B CELL ACTIVATION AND THE ESTABLISHMENT OF EPSTEIN-BARR VIRUS LATENCY

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All herpesviruses are capable of remaining latent in host cells subsequent to primary infection. Understanding the mechanism by which these viruses establish and maintain latency and undergo reactivation is critical to understanding the biology and clinical consequences of herpesvirus infection. EBV is a lymphotropic herpesvirus that infects the majority of the human population subclinically. The virus is the causative agent of infectious mononucleosis and is associated with nasopharyngeal carcinoma and the endemic form of Burkitt's lymphoma (BL)[1] (1, 2). In addition, EBV-containing B cell lymphomas are known to occur in immunosuppressed persons, such as organ transplant recipients treated with cyclosporin A, and individuals with AIDS (3, 4). EBV can also infect human peripheral blood B cells in vitro, and this system currently provides the most accessible model for the study of herpesvirus latency.

In vitro latent infection with EBV causes B cells to become activated. It has been shown that EBV-infected cells express the B cell activation antigen CD23 (Blast-2) and show increased RNA content and synthesis by 24 h. The expression of CD23 is predictive of subsequent proliferation that occurs at 2–3 d post-infection (5). Unlike other agents of B cell activation, EBV does not induce the cells to terminally differentiate; rather, the infected cells maintain a blastoid state and proliferate permanently. The derivation of lymphoblastoid cell lines that are latently infected with EBV is, therefore, a two-step process involving activation and immortalization.

The latently infected B cells also express characteristic viral markers. These include the Epstein Barr nuclear antigens (EBNAs) (6–10), and the latent membrane protein, which is capable of transforming established rodent cell lines (11).

The onset of latency is also accompanied by structural changes within the viral genome. EBV is a dsDNA virus of ~170 kb, the genome of which is linear in the virion (12). In latently infected cells, however, the viral DNA is present as a covalently closed, circular episome (CCC) (13, 14). The termini of the linear EBV genome are composed of a variable number of directly repeated sequences. The process of circularization most likely involves recombination between repeat units on the opposing termini, and the number of terminal repeats (TR) used to form a CCC

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[1] *Abbreviations used in this paper:* ACV, Acyclovir; Ara C, Arabinoside C; BL, Burkitt's lymphoma; CCC, covalently closed, circular episome; EBNA, Epstein-Barr nuclear antigen; TR, terminal repeats.

is characteristic of any circularization event (15, 16). Latently infected cells contain multiple copies of the circular viral genome due to amplification of viral relative to cellular DNA (17). The production of a circular EBV genome is a crucial step in the immortalization process as it establishes the genome in the state in which it can persist in the infected B cell and thus ensures continued cellular proliferation. In this paper we have examined the circularization event in newly infected B cells in vitro in relation to the processes of B cell activation and immortalization.

Materials and Methods

Cell Lines. The BL cell lines Raji and Ramos were obtained from American Type Culture Collection (Rockville, MD), and BL2 was a kind gift of Dr. G. Lenoir (IARC, Lyon, France). These lines were maintained in RPMI 1640 supplemented with 10% FCS and antibiotics at 37°C and split to 0.3 × 10^6/ml twice weekly. The EBV-producing marmoset cell line B958 was a kind gift of Dr. E. Kieff (Harvard University Medical School, Boston, MA) and maintained as above, except that it was fed with 8% FCS. The P3HRI cell line, a kind gift of Dr. M. Nonoyama (Showa University Research Institute, St. Petersburg, FL), was maintained in RPMI 1640 with 5% FCS and antibiotics at 34°C.

Virus. B958 or P3HRI cell lines were seeded at 0.3–0.4 × 10^6/ml. Supernatant fluids from these cultures were harvested 7 d later by centrifugation at 2,000 rpm for 15 min at 4°C. Aliquots were frozen at −70°C and used as a source of infectious virus. The TD_{50} of B95-8 stocks was estimated to be 5 × 10^5/ml.

Purification of B Cells. Whole peripheral blood cells, obtained by venipuncture of healthy donors, or by-products of platelet pheresis, obtained from the Blood Bank of the Dana Farber Cancer Institute (Boston, MA), were centrifuged over Ficoll-Hypaque to obtain PBL. Enrichment for B cells was accomplished by either of two methods. In some instances, the B cells were purified by positive selection on columns of Sephadex-coupled rabbit anti-human Fab and eluted with 1% human gamma globulin, as described previously (18). An alternative method used was negative selection of B cells by incubation of PBL with SRBCs treated with Neuraminidase (Gibco Laboratories, Grand Island, NY). PBL were incubated with SRBCs at 4°C for 1 h, centrifuged over Ficoll-Hypaque, and the nonrosetting fraction, containing B cells and macrophages, was subjected to adherence on plastic or glass at 37°C for 1 h or more. Nonadherent cells were then decanted and used as a source of B cells. A second rosetting did not appreciably enrich for B cells. Both of these methods yielded <1% T cells and <10% macrophages, as determined by indirect immunofluorescence using mAbs specific for T cell (OKT3) or macrophage (MO2) markers. Greater than 90% of these cells reacted with antibody to the B cell-specific marker B1.

Infections. For infections using undiluted viral supernatant (all infections unless otherwise noted), 4 × 10^6 purified B cells were suspended in 1 ml viral supernatant and incubated at 37°C. At 2 h post-infection, an equal volume of RPMI 1640 containing 20% FCS and antibiotics was added. Cells were fed approximately every 3 d by diluting the culture with RPMI 1640/20% FCS.

