in medium, again supplemented with 5 μg of deoxycytidine per ml. One-third of the culture was immediately processed for isolation of DNA (see below). Sixteen portions (2.5 ml each) were quickly transferred to individual sterile plastic tubes, and these, together with the remaining bulk culture, were incubated at 37°C. At hourly

* Corresponding author.
† Present address: Department of Microbiology and Immunology, University of Minnesota, Duluth, MN 55812.

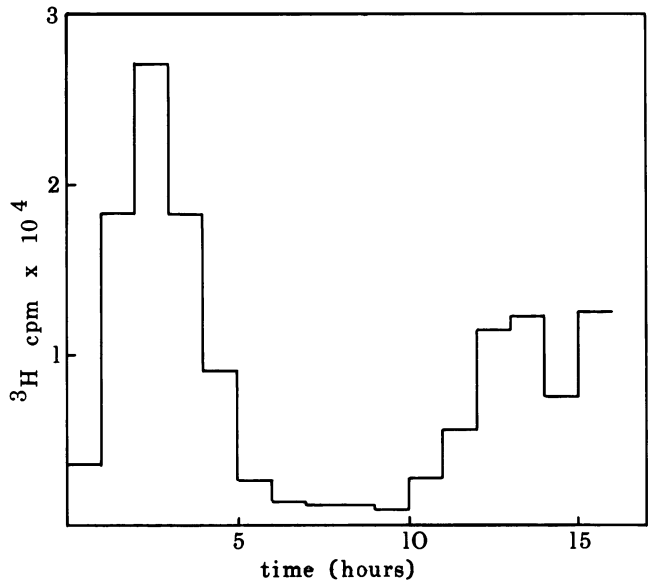

FIG. 1. DNA synthesis in the synchronized culture of Raji cells at various times. After an 1-h incubation with [^{3}H]thymidine, the cells in the 2.5-ml cultures (initially containing 10^6 cells per tube) were mixed, two 1-ml samples were transferred to small tubes, and the cells were pelleted, washed once with sodium phosphate-buffered saline, suspended in a small volume of buffer, and precipitated with trichloroacetic acid. The trichloroacetic acid precipitates were collected on individual glass fiber filters, dried, and counted. The plotted amount of [^{3}H]thymidine incorporated into acid-insoluble material is the mean of duplicate samples. At 10 h, the number of cells per milliliter was determined on a small sample of one cell suspension and found to be twice that present at zero time.

intervals, 1 μCi of [^{3}H]thymidine (specific activity, 40 to 60 Ci/mmol; New England Nuclear Corp.) was added to one of the 16 tubes, and the degree of cell synchrony was followed by measuring the amount of isotope incorporated into acid-insoluble material after an additional 1-h incubation at 37°C (Fig. 1).

The control culture consisted of a second batch of cells which was washed and transferred to fresh medium (without any nucleoside additions) at those times when synchrony-inducing medium changes were made on the parallel culture. The control culture doubled once during the first 24-h incubation and again during the combined 8- plus 16-h incubation periods.

Cell viability was periodically determined by the trypan blue exclusion test and found to be greater than 95% in both the synchronized and control cultures throughout the experiment. In addition, both cultures were carefully monitored for evidence of induction of the productive viral cycle. Acetone-fixed cell smears were prepared from washed cell samples taken at the time of all medium changes, as well as 24 h after the last cell sample was removed for DNA isolation. At no point were early antigen-positive cells, indicative of lytic EBV infection, observed by direct immunofluorescence with fluorescein isothiocyanate-conjugated anti-early antigen sera of high titer.

Isolation and analysis of EBV DNA. At 0, 1.5, and 4 h after the synchronized cells had been suspended in the final deoxycytidine-containing medium, 5×10^7 to 6×10^7 cells were removed, collected, and quickly washed twice with cold sodium phosphate-buffered saline (0.14 M NaCl, 0.01 M sodium phosphate [pH 7.4]). A total of 3×10^7 washed cells were suspended at a concentration of 2×10^7 cells per ml in 2×-concentrated sodium phosphate-buffered saline. An equal volume of 1.8 M NaCl was added, the suspension was mixed, and the cells then were gently lysed by the addition of one-half volume of 3% Sarkosyl in 1 M NaCl–0.075 M Tris-hydrochloride–0.025 M EDTA (pH 9.0). These three DNA samples will, for reasons to be discussed below, be referred to as the early, middle, and late S phase preparations. The control DNA sample was prepared from an equal number of cells taken from the unsynchronized culture at 4 to 5 h after the cells were last suspended in fresh medium.

DNA was isolated in the continued presence of 1 M salt to inhibit both nuclease activities and branch migration at replicating forks. After treatment of the cell lysate with proteinase K, the DNA was enriched for viral DNA sequences by equilibrium density gradient centrifugation in CsCl. The viral DNA-containing fractions were pooled, mixed with ^{3}H-labeled bacteriophage T4 DNA, dialyzed, and concentrated, and the sedimentation properties of the EBV DNA were determined by velocity centrifugation through neutral glycerol gradients. The viral DNA content of a sample of individual fractions was quantitated by nucleic acid hybridization with EBV [^{32}P]cRNA (16). Hybridization over representative CsCl gradients demonstrated that 90 to 95% of the total intracellular EBV DNA was included in the pool analyzed by velocity sedimentation.

