JOURNAL OF VIROLOGY, Jan. 1997, p. 314–324 Vol. 71, No. 1
0022-538X/97/$04.00+0

Selective Switch between Latency and Lytic Replication of Kaposi's Sarcoma Herpesvirus and Epstein-Barr Virus in Dually Infected Body Cavity Lymphoma Cells

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Received 10 July 1996/Accepted 27 September 1996

The BC-1 cell line, derived from a body cavity-based, B-cell lymphoma, is dually infected with Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). In these studies, the relationships between these two gammaherpesviruses and BC-1 cells were characterized and compared. Single-cell cloning experiments suggested that all BC-1 cells contain both genomes. In more than 98% of cells, both viruses were latent. The two viruses could be differentially induced into their lytic cycles by chemicals. EBV was activated into DNA replication and late-gene expression by the phorbol ester tetradecanoyl phorbol acetate (TPA). KSHV was induced into DNA replication and late-gene expression by *n*-butyrate. Amplification of both EBV and KSHV DNAs was inhibited by phosphonoacetic acid. Induction of the KSHV lytic cycle by *n*-butyrate was accompanied by the disappearance of host-cell β-actin mRNA. Induction of EBV by TPA was not accompanied by such an effect on host-cell gene expression. Induction of the KSHV lytic cycle by *n*-butyrate was associated with the expression of several novel polypeptides. Recognition of one of these, p40, served as the basis of development of an assay for antibodies to KSHV in the sera of infected patients. BC-1 cells released infectious EBV; however, there was no evidence for the release of encapsidated KSHV genomes by BC-1 cells, even though *n*-butyrate-treated cells contained numerous intranuclear nucleocapsids. The differential inducibility of these two herpesviruses in the same cell line points to the importance of viral factors in the switch from latency to lytic cycle.

Kaposi's sarcoma (KS) consists of several entities with distinctive clinical and epidemiologic features (14, 39). DNA sequences of a new herpesvirus, designated KS-associated herpesvirus (KSHV) or human herpesvirus 8, have been found in all forms of the disease (5, 8, 9, 11, 13, 17, 32, 40, 45). Furthermore, sequences of the new virus are present in B-cell non-Hodgkin's lymphomas variably known as body cavity-based lymphomas or primary effusion lymphomas (7, 21). When they occur in patients with AIDS, these B-cell lymphomas also harbor another human herpesvirus, Epstein-Barr virus (EBV) (21). Primary effusion lymphomas that are EBV negative in human immunodeficiency virus (HIV)-seronegative individuals have been reported. The presence of KSHV sequences in blood mononuclear cells, skin, prostate and semen of healthy individuals is under investigation (2, 30).

The DNA sequences of KSHV were originally identified in an AIDS-associated KS lesion by means of a PCR-based subtraction hybridization technique known as representational difference analysis (8, 24). Two subgenomic *Bam*HI fragments of DNA, designated KS 330 *Bam* and KS 631 *Bam*, were isolated; these showed extensive similarity to late genes of several members of the gammaherpesvirus family, including EBV, herpesvirus saimiri (HVS), and equine herpesvirus 2 (1, 37, 38). The organization of viral genes on a 20.7-kb lambda phage clone of KSHV that encompasses KS330 *Bam* is nearly identical to that of HVS (1, 31).

Gammaherpesviruses characteristically establish latent infection in lymphoid cells. Two of the group, EBV and HVS, immortalize lymphocytes, causing them to grow continuously in vitro (27, 42, 49). EBV usually immortalizes B cells, and HVS immortalizes T cells (4, 51). It is not yet known whether KSHV possesses immortalizing properties. Immortalization by EBV is the result of actions of several viral genes acting in concert (15, 46). The mechanism of immortalization by HVS is less well understood; a membrane-associated protein with repetitive collagen repeat motifs, called the saimiri transformation-associated protein, is involved (18, 19). Both EBV and HVS induce lymphomas in New World primates (26, 44).

In lymphoid cells, the latent gammaherpesvirus genome expresses a limited gene program. In the case of EBV, up to 11 genes may be expressed; these encode six nuclear proteins (EBNAs), three cytoplasmic membrane-spanning proteins (LMPs), and two small RNAs (EBERs) (15, 46). Lytic gene expression, accompanied by production of replicative-cycle proteins, can be triggered by a variety of agents which act by inducing expression of a viral switch protein, ZEBRA, encoded in the *BZLF1* gene of EBV (10, 28). ZEBRA is a transcriptional activator and an essential factor for lytic viral DNA replication (41). Latent genes of HVS expressed in lymphoid cells include the small RNAs (HSURs) (23, 33). HVS does not have a homolog of ZEBRA (1). Most tumor-derived lymphoid-cell lines harboring HVS contain defective rearranged HVS genomes (12, 16).

A cell line, BC-1, established from an AIDS-related body cavity B-cell lymphoma, like the tumor from which it was derived, harbors both KSHV and EBV (6, 21). The purposes of our study were to determine whether (i) all BC-1 cells contain both viruses, (ii) the KSHV genome in BC-1 cells could be

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switched into lytic gene expression, (iii) the EBV and KSHV genomes are under coordinate or separate control, (iv) they mutually cross-activate or cross-repress each other, (v) KSHV activation is accompanied by the formation of viral particles, and (vi) encapsidated KSHV DNA and infectious KSHV are released from BC-1 cells.

For these studies, we used two chemical inducing agents that are known to activate EBV gene expression. One chemical, the phorbol ester tetradecanoyl phorbol acetate (TPA), acts via the protein kinase C pathway and the AP-1 family of transcription factors to stimulate gene expression (3, 52). The second inducing agent, *n*-butyrate, is thought to act by inhibiting the deacetylation of histones, thereby alleviating repression by chromatin (25). We found that *n*-butyrate was a strong inducer of KSHV lytic gene expression in BC-1 cells but had little effect on EBV gene expression. This selective activation of KSHV gene expression by *n*-butyrate permitted development of first-generation serologic assays for antibodies to KSHV lytic-cycle proteins (29).

MATERIALS AND METHODS

Cell cultures. BC-1 cells were cultured in RPMI medium plus 15 to 20% fetal bovine serum (FBS). HH514-16 (C1.16) cells, in which the P3J-HR-1 strain of EBV is tightly latent but highly inducible by butyrate, were grown on RPMI medium plus 8% FBS (35). All cultures contained penicillin and streptomycin (50 U/ml) and amphotericin B (1 μg/ml).

Cloning of BC-1 cells. Suspensions, containing 100, 10, 1, and 0.5 BC-1 cells per 0.1 ml of RPMI medium with 15% FBS and antibiotics, were placed into 96-well plates covered with a confluent monolayer of MRC-5 cells. The plates were incubated in a humidified 5% CO_2 atmosphere at 36°C. At day 7 after plating, all wells containing 100 and 10 cells per well showed growth while no growth was seen in wells containing 1 or 0.5 cell per well. The plates with 100 and 10 cells per well were discarded. At day 14, cell growth was observed in 16 of 96 wells originally plated with 1 cell per well and in 2 of 44 wells plated with 0.5 cell per well. When BC-1 cells had multiplied sufficiently, they were transferred to 24-well plates with MRC-5 cells and then to 25-cm^2 flasks without a feeder layer. Fifteen clones, all derived from the 1-cell/well dilution, were propagated free of feeders.

Chemical induction. BC-1 cells were subcultured into 50-ml volumes, and inducing agents were added 24 to 48 h later. TPA was added to a final concentration of 20 ng/ml, and *n*-butyrate (catalog no. 5887; Sigma) was added to a final concentration of 3 mM. One flask was left untreated as a control. Cells were exposed to inducing agents for 24 to 48 h.

Effects of PAA. Twenty-four h after cells were subcultured, they were resuspended in medium containing 0.4 mM phosphonoacetic acid (PAA) or in medium without PAA. Chemical inducing agents (TPA or *n*-butyrate) were added 48 h later; cells were harvested for analysis of viral DNA content 48 h after addition of chemical inducing agents.