Gardella Gel Technique. Analysis of viral DNA was carried out by the method of Gardella et al. (19), with modifications. This technique allows the resolution of linear and circular viral DNA from EBV-infected cells. Horizontal gels of 0.75% agarose were poured. The area above the wells was removed and replaced with 0.8% agarose containing 2% SDS and 1 mg/ml pronase. 10^6 or 2.5 × 10^6 whole cells were placed in each well. Electrophoresis was carried out at 4°C at 30 V for 2–3 h, at which time the voltage was increased to 160 V for an additional 14–18 h. Gels were then stained with ethidium bromide (0.5 μg/ml) for 0.5–1 h, depurinated with HCl, denatured with NaOH,and neutralized as described. Transfer was accomplished by the method of Southern (20) onto ζ probe membranes (Bio-Rad Laboratories, Richmond, CA). Blots were baked for 1–2 h at 80°C in a vacuum oven. Hybridization to nick-translated fragments of the EBV genome was performed according to standard methods (21). In many of these experiments, the Bam HI W fragment of the genome was used as a probe. However, if blots were erased and reprobed with the Bam HI L, Bam HI K, or

Mlu subfragment of the Bam Nhet (see below) fragment of the genome, the signals obtained were similar in relative intensity. In their initial report, Gardella et al. (19) could detect as few as 0.25 EBV genome equivalents using this technique. By optimizing conditions, we have detected as few as 0.05 genome equivalents per cell.

Quantitation of EBV Genomes within Infected Cells. The number of circular and linear viral genomes within EBV-infected cells was estimated by comparison to serial dilutions of the Raji BL cell line. Raji cells, which contain ~60 (published estimates give an average of 56 ± 8) EBV genome copies per cell (22), were serially diluted with EBV^- BL cells, either Ramos or BL2. The diluted Raji cells were subjected to electrophoresis on Gardella gels, blotted, and hybridized in parallel with equal numbers of the experimental cells (for example, see Fig. 2). The signals obtained upon autoradiography were scanned with a scanning densitometer (LKB Instruments, Inc., Gaithersburg, MD). The area under each curve obtained by densitometry was plotted against genome number (typically, from 60 down to 1/16 of a genome equivalent) and used to generate a standard curve. Experimental values were then determined by measuring the area under the curve obtained by densitometry of the experimental samples and calculating a genome number per cell from the Raji cell standard curve. A similar method has been used to estimate EBV genome numbers in infected cells by whole cell DNA dot blot (23). In various established cell lines, the estimated genome numbers we obtain using this system are concordant with published values to ~20–30%.

Inhibitors. For mitomycin C treatment, B cells were purified from peripheral blood, washed, and resuspended at 10^7 cells/ml in RPMI 1640 containing 50 μg mitomycin C (Sigma Chemical Co., St. Louis, MO). This concentration is sufficient to completely inhibit proliferation of mitogen-treated B cells. The cells were incubated at 37°C for 0.5 h, washed four times, and resuspended at 4×10^6/ml in B958 viral supernatant. Control cells were treated in the same manner, except mitomycin C was excluded. Cells were harvested on days 1, 2, and 3 post-infection.

For experiments with other inhibitors, cells were treated with 100 μM Acyclovir (ACV; Burroughs Wellcome Co., Greenville, NC) (90% inhibition of viral capsid antigen production in P3HR-1), 1 or 10 μg/ml actinomycin D (Sigma Chemical Co.) (>99% inhibition of [^{3}H]thymidine incorporation at both concentrations), 5, 20, or 50 μg/ml cycloheximide (Sigma Chemical Co.) (98% inhibition of [^{3}H]leucine incorporation at all three concentrations), or 20 or 50 μg/ml Arabinoside C (Ara C; Upjohn Co., Kalamazoo, MI) (92% and 96% inhibition of [^{3}H]thymidine incorporation, respectively), for 1 h before infection, and the drugs were maintained in the culture throughout the course of the infection. Cells were harvested at 2–3 d post-infection. Ficoll-Hypaque density centrifugation was performed before harvesting, and cells were >90% viable as judged by trypan blue exclusion.

Isolation of Nuclei. Nuclei were extracted from cells at 48 h post-infection using 0.5% NP-40, as described by Benz and Strominger (24). Upon Wright-Giemsa staining, <10% of the nuclei showed associated cytoplasmic staining.

EBNA Staining. EBNA staining was carried out by anti-complement immunofluorescence as described by Reedman and Klein (25). Positive serum was obtained from a donor seropositive for EBV, and complement and negative serum were obtained from an EBV seronegative donor.

Cytofluorographic Analysis. For analysis, ~10^6 cells were washed in RPMI 1640 containing 1% FCS. The cells were then resuspended in 100 μl of hybridoma ascites of various antibodies and isotype-matched negative controls, diluted 1:100. For cell sorting, 10^7 cells per tube (total of 10^8) were washed and resuspended in 200 μl EBVCS hybridoma ascites; EBVCS recognizes the CD23 (Blast-2) B cell activation antigen, and was a kind gift of Dr. B. Sugden (University of Wisconsin, Madison, WI). Cells were incubated with the antibodies for 20 min at 4°C, washed twice, and resuspended in 100 μl (for analysis) or 200 μl (for sorting) FITC-labeled goat anti-mouse Ig (Cappel Laboratories, Cochranville, PA), diluted 1:20. Cells were incubated another 20 min at 4°C, washed twice, and resuspended at 10^6/ml (for analysis) or 8×10^6/ml (for sorting). Analysis was performed on a FACS IV. Sorting was carried out on an EPICS cell sorter. For sorting experiments, only small lymphocytes were collected to avoid contamination by lymphoblasts or monocytes. The 20% most and least fluorescent cells were collected as $CD23^+$ and $CD23^-$ populations, respectively.

Cloning. Cells were cloned on day 6 post-infection by limiting dilution. Microtiter plates containing 1, 10, and 100 cells per well were established on a feeder layer of irradiated human foreskin fibroblasts (350 Q). Wells were fed approximately once every week by removing half the volume and replacing it with fresh medium (RPMI, 20% FCS, antibiotics). Clonality of the cell lines was determined by analysis of Ig gene rearrangements on Southern blots.