Sedimentation values were calculated relative to the distance that the 61.8S T4 DNA marker, included on all gradients as an internal standard, had moved from the meniscus (8). DNA from selected glycerol gradient fractions was spread for electron microscopy by the microdiffusion method in the absence of formamide (11). The open circular form of bacteriophage PM2 DNA (Boehringer Mannheim Corp.) was added as a size reference, but no attempt was made to relax any supercoiled molecules in the test samples before spreading.

Washed cell pellets from a minimum of 1.5×10^7 synchronized cells taken at 0, 0.75, 1.5, and 4 h after their release from the G1-S phase boundary and control cells collected at the time of DNA isolation and 24 h later were frozen for subsequent analysis of their relative viral DNA content. DNA was prepared from these samples, the EBV DNA content was determined in triplicate by filter hybridization with EBV [^{32}P]cRNA, and total DNA was determined by the diphenylamine test (6). It was previously reported that at 60 and 90 min after the release of Raji cells from the second thymidine block, the relative EBV DNA concentration was twice that seen with unsynchronized and late S phase cells (13). Compared with the two control DNA samples, the relative amount of EBV DNA at various times during the S phase in the present investigation was measured to be 1.0 at zero time, 1.3 at 45 min, 0.9 at 90 min, and 1.1 at 4 h. Thus, only at 45 min was the relative DNA concentration found here to be significantly different from 1.0. One possible reason for our failure to reproduce the published results is that we did not achieve the same degree of synchrony. The time between successive periods of DNA synthesis is only 6 to 7 h (Fig. 1). Therefore, cells arrested late in the S phase during the first thymidine block could have divided and entered a second S phase during the 8-h release period employed. The broadness of the second peak of DNA synthesis seen in Fig. 1, the above hybridization data, and the subsequent results are consistent with some of our Raji cells having been in the early S phase when the second thymidine block was added. Because some cells were proba-

bly already in the early S phase at zero time, we prefer to refer to the three DNA preparations characterized here by the more general terms of early, middle, and late S-phase samples.

RESULTS

Sedimentation properties of replicating EBV DNA. Replicating DNA forms should have altered sedimentation properties from the parental, nonreplicating molecules from which they are derived. The circular EBV genomes, isolated from Raji cells in the late log or stationary phase, sediment at either 104 ± 1 or 65S depending on whether they are in the covalently intact, supercoiled configuration or the relaxed, open circular form, respectively (16). Supercoiled dimers of EBV DNA circles sediment, in our hands, between 140 and 150S (11), which probably places the upper limit on the sedimentation value that a replicative intermediate of circular EBV genomes might attain. The linear EBV genome, as isolated from virus particles, has a sedimentation coefficient (relative to 61.8S T4 DNA) of 58 ± 1S (10, 23), and a molecule twice that length should sediment at 75S.

The sedimentation properties of the EBV DNA isolated from cells in the early, middle, or late S phase were determined. The nucleic acid hybridization profiles measured over these three neutral glycerol gradients are presented in Fig. 2, together with that obtained with DNA isolated from exponentially growing, unsynchronized Raji cells. The major peak of EBV-hybridizing DNA sequences sediments slightly faster than the 61.8S internal size marker (black arrow) at the 65S position of all four gradients. However, a number of differences which appear to be correlated with DNA replication may be noted in the fast-sedimenting material in fractions 6 through 22 of Fig. 2A, B, and C.

At early times in the S phase (Fig. 2A and B), there is a distinct shoulder of EBV-hybridizing DNA sequences on the forward (left) edge of the 65S peak. Moreover, no distinct peak at 104S (the position of the open arrow) is seen on either gradient. Instead, a number of fast-sedimenting forms, the distribution of which is particularly heterogeneous in the middle S phase sample (Fig. 2B), are seen. Early in the S phase (Fig. 2A) the fast-sedimenting material is concentrated at the 130 and 92S regions of the gradient.

Late in the S phase (Fig. 2C) the forward edge of the 65S peak sharpened considerably, and a clear peak can be seen just to the left of the 104S position (open arrow). Although 104S mature molecules would thus appear to be forming, nearly twice as much EBV DNA is found at 90 to 94S in fractions 17 through 19. Some material is also located at 130S in fractions 9 and 10 of the glycerol gradient shown in Fig. 2C.

Finally, in Fig. 2D the DNA from exponentially growing cells can be seen to give a sharp, nearly symmetrical peak at 65S and a small but distinct peak at 104S. The proportion of the 104 to 65S forms is unusually low in this DNA sample. We routinely isolate better than 30% of the intracellular EBV DNA of Raji cells in the covalently intact form, when the