Immunoblotting assays. Cells, suspended in sodium dodecyl sulfate sample buffer at a concentration of 5×10^7 cells/ml, were sonicated and boiled prior to electrophoresis through a 10% polyacrylamide gel. Each lane was loaded with 10 μl of antigen. After electrophoresis, gels were transferred to nitrocellulose at 0.4 mA for 2 h and blocked overnight with 5% skim milk. Identical blots were probed with 1:100 dilutions, in skim milk, of three different human sera, with three monospecific rabbit antisera prepared against the EBV gene products BZLF1, an immediate-early product, and BLRF2 and BFRF3, late-gene products (43, 50). The human sera were SJ ($EBV^+/KSHV^-$), RM ($EBV^+/KSHV^-$), and 01-03 ($EBV^+/KSHV^+$). Antigen antibody reactions were detected by addition of 1 μCi of ^{125}I-protein A in 10 ml. Blots were exposed to XAR film to produce an autoradiograph.

Immunofluorescence assay. BC-1 cells were treated with inducing agents for 48 h, washed once with phosphate-buffered saline (PBS), resuspended at 5×10^5/ml in PBS, and dropped onto microscope slides. When dry, cells were fixed in acetone-methanol (2:1) for 5 min and stored at −20°C. Cells were stained by indirect immunofluorescence with human patient sera at 1:10 followed by fluorescein-conjugated sheep anti-human immunoglobulin (MF01; Wellcome) at 1:30. The human sera were from three donors. One was $EBV^+/KSHV^-$, one was $EBV^+/KSHV^+$, and one was $EBV^-/KSHV^-$.

Southern blot analysis for KSHV and EBV DNAs. To prepare total cellular DNA, 2×10^7 cells from each experimental group were washed once in PBS with no Mg^{2+} or Ca^{2+} and resuspended in 5 ml containing 0.2 M Tris, 0.1 M EDTA, 1 mg of pronase per ml, and 1% sodium dodecyl sulfate. The samples were incubated at 60°C for 2 h. One milliliter of 5 M potassium acetate was added, and the samples were held on ice for 30 min. The samples were centrifuged for 20 min at 8,000 rpm at 2°C in a Sorvall SA600 rotor, and the supernatants were harvested. RNase was added to a final concentration of 20 μg/ml. The DNA was precipitated by addition of 2 volumes of 95% ethanol; the pellets were dried and resuspended in 500 μl of 0.01 M Tris (pH 8)–1 mM EDTA. The concentration of DNA was estimated by optical density at 260 nm. For Southern analysis, 5 μg of cellular DNA was digested with *Bam*HI and electrophoresed in a 1% agarose gel. The gel was transferred to nitrocellulose by the Southern method. DNA probes, labeled with [α-^{32}P]dCTP by the random prime method, contained about 10^7 cpm. The EBV probes were *Bam*HI Z and *Xho*I 1.9; the KSHV probes were KS 631 *Bam* and KS 330 *Bam*. All probes were excised from their vectors. The hybridized blots were exposed to XAR film overnight. To compare the relative inducibility of the two viral DNAs, serial twofold dilutions of cellular DNA were prepared, beginning at 5 μg, digested with *Bam*HI, and analyzed by Southern blot hybridization.

PCR analysis for KSHV and EBV DNAs. The primers used to amplify KSHV DNA were from a region of KSHV DNA that encompasses KS 330 *Bam*. The length of the expected amplified product is 1,851 bp. The sequences of the KSHV primers were 5′ CGGAATTCCCTGCGAGATAATTCCCACGCCGGTC and 5′ CGGGATGCAGAACAGGGCTAGGTACACACAATTTTCAAG. The primers used to amplify EBV DNA from the EBV *BMLF1* open reading frame produce a product of 304 bp. The two primers were 5′ CACCACCTTGTTTT GACGGG and 5′ GTCAACCAACAAGGACACAT. Each reaction mixture contained 200 to 300 ng of total cell DNA, 100 ng of each primer, and 2.5 U of *Taq* polymerase. The PCR conditions were 1 cycle at 94°C for 3 min; 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min. Aliquots of each reaction were electrophoresed in a 1% agarose gel which was stained with ethidium bromide. The PCR products were also detected by Southern analysis.

Northern (RNA) analysis. Cells were lysed in 150 mM NaCl–10 mM Tris (pH 8.0)–0.1% Nonidet P-40 (NP-40), and the nuclei were deposited by centrifugation. RNA was isolated in 7 M urea–350 mM NaCl–10 mM Tris (pH 7.5)–20 mM EDTA. The RNA was electrophoresed in a 1% agarose–6% formaldehyde gel in 20 mM MOPS (morpholinepropanesulfonic acid) (pH 7). Each lane received RNA from 2.5×10^6 cells. The gel was transferred to Nytran (S and S) and hybridized with a probe radiolabeled with [α-^{32}P]dCTP by the random prime method. Probes were derived from the EBV late gene *BFRF3*, a 754-bp fragment representing the start of the open reading frame, and KS 330 *Bam* (8, 50). RNA loading equivalence was estimated by probing with a 1.8-kbp fragment of β-actin cDNA.

Transmission electron microscopy. Cell pellets from BC-1 cells that were untreated or treated for 48 h with *n*-butyrate were fixed in glutaraldehyde and osmium tetroxide and embedded in Epon as previously described (48). Ultrathin sections were viewed in a Philips EM400T electron microscope at direct magnifications of ×17,000 to ×60,000.

Examination of extracellular viral genomes for DNase sensitivity. Seventy-five milliliters of BC-1 cells at 10^6 per ml was treated with *n*-butyrate for 48 h. Cells were deposited by two low-speed centrifugations at 5,000 rpm for 5 min, and the supernatant fluid was centrifuged at 35,000 rpm for 30 min at 4°C in a Beckman SW41 rotor. Viral pellets were resuspended in 0.5 ml of PBS–10 mM $MgCl_2$–50 μg of DNase (Worthington) per ml and incubated at 37°C for 3 h. NP-40 was added to a final concentration of 1%, and incubation was continued at room temperature for 5 min. DNase (110 μg/ml) was added, and incubation was continued at 37°C for 2 h. The incubation buffer was adjusted to contain 0.1 M NaCl–50 mM Tris (pH 8.0)–25 mM EDTA to inactivate the DNase. Pronase was added to a final concentration of 140 μg/ml, and incubation was continued overnight at 50°C. The mixture was extracted once with phenol and once with phenol-chloroform, and the DNA was precipitated with ethanol. The DNA was solubilized in 100 μl of 10 mM Tris (pH 8)–1 mM EDTA. Two microliters of DNA was used in each PCR for detection of EBV and KSHV DNAs.

Assay for infectivity in BC-1 cell supernatants. Ten milliliters of BC-1 cell cultures at 10^6/ml was treated with TPA, *n*-butyrate, or a mixture of TPA and *n*-butyrate or left untreated for 24 h. Cells were washed and resuspended in fresh culture medium. After 48 h of additional incubation at 37°C, cells and fluids were frozen and thawed three times. The suspension was centrifuged at 1,000 rpm for 5 min in an IEC 269 rotor, and the supernatant was centrifuged again. Two milliliters of supernatant was added to 8 ml of human umbilical cord lymphocytes (HUCL) at 10^6 cells/ml. Cells were morphologically transformed after 2 weeks. Three months after infection, cellular DNA from each culture was analyzed for KSHV and EBV DNAs by PCR.