Analysis of EBV-terminal Fragments in Clonal Cell Lines. High molecular weight DNA from EBV-infected B cell clones or lines was extracted essentially as described (21). 1–10 μg of purified cellular DNA was digested with 10–20 U of Bam H1, subjected to electrophoresis on 0.6% agarose gels, and Southern blotted onto ζ probe membranes. Blots were then hybridized to an Mlu subfragment of the Bam H1 Nhet fragment of EBV, which corresponds to the right-hand end of the genome (11). The Mlu fragment was obtained by Bam H1 digestion of plasmid pHS1-LM, a kind gift of Dr. E. Kieff (Harvard University Medical School, Boston, MA). This fragment was labeled with ^{32}P by the random priming method according to the manufacturer's specifications (Boehringer Mannheim Biochemicals, Indianapolis, IN). Upon circularization of the linear viral genome, Bam Nhet is linked to sequences in the left-hand end of the genome (Bam Jhet), to form CCC containing a new restriction fragment, Bam NJhet. When CCC are digested with Bam HI, Southern blotted, and hybridized to the Mlu probe, the size of Bam NJhet varies from 8 to 11.5 kb, depending on the number of TR used to form the CCC. Bam NJhet contains 7.5 kb unique DNA and its total molecular weight varies in increments of 500 bp, the size of the repeat unit (26).

Results

Circular Viral Genomes Can be Detected within EBV-infected Cells by 20 h After Infection. The EBV genome in the virion is linear; however, in stably transformed LCL, the latent genome persists as multiple covalently closed circles. To determine when the transition from the linear to the circular form occurs, peripheral blood B cells were infected with the B95-8 strain of EBV and harvested at various times after infection. Linear and circular viral genomes were then separated using the Gardella gel technique and detected by Southern blotting and hybridization to EBV-specific probes. As shown in Fig. 1, CCC were apparent by 20 h post-infection; however, in some experiments they were detected as early as 16 h post-infection. Only a small amount of the total EBV DNA within the infected cells assumed a circular configuration; the majority of the viral DNA retained the linear form.

Distribution of EBV Genomes within Infected Cells from 0 to 48 h Post-infection. To analyze the fate of EBV DNA in a quantitative fashion, we measured the amount of linear and circular DNA present at various times after infection. A summary of the results is presented in Table I. In this experiment, the highest multiplicity of virus obtainable was used in order to illustrate the relevant events most clearly; however, the distribution of viral genomes within newly infected cells was always similar, regardless of the multiplicity used. When we analyzed an aliquot of our virus preparation, we found that under the experimental conditions used, B cells were exposed to ~170 linear genomes per cell. By 6 h post-infection, an average of 30-40 linear genomes were associated with each cell, of which, seven remained cell associated by 48 h. At this time approximately one-fourth of the linear genomes were found in the nuclear fraction. Analysis of CCC at 48 h revealed an average of 0.16 per cell. Therefore, we may conclude that at most 16% of the cells exposed to EBV contained a viral genome in its latent form, and that only 0.1% of the original input genomes and 5% of the cell-associated genomes became CCC.

Production of a Circular EBV Genome Can Be Correlated with a Specific Stage in B Cell Activation. The first detection of CCC at 16–20 h post-infection suggests that cir-

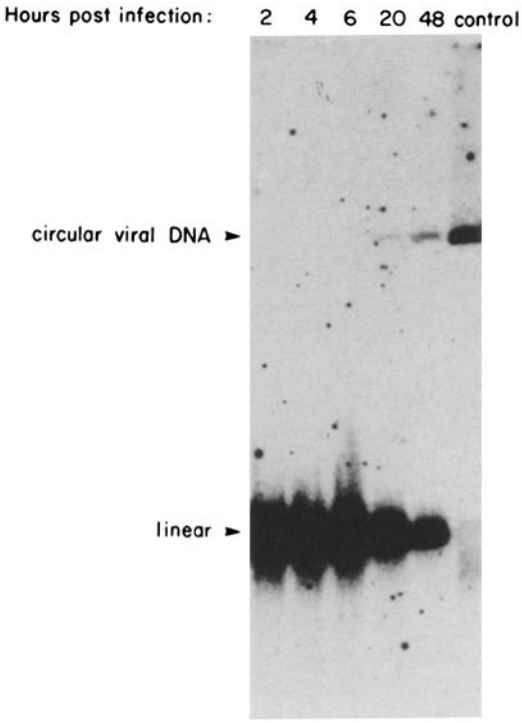

FIGURE 1. Kinetics of appearance of CCC within newly EBV-infected B cells. B cells were harvested at 2, 4, 6, 20, and 48 h post-infection, analyzed by Gardella gels, Southern blotted, and hybridized to an EBV-specific probe.

cularization of EBV genomes occurs sometime before the activated B cells begin proliferating. To better define the level of cellular activation required for the genome to circularize, we blocked the infection process at various stages.

To determine if cellular DNA synthesis was necessary for the production of CCC, cells were treated with the DNA synthesis inhibitor mitomycin C and then extensively washed to remove excess drug before infection. These cells showed no reduction in circular viral genome number at 2 d post-infection (Fig. 2 *A*) despite their

TABLE I

Fate of Infectious Virus from 0 to 48 h Post-infection

Time post-infection	Location	Linear viral genomes per cell	Circular viral genomes per cell
h			
0	Input genomes	170	<0.10
6	Whole cell	30–40	<0.10
48	Whole cell	6.7	0.16
48	Nuclei	1.8	0.16

Cells were harvested at 0, 6, and 48 h post-infection, analyzed on Gardella gels, Southern blotted, and hybridized to EBV-specific probes. Numbers of viral genomes were estimated by scanning densitometry and comparison to populations of Raji cells that were subjected to the same procedure. See Fig. 2 *A* for representative Raji cell serial dilution with EBV^- BL cells.