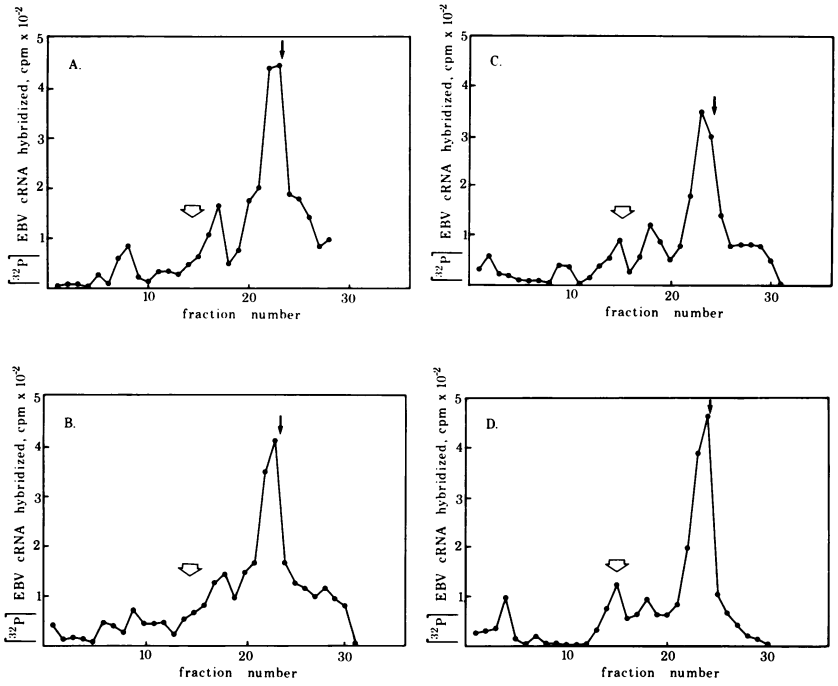

FIG. 2. Glycerol gradient centrifugation of high-molecular-weight DNA from Raji cells. High-molecular-weight DNA banding at 1.714 to 1.722 g/cm^3 from several CsCl gradients was pooled, mixed with bacteriophage T4 [^{3}H]DNA, dialyzed in such a way as to remove CsCl but retain a 1 M salt concentration, concentrated, dialyzed against 1 M salt, layered on a 36-ml glycerol gradient (10 to 30% [wt/vol] glycerol in 1 M NaCl, 0.02 M Tris-hydrochloride, 0.002 M EDTA [pH 8.0]), and centrifuged for 220 min at 25,000 rpm at 20°C in a Spinco SW27 rotor. Fractions (1 ml) were collected from the bottom of the tube, and the position of the T4 DNA marker (solid arrow) was determined by measuring the radioactivity of a small sample. The remaining DNA from each fraction of the gradient shown in (D) and 750-μl samples of each fraction of the gradients shown in (A), (B), and (C) were fixed on individual nitrocellulose filters and hybridized with EBV [^{32}P]cRNA. The 104S position was determined relative to the 61.8S T4 DNA (8) and is marked with the large open arrow. The DNA samples were isolated from the following cell cultures: synchronized cells in the early S phase (A), synchronized cells in the middle S phase (B), synchronized cells in the late S phase (C), and an unsynchronized cell population (D).