RESULTS

Selective amplification of EBV and KSHV DNAs in BC-1 cells treated with inducing chemicals. To determine whether the two gammaherpesvirus genomes harbored by BC-1 cells were under coordinate or separate control, cells were treated with chemical inducing agents known to effect a switch between latency and lytic-cycle gene expression of EBV. TPA and *n*-butyrate were added singly and in combination; the effects of this treatment on the contents of EBV and KSHV DNAs were monitored by Southern analysis. For comparison,

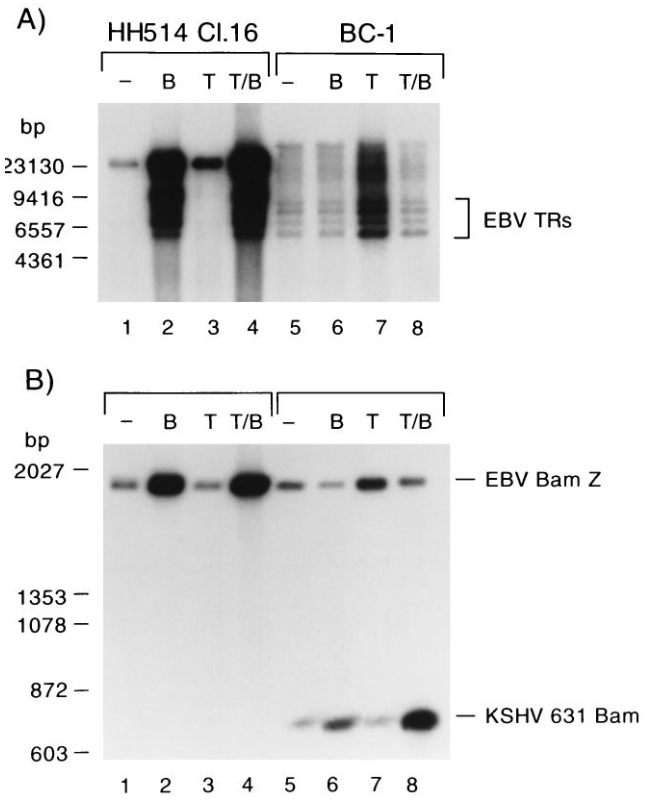

FIG. 1. Selective amplification of EBV and KSHV DNAs in BC-1 cells by inducing chemicals. DNA was prepared from HH514-16 (C1.16) (HH514 C1.16) and BC-1 cells that had been treated with *n*-butyrate (B), TPA (T), or both chemicals (T/B) for 48 h or left untreated (−). The DNA was digested with *Bam*HI and electrophoresed in a 1% agarose gel. A Southern blot was sequentially probed with EBV *Xho*I 1.9, which detects the EBV terminal repeats (TRs) (A), and EBV *Bam*HI Z and KS 631 *Bam* (B).

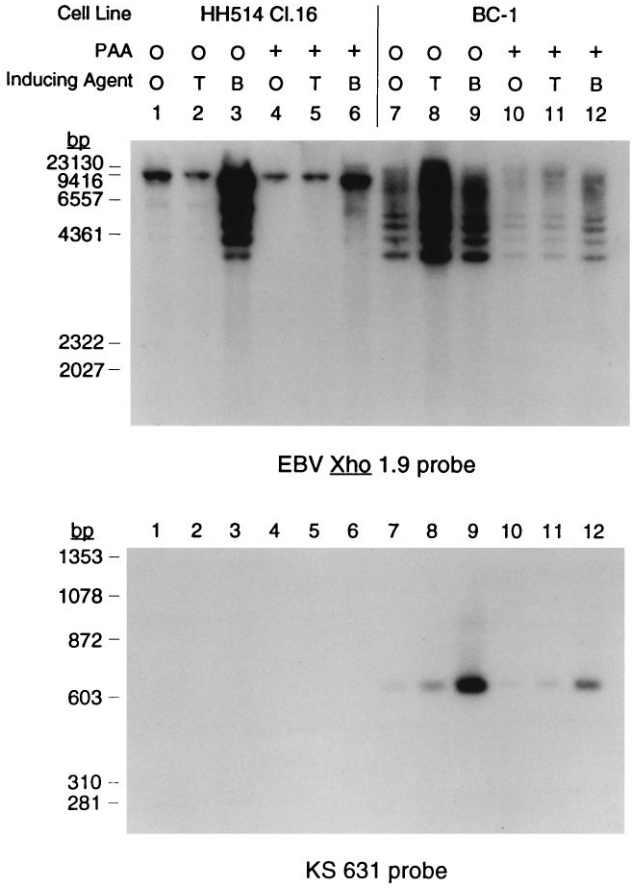

FIG. 2. Effects of PAA on *n*-butyrate-induced amplification of KSHV DNA. HH514-16 (C1.16) (HH514 C1.16) and BC-1 cells were pretreated for 48 h with medium without (O) or with (+) (PAA); an inducing chemical, TPA (T) or *n*-butyrate (B), was added; and DNA was prepared after 48 h. Southern blots were probed with EBV *Xho*I 1.9 (top) or KS 631 *Bam* (bottom).

HH514-16 (C1.16) cells, in which EBV is tightly latent but highly inducible by *n*-butyrate, were studied in parallel.

EBV underwent spontaneous lytic replication in BC-1 cells. There was a ladder of restriction fragments heterogeneous in size containing the EBV terminal repeats (Fig. 1A, lane 5). This ladder is characteristic of linear EBV DNA destined for encapsidation (20, 34). Treatment of BC-1 cells with TPA caused an increase in the abundance of EBV DNA, as evidenced by an increased signal from the ladder of terminal repeats (Fig. 1A, lane 7) or from an internal region of EBV DNA detected with the *Bam*HI Z probe (Fig. 1B, lane 7). However, surprisingly, treatment with the combination of TPA and *n*-butyrate did not increase the EBV DNA content in BC-1 cells (see Discussion).

HH514-16 (C1. 16) cells, in which EBV is tightly latent, lacked the ladder of terminal repeats; instead, only a single restriction fragment representing fused termini was seen in untreated cells (Fig. 1A, lane 1). In C1.16 cells, *n*-butyrate by itself or in combination with TPA caused an increase in the abundance of EBV DNA (Fig. 1, lanes 2 and 4). TPA alone had little or no effect on the EBV DNA content in C1.16 cells. Thus, the response of the EBV genome to chemical induction differed between the two cell lines. In BC-1 cells, EBV was induced by TPA, while in C1.16 cells, EBV was induced by *n*-butyrate.

In BC-1 cells, *n*-butyrate alone or in combination with TPA caused an increase in the KSHV DNA content (Fig. 1B, lanes 6 and 8). The content of the KSHV genome in BC-1 cells was minimally affected by TPA (Fig. 1B, lane 7). The same results were obtained when the blots were probed with KS 631 *Bam* (Fig. 1) and KS 330 *Bam* (not shown). These results indicated that DNA amplification of these two gammaherpesviruses carried by BC-1 cells was under separate control. TPA preferentially stimulated EBV DNA replication, while *n*-butyrate triggered an increase in KSHV-specific DNA.

Effects of PAA. Treatment with PAA, a compound that predominantly affects lytic-cycle herpesvirus DNA polymerase (47), reduced the level of KSHV DNA in BC-1 cells treated with *n*-butyrate (Fig. 2; compare lanes 9 and 12). PAA also inhibited spontaneous and TPA-induced EBV DNA synthesis in the same cells (Fig. 2; compare lanes 7 and 10 and lanes 8 and 11). Titration experiments showed that PAA caused about a twofold inhibition of chemically induced amplification of KSHV and EBV DNAs in BC-1 cells. For comparison, treatment of C1.16 cells with PAA produced a fourfold inhibition of *n*-butyrate-induced EBV DNA amplification (Fig. 2, lanes 3 and 6). These results are consistent with the idea that increases in the DNA contents of both gammaherpesviruses were mediated by viral DNA polymerases.

Magnitude of induction. To quantitate the magnitude of the effects of chemical induction on the contents of KSHV and EBV DNAs, the DNA samples from chemically treated cells were serially diluted and compared with DNA from untreated

cells. In the samples from the experiment depicted in Fig. 2, TPA induced a fourfold increase in EBV DNA content in BC-1 cells while the content of KSHV DNA was increased less than twofold (not shown). *n*-Butyrate induced an 8- to 16-fold increase in KSHV DNA content and less than a 2-fold increase in EBV DNA content (not shown). For comparison, in C1.16 cells, *n*-butyrate induced an 8- to 16-fold increase in EBV DNA content and TPA did not alter the abundance of EBV DNA (not shown). Thus, the level of inducibility of KSHV DNA in BC-1 cells by *n*-butyrate was comparable to the magnitude of induction of EBV DNA in highly inducible C1.16 cells.