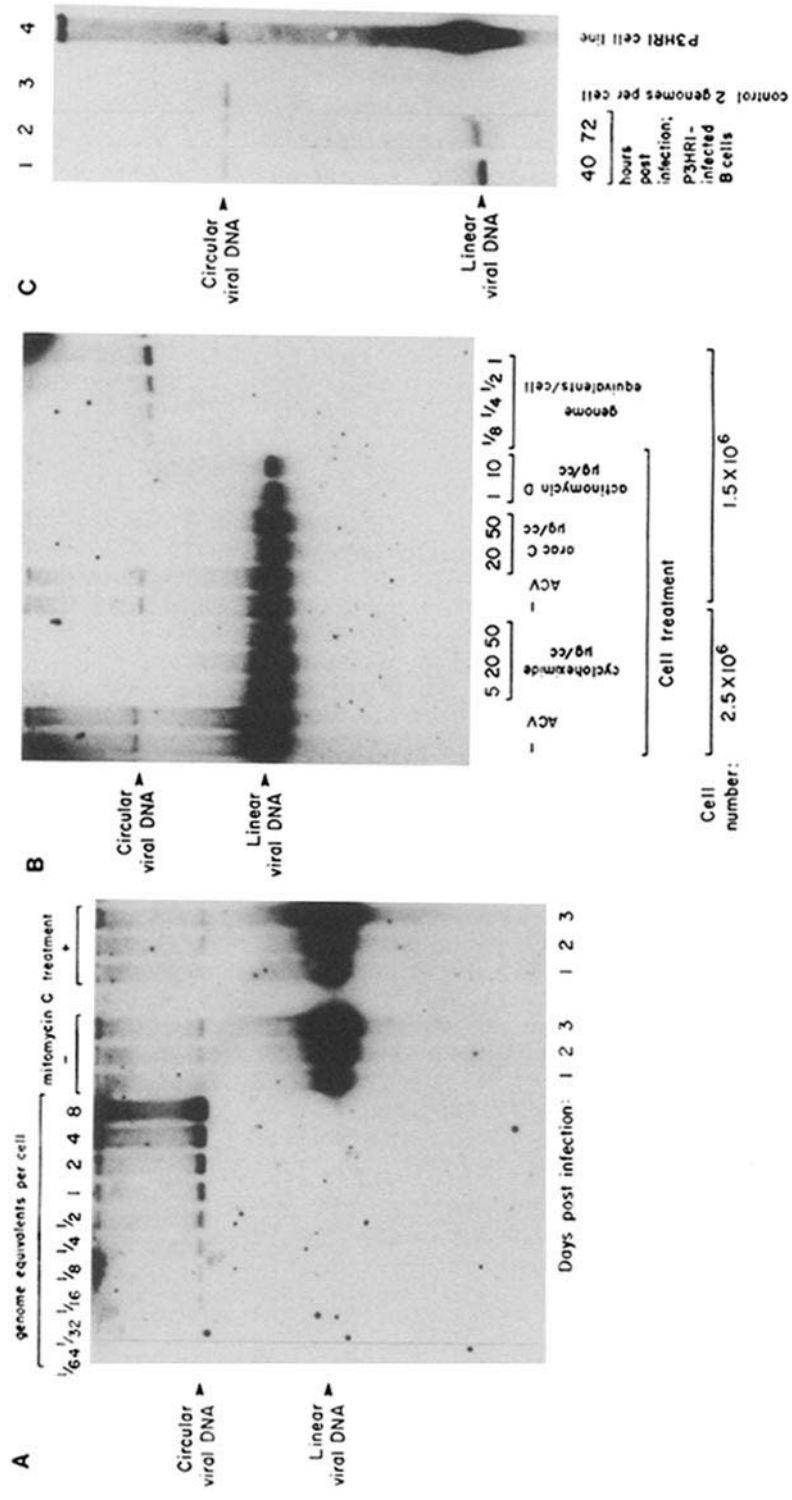

FIGURE 2. (*A*) Effect of mitomycin C on circularization of EBV genomes. Cells were treated with mitomycin C before infection, then harvested in parallel with untreated cells on days 1–3. Also shown is a representative serial dilution of Raji cells with EBV$^-$ BL cells. Signals corresponding to 1/64 up to 8 genomes per cell are shown. (*B*) Effects of inhibitors of protein, RNA, and DNA synthesis on circularization of EBV genomes. Cells were treated with ACV, cycloheximide, Ara C, or actinomycin D before infection, harvested at 2–3 d post-infection, and analyzed via the Gardella technique. 2.5×10^6 or 1.5×10^6 cells were analyzed. (*C*) Analysis of viral DNA in B cells infected with P3HR1 virus. B cells were infected with P3HR1 virus and analyzed at 40 and 72 h post-infection (lanes *1* and *2*) via the Gardella gel technique. Lane *3* shows Raji cells serially diluted with EBV$^-$ BL cells to generate a signal corresponding to two EBV genomes per cell. Lane *4* shows an analysis of the P3HR1 cell line from which the infecting virus was derived.

inability to proliferate. Entry of infected cells into S phase is, therefore, not a prerequisite for the production of CCC.

Inhibitors of the viral and cellular DNA polymerases were also examined for their ability to inhibit production of circular EBV genomes. In these experiments, cells were treated with the inhibitors before and during the infection process and examined for the presence of circular genomes at days 2–3 post-infection. When cells were treated with the viral DNA polymerase inhibitor, ACV, there was no effect on the number of CCC detected (Fig. 2 *B*), indicating that the viral polymerase is not necessary for production of CCC. Cells were also exposed to the DNA polymerase inhibitor Ara C, which inhibits cellular as well as viral DNA polymerase activities. This treatment resulted in abolishment of detectable circularization (Fig. 2 *B*). Since cellular DNA synthesis per se is not required for circularization, it therefore appears that cellular DNA polymerase may be required for the transition of the virus from the linear to the circular form.

As mentioned above, infected cells do not need to enter S phase in order for circularization of the EBV genome to occur. To determine if progression through the G_1 phase of the cell cycle is necessary, cells were infected with the P3HRI strain of EBV. P3HRI is a nontransforming strain of EBV that lacks the gene for the latent viral nuclear protein EBNA2, and P3HRI-infected B cells show no reactivity with anti-EBNA sera (27, 28). Infection with P3HRI induces B cells to progress through G_0 and enter the early G_1 phase (G_{1A}) of the cell cycle but not late G_1 (G_{1B}) or S phases (29). As shown in Fig. 2 *C*, P3HRI-infected B cells contained CCC at 2–3 d post-infection. Therefore, neither entry of infected cells into G_{1B} nor expression of the EBNA gene products are necessary for the production of CCC.