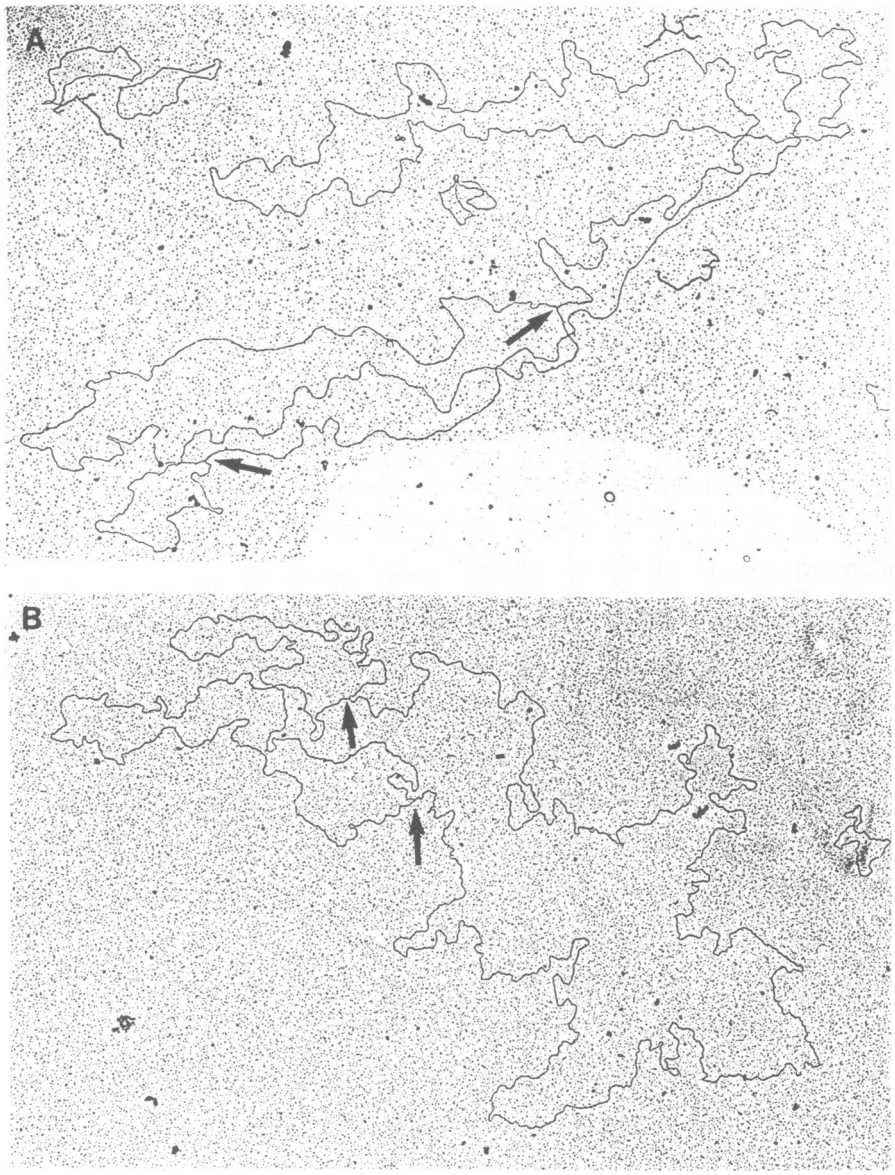

FIG. 3. Electron micrographs of replicating EBV genomes in the theta structure. DNA samples were spread by the microdiffusion technique as described (11). Both molecules are from the glycerol gradient shown in Fig. 2B. The molecule shown in (A) is from fraction 18, and the molecule shown in (B) is from fraction 20. The small DNA circles are bacteriophage PM2 DNA, and the arrows point to the forks of replication on the two large circular EBV DNA molecules.

cells are taken from either late-log- or stationary-phase cultures. Using the same high-salt method as employed here for DNA isolation, we have on occasion recovered over 80% of the intracellular EBV DNA of Raji cells in the covalently closed supercoiled form. The precise reason for the poor yield of covalently closed, supercoiled EBV DNA circles in the present investigation is unknown, but it could perhaps be related to the unusually active physiological state of the culture at the time of DNA isolation. Nonspecific nuclease digestion of the DNA circles before centrifugation would seem unlikely in view of the virtual absence of viral DNA sequences sedimenting slower than the 65S, open circular form.

Electron micrographs of replicating EBV DNA circles. EBV DNA can be identified visually as the only 50- to 55-μm DNA circle present in Raji cells (9, 16). Grids spread with material from selected glycerol gradient fractions were screened in the electron microscope for those large DNA circles with one or more replicating forks. Two types of replicating intermediates were seen, depending on whether the two parental DNA strands were covalently intact or nicked in specific regions.

Relaxed DNA circles with two replicating forks in the so-called theta structure were present on grids prepared from the 130 and 90S regions of the gradients shown in Fig. 2A and B. Two such replicating EBV DNA molecules are shown in Fig. 3. The opened, theta-shaped molecules found in the 130S region were probably in a covalently closed, supercoiled structure at the time of sedimentation but were relaxed by the inadvertent introduction of one or more

single-strand breaks within the nonreplicated segment of the parental DNA molecule before being spread on grids. A total of eight theta-structure molecules similar to those in Fig. 3 were sufficiently well spread to permit contour measurements, and the combined length of the unreplicated region plus one of the two newly replicated segments was between 50 and 55 μm for all molecules. The size of the replicated segment varied from 5 to 80% of the EBV genome, and the