Induction of KSHV lytic-cycle mRNAs by *n*-butyrate. To determine whether inducing chemicals promoted lytic-cycle gene expression of these two gammaherpesviruses in BC-1 cells, Northern blots were hybridized with probes representing genes encoding capsid components of KSHV and EBV (Fig. 3). BC-1 subclone D5, treated with *n*-butyrate or a combination of TPA and *n*-butyrate, expressed a prominent 2.0-kb mRNA and a less abundant 6.9-kb mRNA that were detected by KS 330 *Bam*, a region homologous to a gammaherpesvirus capsid protein gene (Fig. 3A, lanes 5 and 7). These two mRNAs were not identified in untreated BC-1 cells (Fig. 3A, lane 4) and were present only in trace amounts in BC-1 cells that had received TPA alone (Fig. 3A, lane 6). Although KS 330 *Bam* has homology with EBV, this probe did not detect EBV mRNAs in C1.16 cells induced into the lytic cycle (Fig. 3A, lanes 1 and 2).

BC-1 cells treated with TPA expressed three prominent EBV mRNAs of 0.9, 3.3, and 4.1 kb and a less prominent 3.65-kb mRNA, all of which were detected by a probe for *BFRF3*, which encodes a p21 capsid component (Fig. 3B, lane 6). Recent studies have shown that only the 0.9-kb mRNA is a true late transcript; the other mRNAs are expressed early (43, 50). Traces of these EBV lytic-cycle mRNAs were detected in untreated and *n*-butyrate-treated BC-1 cells (Fig. 3B, lane 7).

Whenever *n*-butyrate was present in BC-1 cultures, the cellular β-actin signal was markedly diminished (Fig. 3C, lanes 5 and 7). In the same RNA samples in which there was no detectable cellular β-actin mRNA, the KSHV mRNAs detected by the KS 330 *Bam* probe were abundant (Fig. 3A, lanes 5 and 7). A titration of *n*-butyrate showed that the 3 mM dose of *n*-butyrate, which maximally induced the 6.9- and 2.0-kb mRNAs detected by KS 330 *Bam*, caused the loss of detectable steady-state mRNA for cellular β-actin.

The presence of *n*-butyrate in combination with TPA in BC-1 cultures was also associated with inhibition of EBV lytic gene expression. The EBV mRNAs present in BC-1 cells treated with TPA were not detected after treatment with a combination of TPA and *n*-butyrate (Fig. 3B, lane 7). This result mirrored the failure to detect amplification of EBV DNA in BC-1 cells treated with a combination of TPA and *n*-butyrate (Fig. 1A, lane 8). However, in C1.16 cells, the combination of TPA and *n*-butyrate did not result in inhibition of either EBV late-gene expression (Fig. 3B, lane 2) or cellular β-actin expression (Fig. 3C, lane 2). This differential sensitivity of KSHV, EBV, and host cell mRNA expression to the action of *n*-butyrate is consistent with the hypothesis that KSHV lytic gene expression is associated with a mechanism that shuts off host cell and EBV mRNA expression (see Discussion).

Comparing induction of EBV and KSHV lytic-cycle polypeptides. BC-1 cells spontaneously expressed both latent and lytic-cycle products of EBV. Two polypeptides, one of about 95 kDa corresponding to EBNA1 and the other (p21) a late capsid antigen complex encoded by EBV genes *BFRF3* and *BLRF2* (43), were detected on immunoblots by human antisera to

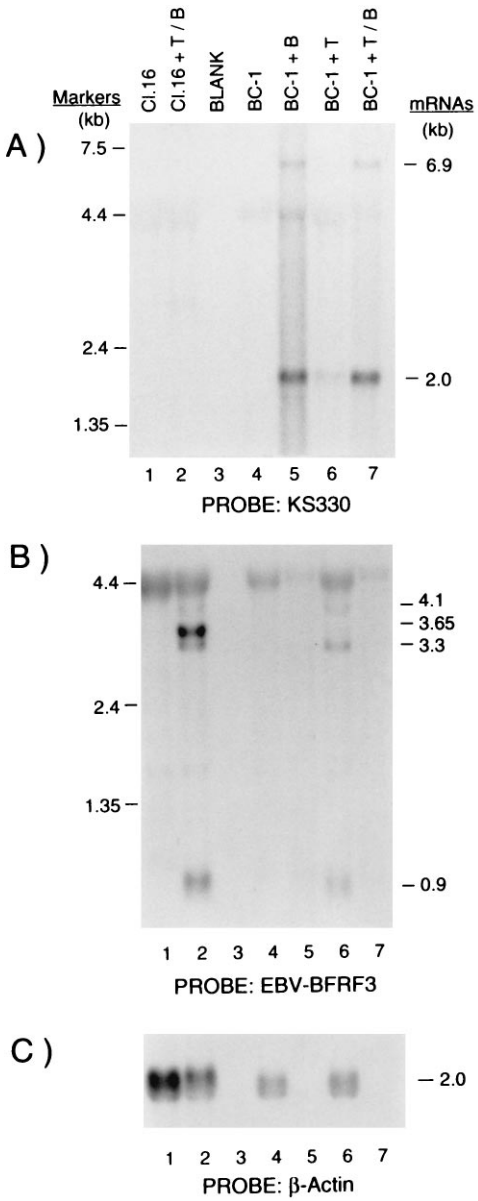

FIG. 3. Induction of KSHV lytic-cycle mRNAs by *n*-butyrate. Northern blot of cytoplasmic RNAs derived from HH514-16 (C1. 16) (lanes 1 and 2) and BC-1 clone D5 (lanes 4 through 7) cells. Cells were untreated or treated 48 h previously with the indicated inducing agent, *n*-butyrate (B), TPA (T), or a mixture (T/B). The blot was probed sequentially with KS 330 *Bam* (A), EBV *BFRF3* (B), and β-actin (C).

EBV (Fig. 4A). The abundance of the p21 complex was minimally altered by *n*-butyrate treatment and induced about fivefold by treatment with TPA. Replicate immunoblots (Fig. 4B) probed with monospecific antisera showed that all the EBV lytic-cycle products encoded in the *BZLF1*, *BFRF3*, and *BLRF2* open reading frames were selectively induced by TPA and relatively unaffected by *n*-butyrate.

To determine whether BC-1 cells also expressed polypeptides related to KSHV, either spontaneously or after chemical induction, sera from patients with KS were used as a source of antibody on immunoblots. Some of the most reactive of these human antisera (Fig. 4A) detected at least three polypeptides, p27, p40, and p60, in chemically treated BC-1 cells. p27 and