To assess whether RNA or protein synthesis were required for circularization to occur, cells were treated with the RNA synthesis inhibitor, actinomycin D, or the protein synthesis inhibitor, cycloheximide. Infected cells maintained on these agents do not express the B cell activation antigen CD23 (Blast-2) or enter G_1 (5). At 2–3 d post-infection, cells treated with these inhibitors do not contain CCC (Fig. 2 *B*). This result indicates that the *de novo* protein and RNA synthesis required for CD23 expression and entry into G_1 are also required for the production of CCC. Therefore, it appears that B cells must be activated to late G_0 (G_{0B}) or G_{1A}, but not G_{1B} in order for circularization to occur.

The Presence of Circular Viral Genomes Correlates with Expression of the B Cell Activation Antigen CD23 (Blast-2). Given that circularization occurs at an early stage of B cell activation, we reasoned that the presence of CCC should correlate with the expression of early surface markers of infection or activation. It has been shown previously that more cells express EBNA upon infection than actually become immortalized. Only those $EBNA^+$ cells that simultaneously express the B cell activation antigen CD23 proceed to immortalize (5). In any given experiment, we found that the number of CCC correlated closely with the number of cells expressing CD23, rather than the number expressing EBNA. This correlation suggested the possibility that the same cells express CD23 and contain a single CCC. If this were true, then the presence of CCC would be predictive of immortalization. To test whether this was the case, B cells were separated by FACS into $CD23^+$ and $CD23^-$ populations at 36 h post-infection and analyzed for the presence of CCC. Although the $CD23^+$ cells were activated, they should not have become proliferating blasts until 48–72 h post-

infection. However, to avoid contamination by B cells that were already activated at the time of infection, blastoid cells were excluded by selecting for small cells before CD23 selection. When the unselected and $CD23^+$ populations were analyzed for CCC, it was found that there were 1.3 CCC/$CD23^+$ cell in the unselected population, and 1.0 CCC/$CD23^+$ cell in the selected population (Fig. 3, Table II). As these two numbers are not significantly different, this result suggests that there is direct concordance between CD23 expression and the presence of a single CCC. This conclusion was further corroborated by the observation that no CCC were detected (<0.10 per cell) in the $CD23^-$ population. Thus, only $CD23^+$ cells that contain CCC at 36 h post-infection proceed to immortalize.

Amplification of Circular Viral DNA Is not Essential for the Establishment of Latency. Amplification of CCC relative to cellular DNA has been demonstrated in cell lines latently infected with EBV (17). To examine the kinetics of amplification of the one CCC present in $CD23^+$ cells at 36 h, we performed extended time course analyses from days 2–12 post-infection. Results of a typical experiment, in which two dilutions of viral supernatant were used, are shown in Fig. 4. The number of CCC detected early after infection was proportional to the amount of input virus (Fig. 4 and data not shown), indicating that this, rather than the fraction of infectable cells, was limiting on the number of CCC detected. Cultures exposed to undiluted viral supernatant contained essentially 100% $EBNA^+$ cells by day 8 (data not shown), indicating that they were all infected. In addition, all cells expressed CD23 (Fig. 5), indicating that they were immortalized B lymphoblasts. At this time, there was an average of one CCC per cell, with only residual linear viral DNA remaining. Since all of these cells were infected and immortalized, each must contain a viral genome, as opposed to a few cells containing multiple viral genomes and others containing none. Amplification of the viral genome in the cells infected with undiluted

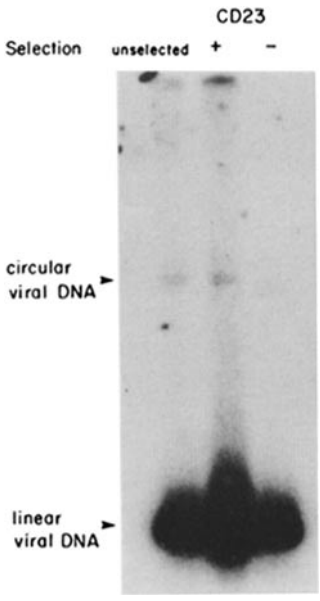

FIGURE 3. Analysis of viral DNA within $CD23^+$ and $CD23^-$ populations. Infected cells were separated by FACS at 36 h into $CD23^+$ and $CD23^-$ populations. These were then analyzed by the Gardella gel technique along with unselected cells.

TABLE II

Quantitation of Circular Viral DNA within CD23$^+$ and CD23$^-$ Cells

CD23 selection	Circular genomes per cell	Percent CD23$^+$ cells	Circular genomes per CD23$^+$ cell
None (unseparated)	0.5	40	1.3
Negative	<0.13	<0.1	-
Positive	0.8	83	0.96

The number of circular viral genomes per cell was determined by scanning densitometry of the bands shown in Fig. 3, and comparison to a Raji cell standard curve. The percentage of CD23$^+$ cells was obtained by post-sorting analysis of the individual fractions.

viral supernatant was not detected until day 10, when two CCC per cell were detected. Interestingly, we observed that the initiation of amplification could consistently be correlated with the loss of incoming linear viral DNA (Fig. 4 and data not shown). The fact that amplification did not occur until 1 wk after all of the markers characteristic of latent infection had appeared indicates that amplification of circular viral DNA is not necessary for B cell activation or the establishment of latent infection by EBV.