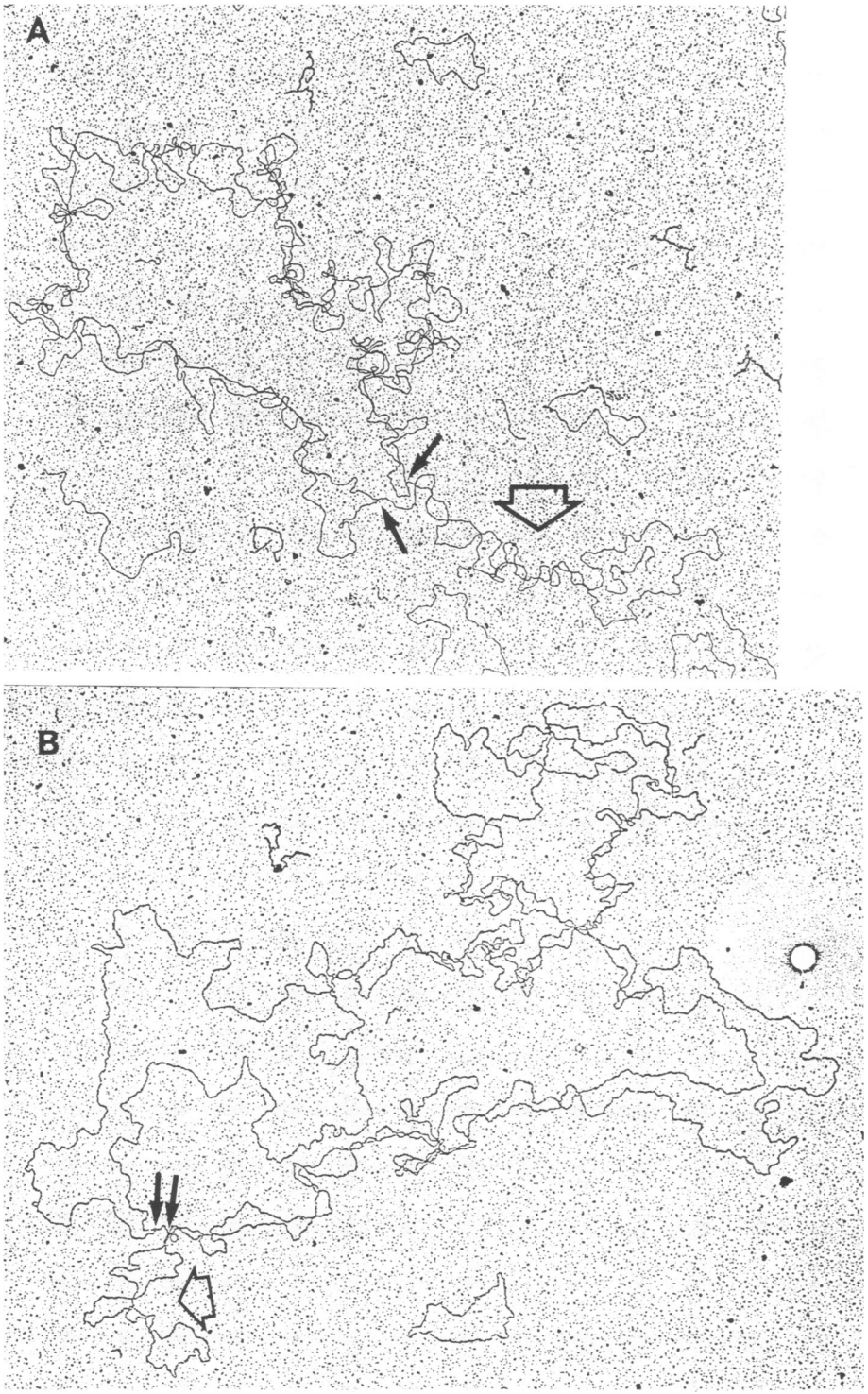

FIG. 4. Electron micrograph of replicating EBV genomes of complex structure. The molecule shown in (A) is from a pool of fractions 7 and 8 of the gradient shown in Fig. 2A, and the molecule shown in (B) is from fraction 9 of the glycerol gradient shown in Fig. 2B. The solid arrows point to the forks of replication; three of these are quite clear, but the fourth is masked in the twined region of the molecule shown in (B). The large open arrows point to the unreplicated regions of parental DNA.