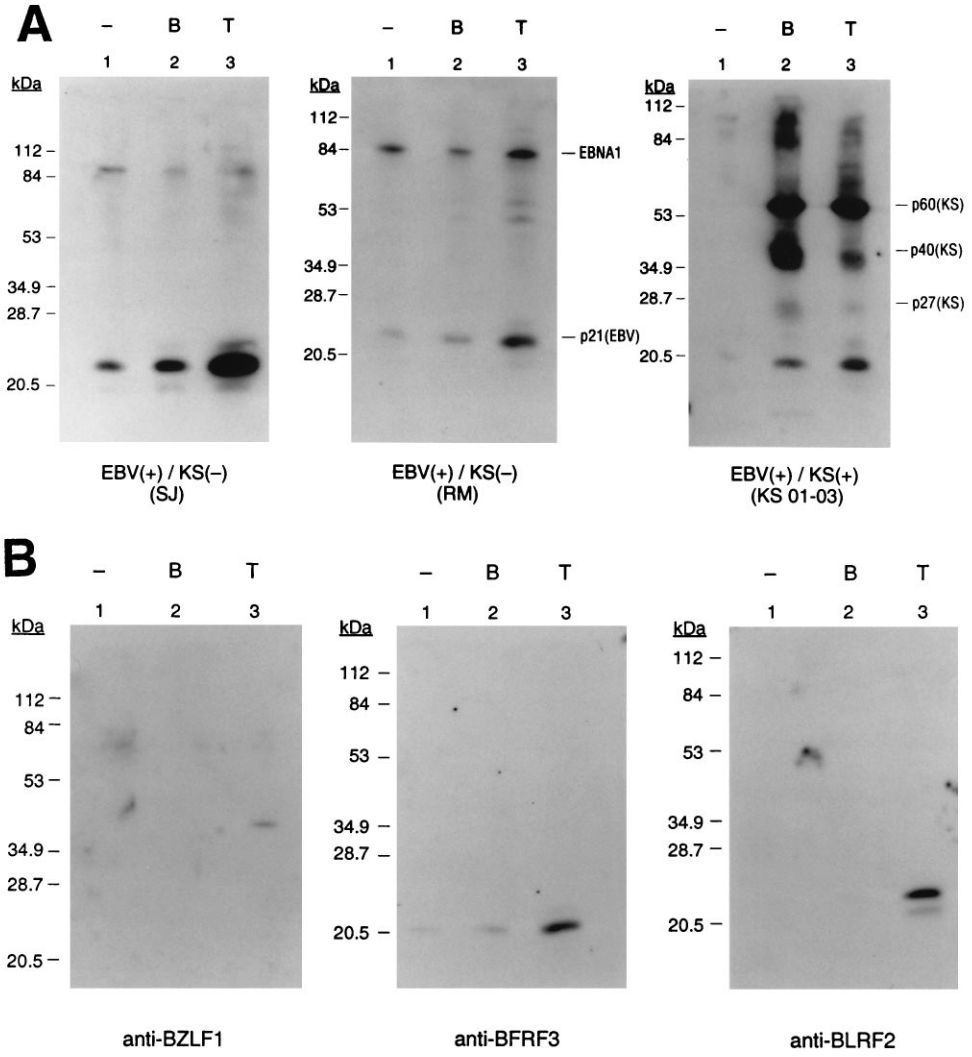

FIG. 4. Immunoblot detection of EBV and KSHV polypeptides. BC-1 cells were untreated (−) or treated with *n*-butyrate (B) or TPA (T). (A) Reactions with three human sera, RM and SJ from healthy EBV-seropositive adults and 01-03 from a patient with KS; (B) reactions with three monospecific rabbit antisera to EBV lytic-cycle products, BZLF1, an immediate-early gene product, and BFRF3 and BLRF2, late-gene products.

p40 were maximally induced by *n*-butyrate treatment. p60 was induced to similar extents by TPA and *n*-butyrate. These proteins were not detected by reference human antisera containing antibodies to EBV early and late antigens.

p27, p40, and p60 were not detected by sera from patients with KS in EBV-infected producer cell lines, HH514-16 and B95-8 (not shown), that were maximally induced into the lytic cycle. The proteins were not present in EBV-negative cells, such as BJAB and BL41, which lack both KSHV and EBV genomes. Thus, this group of proteins was specific to chemically induced BC-1 cells. Their pattern of induction by chemicals was parallel to the induction of KSHV DNA and mRNA in BC-1 cells (Fig. 1 to 3). Therefore, these polypeptides are likely to be encoded by KSHV or less likely to be cellular proteins that are induced during the KSHV lytic cycle.

Presence and expression of both KSHV and EBV genomes in single-cell clones of the BC-1 cell line. Differential induction of EBV and KSHV expression in BC-1 cells could result from the differential response to chemical induction of two different populations of cells, one infected with KSHV and the other infected with EBV. Alternatively there could be two populations of BC-1 cells, both dually infected with KSHV and EBV, which differ in their responses to chemical induction. To explore these possibilities, single-cell clones of BC-1 cells were obtained by limiting dilution. Each of the 15 clones analyzed contained both genomes (Fig. 5A and data not shown). EBV spontaneously replicated in all clones (not shown). Each clone expressed latent EBNA1 and the EBV late lytic replicative p21 complex (Fig. 5B and data not shown). The abundance of EBV p21 was unaffected by *n*-butyrate treatment. However, in all clones, *n*-butyrate treatment induced the p40 polypeptide detected with antiserum from a patient with KS (Fig. 5B). There was variability among the clones in the level of p40 expression; e.g., clone D5 expressed more of this antigen than did clone D10.

Cell-by-cell assays for EBV and KSHV lytic-cycle antigens. The increased expression of p40 and other KSHV polypeptides induced by *n*-butyrate could result from amplified levels of viral polypeptide expression within a few cells that are spontaneously in the KSHV lytic cycle or could be due to recruitment of additional cells to enter the lytic cycle. To distinguish between these possibilities, the proportion of cells expressing

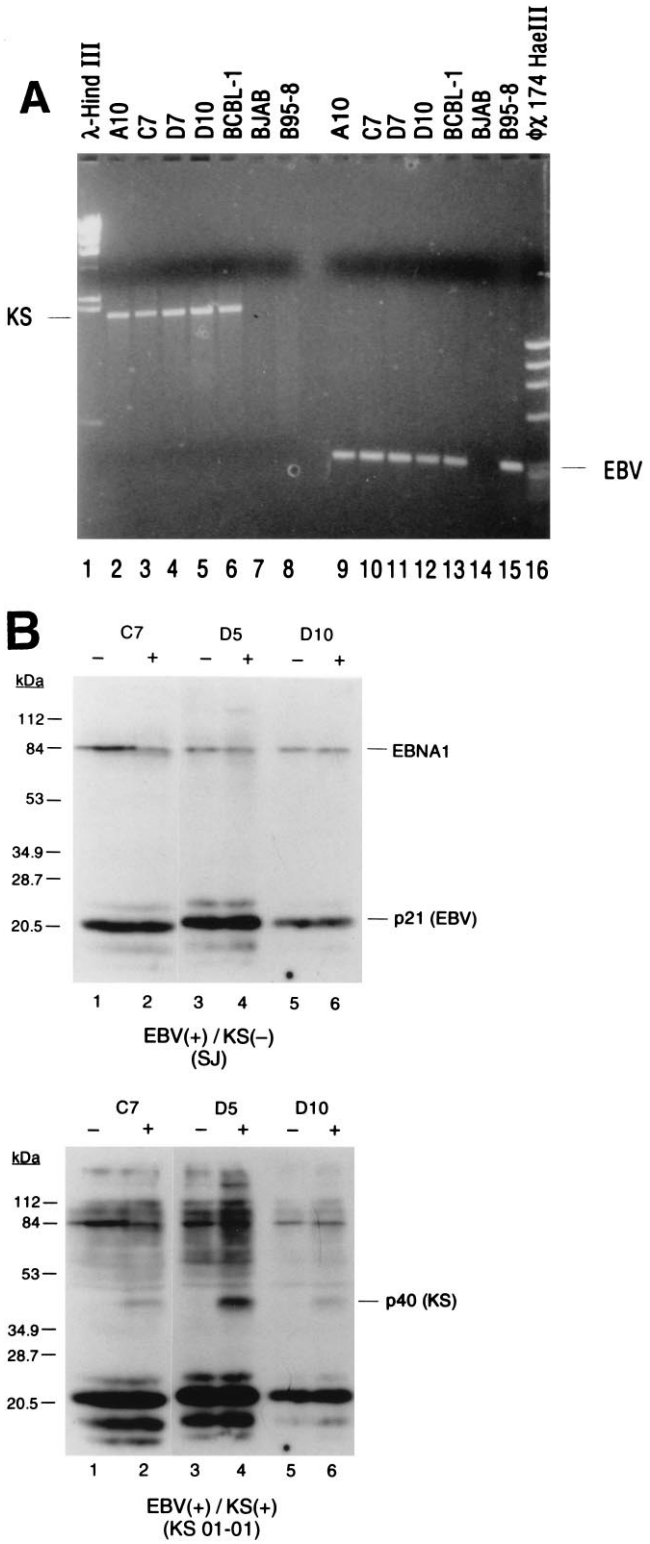

FIG. 5. Single-cell clones of BC-1 cells contain both viruses. (A) Presence of KSHV and EBV DNAs in single-cell clones. Lanes 1 and 16 contained size marker DNA fragments. Cellular DNAs from single-cell clones of BC-1 cells were loaded in lanes 2 through 5 and 9 through 12. Lanes 2 through 8 contained PCR mixtures for KSHV DNA; a 1,851-nucleotide fragment (KS) was diagnostic. Lanes 9 through 15 contained PCR mixtures for EBV DNA; a 304-nucleotide fragment (EBV) was diagnostic. (B) Expression of EBV and KSHV polypeptides in three single-cell clones of BC-1 cells. These clones were untreated (−) or treated with sodium butyrate (+) for 48 h. An immunoblot was reacted with an EBV-positive human antiserum (top). The immunoblot was reprobed with antiserum from a patient with KS (bottom).

lytic-cycle EBV and KSHV antigens was measured by indirect immunofluorescence assays.