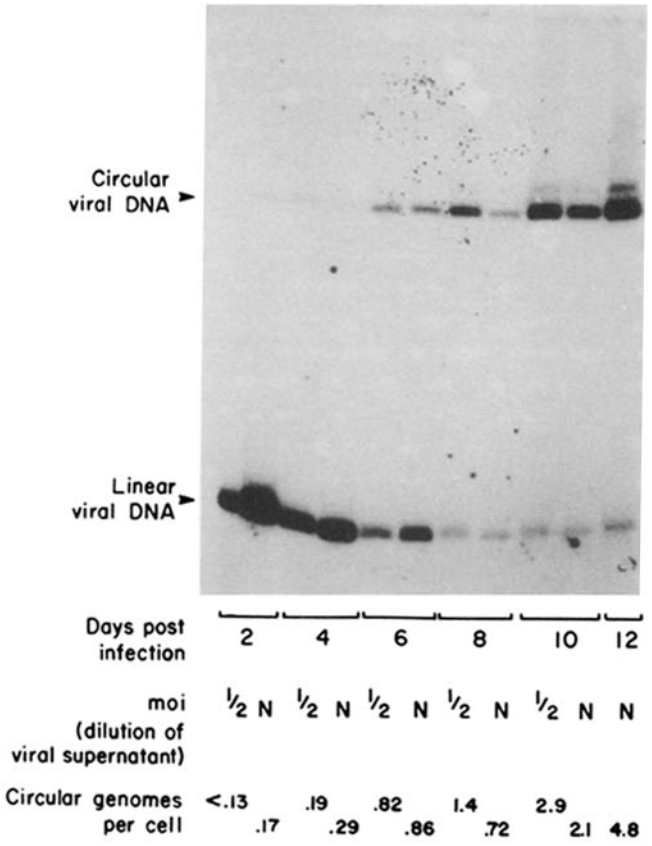

FIGURE 4. Analysis of linear and circular viral DNA from days 2–12 post-infection. Cells infected with undiluted or a half-diluted preparation of viral supernatant were harvested on days 2, 4, 6, 8, 10, and 12 (undiluted only) post-infection and analyzed for the presence of circular and linear viral DNA. Numbers of circular genomes per cell are indicated at the bottom of the figure.

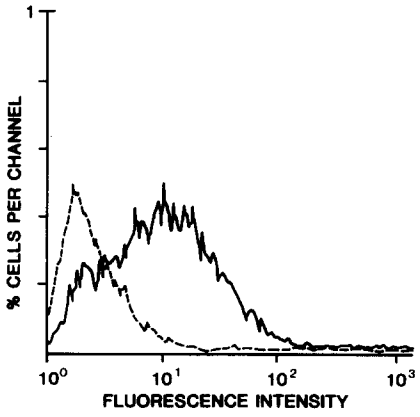

FIGURE 5. CD23 expression on day 8 post-infection. The infected cell population was stained for CD23 expression by indirect immunofluorescence using the EBVCS2 antibody on day 8 post-infection. *Heavy line*, anti-CD23; *light line*, control isotype-matched antibody.

Clonal Cell Lines Contain a Clonal Population of EBV Episomes. Our analysis suggests that a single viral genome circularizes in an infected cell and subsequently proceeds to amplify, giving rise to multiple episomes. If this hypothesis is correct, then clonal cell lines derived from infections, such as those shown in Fig. 4, should contain a clonal population of viral episomes. When a CCC is formed, varying numbers of the 500-bp direct terminal repeat may be used. If multiple circularization events occur in a single cell, the resulting clonal cell line will contain multiple fused terminal fragments, whereas, if a single event occurs, only one predominant fragment will be detected. Therefore, Southern blots of Bam HI-digested DNA from five independent clonal cell lines were hybridized to a probe corresponding to unique DNA contained within the right-most Bam H1 fragment of the EBV genome in order to determine the number of fused terminal fragments each clone contained. Newly established polyclonal cell lines contained eight different EBV-terminal fragments (Fig. 6), varying in size from 8 to 11.5 kb, in increments of 500 bp. In contrast, the cloned cell lines showed either a single sized fragment hybridizing to the probe (clones C2, E5, and H8), or one major hybridizing species, with several minor bands (D9, F8) (Fig. 6). The latter pattern has been observed previously in clonal cell lines that were originally infected with a single genome (16). Furthermore, the size of the hybridizing fragment within each clone was distinct from that within any other clone. This finding indicates that these five clones arose independently. The independent and clonal nature of these lines was also confirmed by analysis of Ig gene rearrangements within the cells (data not shown). Therefore, our results demonstrate that the multiple CCC genomes in our clones arose from a single genome that circularized and subsequently amplified.

CCC Replicate but Do not Amplify Early after Infection. As mentioned above, before the initiation of amplification, CCC were maintained at one per $CD23^+$ cell. We wished to determine whether or not the viral genome was able to replicate during this time. Therefore, we performed a quantitative analysis of total CCC and linear viral genomes in a given culture over time. From days 1 to 11 post-infection, the number of CCC increased exponentially over time (Fig. 7) in parallel with cellular proliferation (data not shown). In contrast, input linear viral DNA was gradually lost, and by day 7 only a constant residual amount of linear viral DNA remained