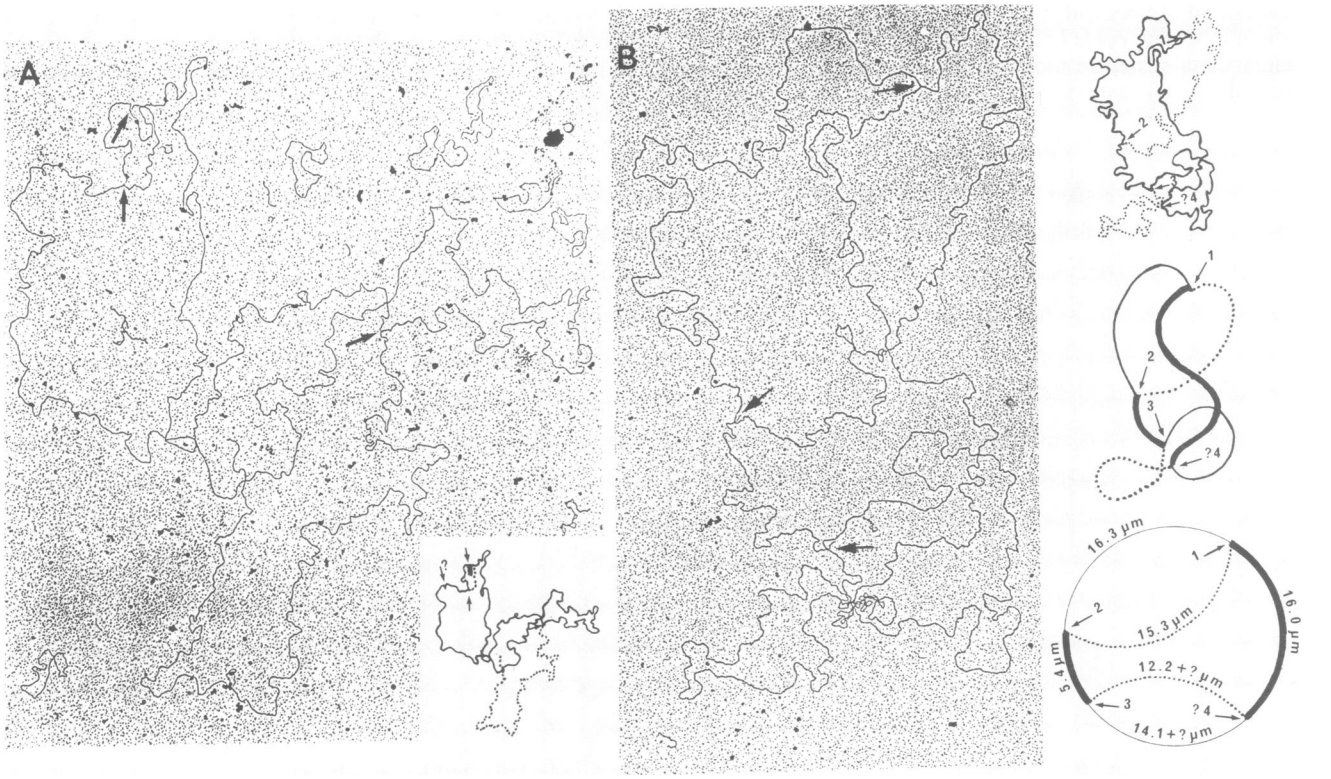

FIG. 5. Electron micrographs of two EBV genomes with more than a pair of replication forks. Three clear forks of replication are shown by arrows on each molecule. The molecule shown in (A) is from the pool of fractions 7 and 8 of the glycerol gradient shown in Fig. 2A, and the molecule shown in (B) is from fraction 18 of the gradient shown in Fig. 2B. The contour of a complete circular EBV genome is traced by a solid line in the small inserts, whereas the dotted lines indicate the extra DNA from the duplicated regions. The length of the solid line of the molecule shown in (A) is 53.8 μm, and that of the molecule shown in (B) is 51.8 μm. The fourth arrow with the question mark in the small insert in (A) is at a site which might appear to be a fourth DNA fork on the print but which on higher magnification of the negative is clearly a loop of the parental DNA thread. The DNA tail extending from the third fork of the molecule shown in (A) is estimated to be 21.8 μm. Below the insert of the direct tracing of the molecule shown in (B) is a schematic representation of this molecule, where the unreplicated regions of the parental genome are shown as thickened solid lines and the daughter strands are shown as either a thin solid line or a dotted line. Finally, the solid line in the schematic drawing has been straightened to form a circle. Assuming that a fourth fork of DNA replication is located in the tangled region of the molecule shown in (B), the diagram shows how the structure is most consistent with DNA replication having initiated at two different sites. The uncertainty about the lengths of the second theta loop is a direct result of the inability to follow the tangled DNA thread near the site of the fourth arrow.

two daughter strands were within a few percent of the same length. Linear molecules with one or more replicating forks were seen on most spreads, but these were heterogeneous in length and probably of cellular DNA origin.

Replicating, circular EBV DNA molecules in a more complex configuration were seen in fractions 7 through 10 of the two glycerol gradients with DNA isolated from cells in the early and middle S phases (Fig. 2A and B). The partially completed daughter DNA strands are free to rotate around their parental template strands, and in the case of small, circular DNA molecules, the two newly replicated regions of covalently closed, supercoiled molecules are seen as two well-separated loops or wings attached to the coiled, unreplicated tail of the parental DNA (15, 24). We have not as yet seen covalently intact, supercoiled EBV DNA molecules in a similar configuration. The newly replicated regions of the two EBV molecules shown in Fig. 4 are instead apparently wrapped around each other many times. Whether this wrapping reflects a difference in the in vivo structure or is a property of large, partially replicated, supercoiled DNA molecules in solution is not known.

In the two early S phase DNA samples, a total of 19 circular EBV DNA molecules were seen, of which 10 were clearly in the process of DNA replication. No molecules with only a single DNA fork, indicative of the rolling-circle mode of DNA replication, have thus far been observed. However, among the 10 EBV DNA molecules with a pair of DNA forks enclosing one theta-type replication loop, two molecules were seen with a third, definite replication fork (Fig. 5). The DNA strand extending from the third fork of the molecule shown in Fig. 5A ends in a free DNA tail, and no indication of a fourth fork could be found on the parental DNA circle. This structure is consistent with a number of possible interpretations, including (i) a double initiation of DNA synthesis at the same origin, (ii) initiation of DNA synthesis at two independent origins, or (iii) a molecule undergoing a combination of both the Cairns and rolling-circle modes of replication. With regard to the molecule shown in Fig. 5B, a fourth fork might be located within the tangled region indicated by the fourth arrow. If this is the case, the structure would be consistent with DNA synthesis having initiated at two independent origins, as depicted diagramatically in the insert. Regardless of the interpretation, the finding of the two molecules shown in Fig. 5 among

a limited population of replicating EBV genomes suggests that the complete duplication of the large circular EBV DNA molecules may be more intricate than that of the simple Cairns type with initiation at a single site.

Analysis of DNA isolated late in the S phase (Fig. 2C) revealed no circular EBV DNA molecules in the process of replication, although some linear DNA forms with branch points were still found. In particular, fractions 9 and 10 and 17 through 19, which correspond, respectively, to the 130 and 90S regions of the gradient where replicating forms were found at earlier times, were searched in detail. What appear to be overlapping pairs of relaxed, open circular EBV genomes were observed in spreads of material sedimenting at ca. 90S. However, due to the exceedingly low concentration of EBV DNA, it is highly unlikely that these forms were simply two independent EBV genomes spread on top of each other, but it is likely instead that they were two physically interlocked, circular DNA molecules or catenated dimers. Catenated dimers of EBV DNA circles have previously been isolated as supercoiled forms from unsynchronized cultures of Raji cells (11). One of the dimers found in the 90S region of the late S phase sample was unusual in that the two monomers appear to be wrapped around each other two times (Fig. 6). The 92S peak at fraction 18 of the gradient shown in Fig. 2D is presumably also composed of relaxed catenated dimers, but because the entire fraction of this, the control gradient, was used for nucleic acid hybridization, it was not possible to confirm this by direct visualization in the electron microscope.

Catenated dimers of two circular EBV genomes were also found in fractions 9 and 10 of the gradient shown in Fig. 2C. Although one fully relaxed dimer, identical to those found to sediment in the 90S region, was observed, one or both of the monomeric EBV genomes of the other pairs isolated as fast-sedimenting material were in the supercoiled configuration. However, to have sedimented at the 130S position, both molecules of all dimers seen in these two fractions were presumably in the supercoiled configuration at the time of centrifugation. It is concluded that late in the S phase, catenated forms of circular EBV genomes are the predominate species in those fractions where replicating molecules were seen at earlier times in the cell cycle.