Human EBV antibodies or monospecific antibodies to EBV capsid antigen components, such as BLRF2, detected EBV capsid antigen (VCA) in 1 to 2% of BC-1 cells. Human antisera and monoclonal antibodies to EBV early antigens detected similar proportions of antigen-positive cells. The percentage of cells expressing EBV lytic-cycle products increased about fivefold after treatment with TPA but was not affected by *n*-butyrate. Thus, the EBV genome was latent in about 98% of BC-1 cells and remained so in 90% of cells even after TPA treatment.

The results of immunofluorescence reactions were dramatically different when sera from patients with KS were the source of antibody. Again about 1 to 2% of untreated cells were antigen positive. Since the sera from patients with KS contained antibodies to EBV VCA, these antigen-positive cells may represent cells that spontaneously enter the EBV lytic cycle. Alternatively, they may represent cells that spontaneously produce KSHV lytic-cycle antigens. However, *n*-butyrate caused a 10- to 20-fold increase in the number of BC-1 cells that expressed antigens detectable by sera from patients with KS. These antigens were cytoplasmic and nuclear. Since *n*-butyrate treatment had no effect on the number of BC-1 cells expressing EBV lytic-cycle antigens, the antigen-containing cells detected with sera from patients with KS were considered to be expressing lytic-cycle KSHV polypeptides. Thus, *n*-butyrate treatment caused 25 to 50% of the BC-1 cell population to switch from latency to the KSHV lytic cycle and produce antigens detectable by immunofluorescence.

These results with the uncloned BC-1 parental line were reproduced in the single-cell clones. The number of cells positive for EBV VCA in these clones varied from <1 to 4% and was not altered by *n*-butyrate treatment. However, *n*-butyrate treatment induced between 10 and 50% of cells in each clone to express lytic-cycle KSHV antigens.

Transmission electron microscopy. BC-1 cells were examined by transmission electron microscopy to determine whether *n*-butyrate induced KSHV to form morphologically recognizable herpesvirus virions. Two preparations of untreated BC-1 cells, examined over an interval of 2 months, contained herpesvirus nucleocapsids in approximately 2.5% of cell profiles (1 of 40 cell profiles examined). However, 48 h after treatment with *n*-butyrate, 50 to 80% of cell profiles contained herpesvirus nucleocapsids. These were almost exclusively intranuclear and predominantly of the herpesvirus b morphologic type. Of 138 nucleocapsids classified on 15 randomly chosen images, 92% were type b, 3% were type a (i.e., lacking a core), and 5% were type c with a dense core (Fig. 6). Since *n*-butyrate had little or no effect on EBV DNA content or EBV late gene expression (Fig. 1 through 4), these nucleocapsids are likely to be KSHV nucleocapsids. Only a single extracellular particle with the appearance of a morphologically complete herpesvirus particle was detected (Fig. 6B). A majority of the *n*-butyrate-treated cells containing herpesvirus nucleocapsids appeared to have been lysed. In addition, infected cells showed extensive nuclear membrane duplication and nuclear formations of electron-dense-oriented fibrillar material (Fig. 6D).

Attempts to demonstrate extracellular KSHV virions. Biochemical and biologic assays were used to assess whether ma-

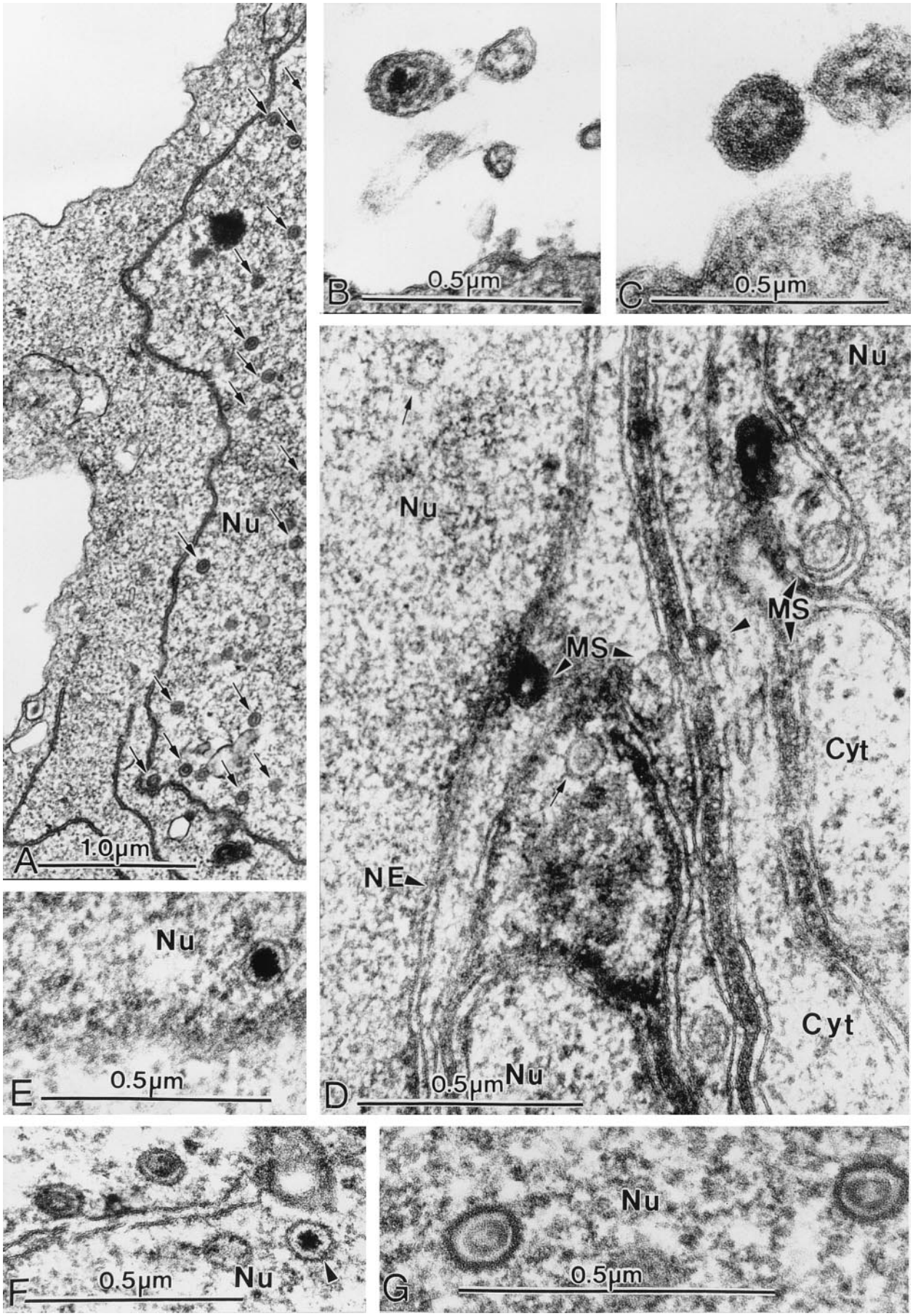
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FIG. 6. Transmission electron microscopy of BC-1 cells. (A) General low-power view of an *n*-butyrate-treated cell. Many herpesvirus nucleocapsids (arrows), mostly of type b, were present in the nucleus (Nu). (B) The only extracellular morphologically mature particle with the appearance of a herpesvirus seen (see text). (C) An extracellular particle lacking an electron-dense core. (D) Area of nucleus (Nu) and cytoplasm (Cyt) with marked reduplication of the nuclear envelope (NE) and associated membrane structures (MS). A viral nucleocapsid (arrow) was associated with these structures. (E, F, and G) Higher-power views of type b and c (arrowhead) nucleocapsids found in the nucleus (Nu) and budding from the nuclear membrane (F).