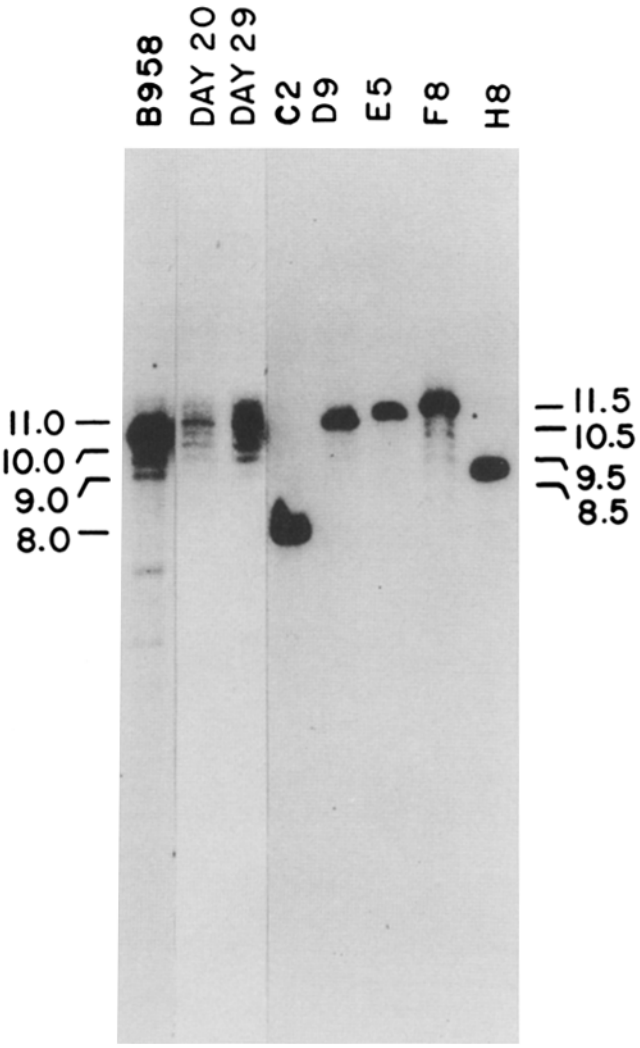

FIGURE 6. Analysis of EBV-fused termini in polyclonal and monoclonal cell lines. High molecular weight DNA was extracted from the B958 cell line, polyclonal cell lines at day 20 or 29 post-infection, and five EBV-infected B cell clones (C2, D9, E5, F8, H8). The DNA was digested with Bam H1, subjected to electrophoresis, and Southern blotted and hybridized to a probe specific for unique DNA contained within the right-most Bam H1 fragment of the EBV genome.

in the population. This amount would not be sufficient to account for the increase in CCC occurring at this time. Therefore, we may conclude that the resident CCC genome replicates as the cell divides, and by extrapolation, commences replication at the onset of cellular proliferation. By extrapolation of the graphs shown in Fig. 7 back to time zero, we also find that 1 of every 1,000 input genomes become a CCC. This result is consistent with the data shown in Table I.

Discussion

The present study was undertaken to identify cellular events critical to the establishment of latency in EBV-infected B cells. Our findings indicate that when B cells were exposed to multiple linear viral genomes only a small fraction reached the nu-

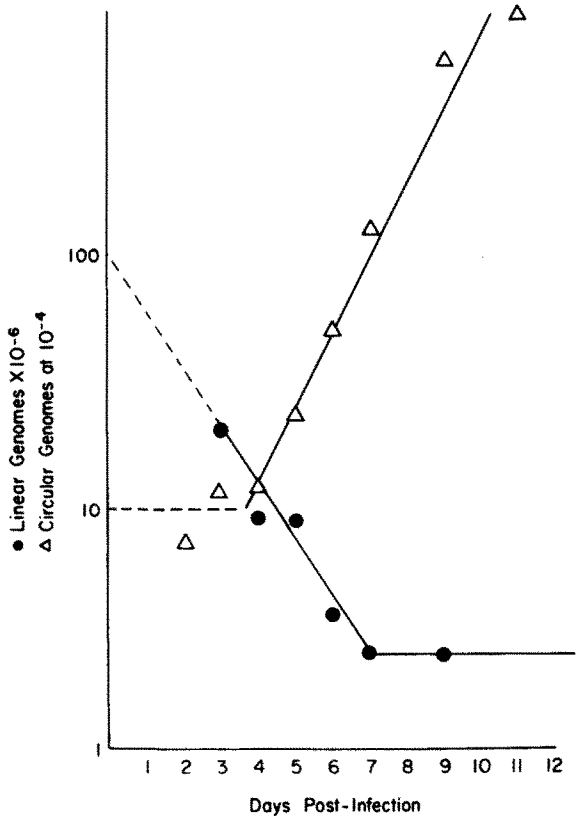

FIGURE 7. Quantitation of linear and circular viral genomes in the infected cell population from days 2–11 post-infection. An arbitrary number of cells was analyzed via the Gardella gel technique and the amounts of linear and circular viral DNA were quantitated by scanning densitometry. The numbers obtained by densitometry were multiplied by the total number of cells in the culture on the day of the analysis to give the total number of circular or linear viral genomes in the infected cell population at that time.

cleus and became circular. The B cells must be activated in order for this to occur; however, the cells do not need to progress further than early G_1 (G_{1A}). This is consistent with the first detection of CCC at 16–20 h post-infection.

We have also observed a concordance between the presence of CCC and the expression of the B cell activation marker CD23. CD23 is one of the earliest markers to appear on activated B cells and is expressed at an elevated level on EBV-transformed B cells (5). It has been reported that membrane triggering through virion binding is sufficient to induce CD23 (29). This occurs within 6 h of infection, even with inactivated or defective viruses. This level of CD23 expression is low, and we have not detected it in our experiments. We do not find CD23 expression until 24 h post-infection. In our experiments the level of expression of CD23 is high, similar to that of transformed B cells, and is only detected on 15–20% of the infected cells. Although this population consists of small resting cells, the presence of CD23 is predictive that they will subsequently blast transform and proliferate (5). Our current study suggests that this subpopulation of cells also contain a single CCC. The relationship between CD23 expression and the presence of CCC is most likely explained by our observation that a certain level of cellular activation is required before CCC can form. However, this level is not characterized by elevated CD23 expression since P3HR-1-infected cells can form CCC but do not express elevated CD23, and cir-

cularization (16–20 h) occurs before elevated CD23 (~24 h). Activation is probably required to produce cellular proteins necessary for circularization, such as the DNA polymerase. Thus, elevated CD23 expression presumably only occurs in the population of infected B cells that already contain a CCC. One explanation for this dependence would be that the CCC form is required for the appropriate viral transcription to cause subsequent elevated CD23 expression. Thus, it has been suggested that one viral gene, EBNA-2, directly upregulates CD23, based on the infection or transfection of EBV^- Burkitt's lymphoma lines (30, 31). However, we favor the notion that EBV latent gene products do not cause atypically high levels of CD23 expression but rather fix the activated B cells at a very specific stage of B cell differentiation that in normal B cells is characterized by elevated CD23 expression and proliferation. In this context it is noteworthy that CD23 itself has been directly implicated in the autocrine growth of both normal and EBV-transformed B lymphocytes (32). This idea predicts that other EBV latent gene products could have a similar effect to EBNA-2 and is supported by the observation that EBV^+ lymphomas tend to change in culture to a lymphoblastoid phenotype, including elevated CD23 (33). A candidate for the normal cellular counterpart of the EBV-activated B cell could be B cells activated by IL-4 plus anti-IgM, which have recently been demonstrated to express equivalent levels of CD23 (34).