DISCUSSION

Circular EBV genomes in the theta configuration, typical of plasmids in the process of DNA replication, have been observed here by electron microscopic examination of DNA isolated from Raji cells during the S phase of the cell cycle. The data, on the basis of (i) the relative ease with which these forms were found in DNA samples prepared at times when the viral DNA was doubling in parallel with the replication of the host genome, (ii) the absence of these structures in the DNA from cells in the late S phase, as well as in all previously analyzed DNA samples isolated from unsynchronized cells, and (iii) the failure to detect any indication that lytic EBV DNA replication had been induced, strongly suggest that the circular EBV genomes, which are found in multiple copies in the nuclei of latently infected and transformed cells, are independent replicons. Because the EBV genome is largely repressed in nonproducer cells like Raji, the viral DNA must predominately employ the DNA synthesis machinery of the host for self-replication. The circular EBV genomes are approximately the same size as the supercoiled loops of chromosomal DNA and could be similarly associated with the matrix complex where DNA replication is thought to occur (26). All intracellular EBV DNA is associated with the chromosomes at metaphase (17), and this system may provide a useful model to probe further chromosome structure and DNA replication in mammalian cells.

Among a total of 10 circular EBV genomes seen in the process of DNA replication, 2 molecules had a complex structure (Fig. 4), which to our knowledge has not previously been described, and 2 molecules indicated that DNA replication had probably initiated more than once (Fig. 5). The newly duplicated regions of the simian virus 40 genomes are apparently not wrapped around each other in partially replicated molecules (15, 24). However, wrapping is introduced during the terminal stages of simian virus 40 DNA replication when the two replicating forks meet, and newly formed catenated dimers have been found to be twined around each other up to 20 times (25). We have thus far only observed the two newly replicated regions of EBV DNA to be twined around each other in molecules where DNA

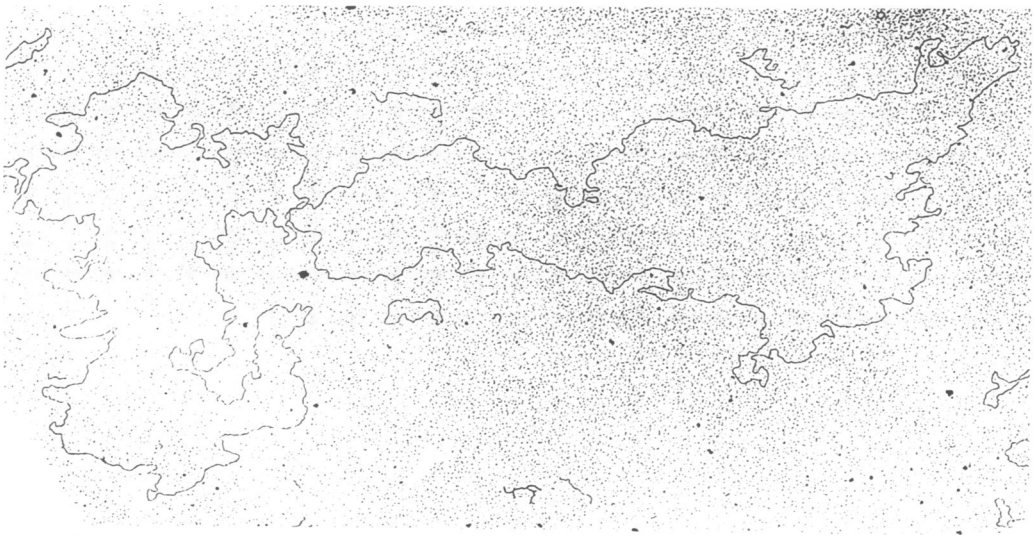

FIG. 6. Electron micrograph of a catenated dimer of two circular EBV genomes. The molecule is from fraction 18 of the glycerol gradient shown in Fig. 2C. At the point of contact the two circular monomers appear to be wrapped around each other twice.

replication is more than 50% complete. This, combined with the possibility that EBV DNA replication may initiate at more than one site on the same DNA molecule, suggests that the wrapping seen could likewise have been introduced when two replicating forks met. With regard to multiple sites for the initiation of DNA synthesis, it is of interest that three potential origins of DNA replication, detected as sequences capable of conferring the property of autonomous replication to a selectable gene in the yeast transfection assay, have been mapped to three widely separated sites on the EBV genome (14). Moreover, the distance between the initiation sites of chromosomal DNA replication in EBV-positive human lymphoid cells is 10 to 20 μm, which would be compatible with the 50-μm EBV genomes employing more than one origin during normal DNA replication (20).

ACKNOWLEDGMENTS

We are grateful to G. Klein (Karolinska Institute, Stockholm, Sweden) for cells and antisera and thank K. Nordström and J. Zabielski (Uppsala University, Uppsala, Sweden) for helpful discussions.

The work was supported by grants from the Swedish Cancer Society and Uppsala University.