ture KSHV was released from BC-1 cells after induction of the KSHV lytic cycle by *n*-butyrate. In the biochemical assay, an extracellular nucleocapsid preparation was obtained by repeated DNase and NP-40 treatment of a high-speed pellet of supernatant fluids from *n*-butyrate-treated BC-1 cells. These preparations contained EBV DNA (Fig. 7, lane 7) but not KSHV DNA in a DNase-resistant form (Fig. 7, lane 4). KSHV DNA was present in a DNase-resistant form inside cells, suggesting that at least a portion of KSHV DNA was encapsidated.

In biologic experiments, supernatant fluids from BC-1 cells that had been untreated or treated with chemical inducing agents were added to cultured HUCL. When HUCL were morphologically transformed into lymphoblastoid cell lines, they were shown to contain EBV DNA (Fig. 8c, lanes 2 through 5) but no KSHV DNA (Fig. 8b, lanes 2 through 5). These results demonstrated no release of encapsidated or infectious KSHV genomes from BC-1 cells, consistent with our observations by transmission electron microscopy in which profiles of *n*-butyrate-treated cells containing large numbers of nuclear and cytoplasmic herpesvirus nucleocapsids rarely revealed mature enveloped extracellular virions.

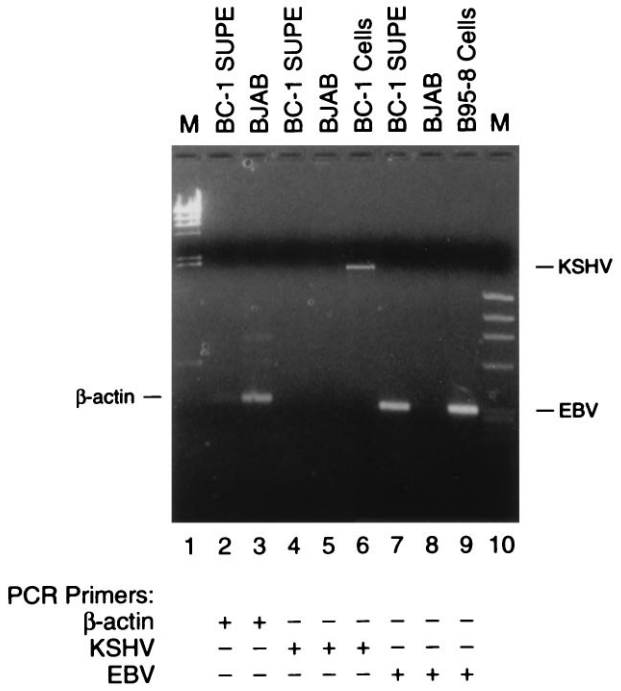

FIG. 7. Detection of EBV DNA but not KSHV DNA in supernatant fluids of *n*-butyrate-treated BC-1 cells. Shown are PCRs with primers present (+) or absent (−) for β-actin (331 nucleotides) (lanes 2 and 3), KSHV DNA (1,851 nucleotides) (lanes 4 through 6), and EBV DNA (331 nucleotides) (lanes 7 through 9). Markers (M) consisted of lambda phage DNA (*Hin*dIII digest) (lane 1) or *phiX* DNA (*Hae*III digest) (lane 10). BC-1 SUPE (lanes 2, 4, and 7) consisted of DNase-treated virion preparations from 75 ml of *n*-butyrate-induced BC-1 cells (see Materials and Methods). DNA prepared from the same BC-1 cells (lane 6) served as a positive control for the KSHV genome; B95-8 cell DNA (lane 9) was a positive control for EBV DNA. BJAB cell DNA (lanes 3, 5, and 8) served as a negative control. −, absent.

DISCUSSION

These studies allow the following general conclusions about cell-virus relationships in the BC-1 cell line, which harbors both gammaherpesviruses. The majority of, if not all, BC-1 cells contain both genomes. In more than 98% of cells, both viruses are latent; in the other 2% of cells, one or both of the viruses spontaneously replicate. Each virus can be independently stimulated into lytic-cycle gene expression by the selective action of inducing chemicals, TPA and *n*-butyrate. Activation of one virus does not activate expression of the other. Both untreated and chemically activated BC-1 cells release infectious EBV. However, unlike EBV, KSHV DNA is not released from BC-1 cells in an encapsidated form that is resistant to the action of DNase.

Both KSHV and EBV are latent in BC-1 cells. Cell cloning experiments (Fig. 6) suggested that the majority of cells harbor both viruses. However, other recently described cell lines established from body cavity lymphomas have been reported to harbor only KSHV (36). Since EBV is not necessary for outgrowth of cell lines from body cavity lymphomas, EBV may be a passenger virus in this disease.

Viral latency is spontaneously disrupted in a few cells. Approximately 2% of untreated BC-1 cells react by immunofluorescence with EBV-positive human sera or with monospecific antisera to EBV late antigens (29, 43) (data not shown). Thus, about 2% of BC-1 cells spontaneously enter the EBV lytic cycle. About 2% of cells are also reactive by fluorescence in situ hybridization with probes for an abundant KSHV-encoded 1.1-kb early RNA (48a). Therefore, a similar number of cells spontaneously enter the KSHV lytic cycle. Transmission electron microscopy confirms that about 2% of untreated BC-1 cells make herpesvirus virions.

Selective chemical activation. TPA markedly activated EBV DNA replication and EBV early- and late-gene expression in BC-1 cells, while the abundance of KSHV DNA and the late mRNA detected by KS 330 *Bam* remained relatively unaffected (Fig. 1 through 4). Although TPA preferentially induced EBV lytic-cycle genes, it also induced the KSHV-associated p60 protein (Fig. 4A). Other studies (not shown) suggest that p60 is a KSHV early protein whose expression is unaffected by inhibitors of viral DNA synthesis. Furthermore, the abundant KSHV 1.1-kb early RNA can also be induced by TPA in BC-1 cells (not shown). These results suggest that in BC-1 cells, KSHV early-gene expression may be activated by TPA but *n*-butyrate is required for KSHV to proceed to lytic viral DNA synthesis and late-gene expression. However, in other body cavity lymphoma B-cell lines in which EBV is not present, TPA is sufficient to drive KSHV all the way to virion production (36).

n-Butyrate by itself strongly activated KSHV gene expression in BC-1 cells but left EBV gene expression relatively unaffected (Fig. 1 through 4). Thus, it is unlikely that a KSHV gene product that is able to cross-activate EBV exists. This finding has permitted the development of first-generation serologic assays using BC-1 cells for detection of antibodies to KSHV lytic-cycle antigens in human sera (29). Sera from 70% of HIV-infected patients with KS recognize a marked increase in the abundance of the p40 polypeptide and in the number of