Previous studies indicated that the inhibition of the EBV transforming function by γ or X irradiation follows one-hit kinetics, leading the authors to suggest that circularization of the viral genome may be necessary for the establishment of latency (35, 36). This observation is consistent with our suggestion that CCC appear before and are required for CD23 expression and subsequent proliferation. Circularization may be essential for several reasons. First, replication of the latent genome presumably requires the CCC form, and failure to replicate will eventually lead to loss of the viral genome and cell death. In addition, circularization could repress genes associated with the lytic cycle, in addition to being required for the expression of latent genes. For example, upon circularization, sequences at the termini, which are separated by >170 kb in the linear form, are brought in close proximity to one another. Indeed, the transcription unit of a recently described EBV latent gene spans the TR, necessitating circularization for its expression (37). Furthermore, the CCC form of the viral genome may be required for the correct transcription of the large transcriptional unit that encodes the EBNA proteins (38).

We have also noted that CCC occur preferentially in the B cells that are in the high density fraction at infection (data not shown). These cells also have more linear genomes associated with them early after infection, suggesting that they simply have an increased probability of receiving an intact genome that can form a CCC and drive the cell on to CD23 expression. This also explains the observation that the $CD23^+$ population at 36 h is enriched for the presence of linear genomes.

For a CCC to form, the viral DNA must be internalized and reach the nucleus of the infected cell. We have found that only 5% of the genomes that become cell associated actually reach the nucleus, indicating that entry into the nucleus is limiting in determining the number of viral genomes that become CCC. In addition, linear viral DNA reaching the nucleus must be full length, containing at least one repeat unit at each end, in order for circularization to occur. It has been suggested that only 12.5% of EBV genomes of the B95-8 strain are sufficiently intact to cir-

cularize in vitro (15). Our results are consistent with this hypothesis, since 9% of the genomes that reach the nucleus become CCC, suggesting that there is a random probability that any viral genome reaches the nucleus, and that the integrity of the viral DNA reaching the nucleus is limiting in the production of CCC. As the cells begin to proliferate, linear viral genomes are gradually lost, while CCC are maintained at one copy per cell due to replication, rather than segregation of input linear viral genomes to daughter cells and further circularization. By day 7–10 post-infection, when only latently infected EBNA and $CD23^+$ cells remained in culture, each cell still contained only one CCC. The conclusion that there was a single viral genome was based on quantitative estimation; however, it was independently confirmed by demonstrating that clonal cell lines derived in our experiments contained a clonal population of viral episomes.

Amplification of the viral genome has been shown to occur in cells latently infected with EBV (17). In contrast to the initial circularization of a linear genome, which determines which cells will immortalize, establishment of the circularized genome at a high copy number is not necessary for the establishment of latency. Amplification was not detected in our experiments until well after the cells had acquired all of the markers characteristic of latent infection, and had been proliferating for >1 wk. Interestingly, the initiation of amplification appears to require a low genome copy number within the infected cell. Over a range of multiplicities of infection, amplification was not seen until all incoming linear DNA was lost (Fig. 4 and data not shown). It is possible that linear and circular viral genomes compete for limiting amounts of viral or cellular factors necessary for amplification. Amplification might commence only when the genome copy number if sufficiently low, and cease when the numbers of CCC are high enough that the levels of these factors are again limiting. Alternatively, *trans*-acting repressors of amplification, perhaps linked to the lytic cycle, could be expressed by linear viral genomes, but not CCC.

We have concluded that, in our experiments, a single viral genome circularizes and is responsible for immortalizing a single cell. That single genome then amplifies, generating the multiple CCC seen in established lines. There are, however, other interpretations of our observations. For example, it could be argued that an undetectable fraction of cells circularize multiple incoming genomes and that only these cells immortalize, giving rise to high copy number cell lines. We have eliminated this possibility by showing that regardless of the genome copy number within a cloned line, each contained only one predominant fused terminal fragment. Thus, each clone initially contained a single circular genome that subsequently amplified to give rise to many genome copies.

Another possibility is that the CCC in a minority of cells undergoes amplification very early after infection, and only these cells are capable of immortalizing. In this case, we would expect that a population of newly infected cells, in which only a small fraction of cells have amplified viral DNA, would have a much lower cloning efficiency than an established cell line, in which most or all of the cells would have amplified viral DNA. However, these two populations of cells clone with the same efficiency, indicating that amplification does not confer a selective growth advantage on the cells.

Another phenomenon that has been suggested to be important in the establishment of cell lines latently infected with EBV is the integration of a viral genome into cellular DNA (39, 40). Although integration has been observed in certain BLs and LCL, it remains unclear whether integration is necessary for the establishment

of latency, or is a fortuitous consequence of long-term association of viral and cellular DNA in a few cell lines.

The γ or lymphotropic herpesviruses commonly assume circular forms during latency (41-43). α herpesviruses such as HSV have also been reported to exist in either a circular or an "endless" form in latently infected brain and ganglia of mice and humans (44, 45). Assumption of the circular form may then be common to the latent state of all herpesviruses. At present, EBV provides the most accessible model for the elucidation of generalized mechanisms for the establishment and maintenance of latency by herpesviruses.

Summary

Linear EBV genomes undergo a transition to the circular form characteristic of latency by 16–20 h post-infection. This transition requires that the infected cells be activated to the G_1 stage of the cell cycle. Cellular proliferation and expression of the activation marker CD23 were not required. Nevertheless, 36 h post-infection, only cells expressing CD23 contained covalently closed, circular episomes (CCC), at an average of one copy per cell. Since the presence of CD23 at this time is predictive that a cell will immortalize, we suggest that the presence of CCC is required for CD23 expression and subsequent immortalization. The one CCC present in each $CD23^+$ cell did not undergo amplification until well after the cells had acquired all of the characteristic phenotypic markers of immortalization. Therefore, while amplification is not necessary for proliferation and immortalization, circularization of a single genome is crucial to the establishment and maintenance of latency by EBV.

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