LITERATURE CITED

1. **Adams, A.** 1979. The state of the EB virus genome in transformed cells and its relationship to host cell DNA, p. 155–183. *In* M. A. Epstein and B. G. Achong (ed.), The Epstein-Barr virus. Springer-Verlag, Berlin.
2. **Adams, A., G. Bjursell, C. Kaschka-Dierich, and T. Lindahl.** 1977. Circular Epstein-Barr virus genomes of reduced size in a human lymphoid cell line of infectious mononucleosis origin. J. Virol. **22**:373–380.
3. **Adams, A. and T. Lindahl.** 1975. Intracellular forms of Epstein-Barr virus DNA in Raji cells, p. 125–132. *In* G. deThé, M. A. Epstein, and H. zur Hausen (ed.), Oncogenesis and herpesviruses II, part 1. International Agency for Research on Cancer, Lyon, France.
4. **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.
5. **Adams, A., T. Lindahl, and G. Klein.** 1973. Linear association between cellular DNA and Epstein-Barr virus DNA in a human lymphoblastoid cell line. Proc. Natl. Acad. Sci. U.S.A. **70**:2888–2892.
6. **Andersson, M., and T. Lindahl.** 1976. Epstein-Barr virus DNA in human lymphoid cell lines: *in vitro* conversion. Virology **73**:96–105.
7. **Benbow, R. M., M. Eisenber, and R. L. Sinsheimer.** 1972. Multiple length DNA molecules of bacteriophage ϕX 174. Nature (London) New Biol. **237**:141–144.
8. **Freifelder, D.** 1970. Molecular weights of coliphages and coliphage DNA. IV. Molecular weights of DNA from bacteriophages T4, T5 and T7 and the general problem of determination of M. J. Mol. Biol. **54**:576–577.
9. **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.
10. **Gussander, E., and A. Adams.** 1979. Intracellular state of Epstein-Barr virus DNA in producer cell lines. J. Gen. Virol. **45**:331–340.
11. **Gussander, E., and A. Adams.** 1980. New intracellular states of Epstein-Barr virus DNA in the Burkitt's-lymphoma-derived Raji cell line. Cold Spring Harbor Conf. Cell Proliferation **7**:23–34.
12. **Hampar, B., J. G. Derge, L. M. Martos, M. A. Tagamets, S.-T. Chang, and M. Chagrabarty.** 1973. Identification of a critical period during the S phase for activation of the Epstein-Barr virus by 5-iododeoxyuridine. Nature (London) New Biol. **244**:214–217.
13. **Hampar, B., A. Tanaka, M. Nonoyama, and J. Derge.** 1974. Replication of the resident repressed Epstein-Barr virus genome during the early S phase (S-1 period) of non-producer Raji cells. Proc. Natl. Acad. Sci. U.S.A. **71**:631–633.
14. **Henry, B. E., II, N. J. Raab-Traub, and J. S. Pagano.** 1983. Detection of autonomous replicating sequences (ars) in the genome of Epstein-Barr virus. Proc. Natl. Acad. Sci. U.S.A. **80**:1096–1100.
15. **Jaenisch, R., A. Mayer, and A. Levine.** 1971. Replicating SV40 molecules containing closed circular template DNA strands. Nature (London) New Biol. **233**:72–75.
16. **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.
17. **Nonoyama, M., and J. S. Pagano.** 1971. Detection of Epstein-Barr viral genome in nonproductive cells. Nature (London) New Biol. **233**:103–106.
18. **Nonoyama, M., and J. S. Pagano.** 1972. Separation of Epstein-Barr virus DNA from large chromosomal DNA in non-virus-producing cells. Nature (London) New Biol. **238**:169–171.
19. **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.
20. **Oppenheim, A., Z. Shlomai, and H. Ben-Bassat.** 1981. Initiation points for cellular deoxyribonucleic acid replication in human lymphoid cells converted by Epstein-Barr virus. Mol. Cell. Biol. **1**:753–762.
21. **Pagano, J. S.** 1974. The Epstein-Barr viral genome and its interactions with human lymphoblastoid cells and chromosomes, p. 79–116. *In* E. Kurstak and K. Maramorosch (ed.), Viruses, evolution and cancer. Academic Press, Inc., New York.
22. **Pritchett, R., M. Pedersen, and E. Kieff.** 1976. Complexity of EBV homologous DNA in continuous lymphoblastoid cell lines. Virology **74**:227–231.
23. **Pritchett, R. F., S. D. Hayward, and E. D. Kieff.** 1975. DNA of Epstein-Barr virus. I. Comparative studies of the DNA of Epstein-Barr virus from HR-1 and B95-8 cells: size, structure, and relatedness. J. Virol. **15**:556–569.
24. **Sebring, E. D., T. J. Kelley, Jr., M. M. Thoren, and N. P. Salzman.** 1971. Structure of replicating simian virus 40 deoxyribonucleic acid molecules. J. Virol. **8**:478–490.
25. **Sundin, O., and A. Varshavshy.** 1980. Terminal stages of SV40 DNA replication proceed via multiply intertwined catenated dimers. Cell **21**:103–114.
26. **Vogelstein, B., D. M. Pardoll, and D. S. Coffey.** 1980. Supercoiled loops and eucaryotic DNA replication. Cell **22**:79–85.
27. **zur Hausen, H.** 1975. Oncogenic herpes viruses. Biochim. Biophys. Acta **417**:25–53.