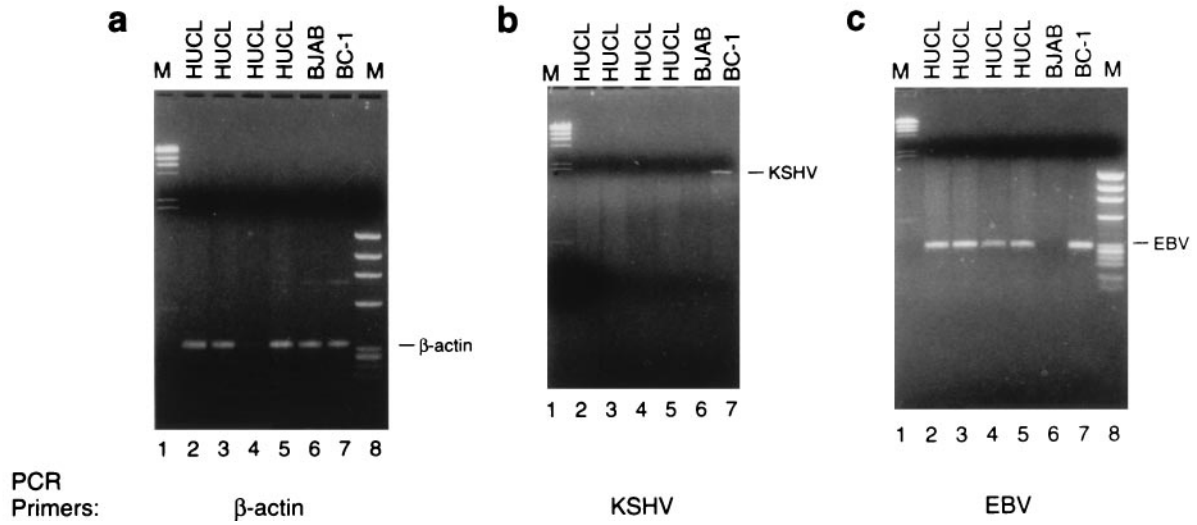

FIG. 8. Detection of EBV DNA but not KSHV DNA in HUCL transformed with BC-1 supernatant fluids. Shown are PCRs for β-actin DNA (a), KSHV DNA (b), and EBV DNA (c). Markers (M) λ *Hin*dIII and ϕX174 *Hae*III were loaded in lanes 1 and 8, respectively. Lanes 2 through 5 contained cellular DNA from HUCL transformed by supernatant fluids from BC-1 cells. The supernatants were from cells that had been untreated (lanes 2) or treated with TPA (lanes 3), *n*-butyrate (lanes 4), or a mixture of TPA and *n*-butyrate (lanes 5). BJAB cells (lanes 6) were a negative control for KSHV and EBV DNAs. BC-1 cells (lanes 7) were a positive control for both viruses.

antigen-positive BC-1 cells in *n*-butyrate-treated cultures. About 12% of HIV-infected patients without KS recognize p40.

Shutoff mechanism accompanying KSHV activation by *n*-butyrate. Treatment of BC-1 cells with *n*-butyrate was associated with a dramatic decrease in the abundance of host cell β-actin mRNA (Fig. 3). The results of experiments so far do not permit a distinction between some nonspecific cytotoxic effect of *n*-butyrate and a KSHV-specific host cell shutoff mechanism. A cytotoxic effect of *n*-butyrate was suggested by the complete disappearance of the cellular β-actin signal, even though KSHV lytic-gene expression and nucleocapsid production was observed in about 50% of cells. However, several pieces of indirect evidence also favor a specific effect mediated by some KSHV gene product(s). Although *n*-butyrate treatment of BC-1 cells inhibited cellular β-actin and EBV late-gene mRNAs, KSHV late-gene mRNAs were unaffected (Fig. 3A). Treatment of HH514-16 cells with *n*-butyrate, which caused 50 to 80% of cells to enter the EBV lytic cycle, was not accompanied by decreased levels of β-actin mRNA. Furthermore, activation of EBV gene expression by TPA in BC-1 and B95-8 cells was not accompanied by a decrease in cellular β-actin mRNA. Thus, *n*-butyrate is not toxic to other lymphoblastoid B cells and activation of EBV by itself is not associated with host-cell shutoff. Moreover, TPA activation of KSHV expression in BCBL-1, a body cavity lymphoma line different from the one we studied in that it does not contain EBV, has also been associated with cell lysis (36). These results are consistent with the hypothesis that induction of KSHV lytic-gene expression by *n*-butyrate is associated with a powerful host cell shutoff mechanism.

The shutoff mechanism may also suppress EBV gene expression. In cells treated with both TPA and *n*-butyrate, there was no increase in EBV DNA replication or late lytic mRNA expression, even though TPA by itself strongly activated these processes (Fig. 1 and 3). This finding suggests that addition of *n*-butyrate and its corresponding activation of KSHV expression shut off EBV lytic-cycle gene expression. However, KSHV gene expression was markedly induced in BC-1 cells treated with both chemicals, suggesting that EBV does not possess a reciprocal shutoff mechanism that impairs KSHV expression.

Lack of release of encapsidated KSHV genomes. *n*-Butyrate treatment caused the appearance of numerous intranuclear herpesvirus nucleocapsids in BC-1 cells (Fig. 6). Since *n*-butyrate did not induce any increase in the content of EBV DNA (Fig. 1 and 2), EBV late mRNA (Fig. 3), or EBV capsid polypeptides (Fig. 4), it is likely that most of these viral particles represent KSHV virions. Nonetheless, there appeared to be a defect in the release from BC-1 cells of particles which contain KSHV genomes in a DNase-resistant form. The explanation that BC-1 KSHV nucleocapsids, unlike EBV nucleocapsids, are leaky and permit the entry of DNase seems unlikely since KSHV DNA can be released in a DNase-resistant state from BCBL-1, another body cavity-based lymphoma cell line (36), and from MH-B2, a cell line recently established in our laboratory (unpublished data). Therefore, B cells can be competent to release encapsidated KSHV. Moreover, intracellular nucleocapsids from *n*-butyrate-treated BC-1 cells contain KSHV DNA in a DNase-resistant form (data not shown). Another explanation is that there is some defect in KSHV maturation in BC-1 cells. Only a single extracellular virion was observed by transmission electron microscopy of BC-1 cells; this was likely to be EBV. It is not yet known whether the KSHV genome in BC-1 cells contains all of the KSHV genes in an intact form or represents a partially defective genome, such as are regularly found in lymphoid tumor cells transformed by HVS (12, 16) and frequently encountered in several EBV lineages (22). BC-1 cells may release infectious KSHV that was not detectable by the biochemical and biologic assays we used. Moore et al. reported that cocultivation of BC-1 cells with Raji cells in communicating chambers separated by a 0.45-μm-pore-size membrane filter resulted in the transfer of the KSHV genome to Raji cells (31). The results of these experiments suggest that infectious KSHV can be released from BC-1 cells. Alternatively, unencapsidated KSHV DNA may have been transferred from BC-1 to Raji cells. The resolution of this problem awaits the development of sensitive infectivity assays for KSHV.

Elements of the switch from latency to lytic cycle of these two gammaherpesviruses in BC-1 cells. There is a classical pattern of EBV gene expression in BC-1 cells. These cells express EBNA1 and other latency products that have not yet been characterized. Treatment with TPA was accompanied within 6 h by increased expression of *BZLF1* mRNA and ZEBRA protein (not shown). Presumably, ZEBRA drives the lytic cascade. The components of the switch in KSHV have not yet been characterized. It is not yet known which KSHV gene products are expressed during latency. KSHV is not known to have a homolog to EBV BZLF1; its closest relative, HVS, does not have such a homolog (1). However, all three gammaherpesviruses do possess variants of the EBV *BRLF1* transactivator (1, 47a). The *R* transactivator, perhaps in combination with other immediate-early genes, is likely to control the latency to lytic cycle switch in KSHV.

A striking biologic observation in the BC-1 cell system was the independent inducibility of these two viruses in the same cell line. Previously, it has been found that in some cell backgrounds, e.g., B95-8 marmoset cells, EBV is preferentially induced by TPA and unaffected by *n*-butyrate; in other cell backgrounds, e.g., HH514-16, EBV is preferentially induced by *n*-butyrate and unaltered by TPA. However, since these different species of cells also harbor different EBV strains, it is not known whether differences in inducibility are a result of viral or cell differences or both. The current observations of different chemical inducibility of two gammaherpesviruses in the same clonal cell background (Fig. 3A and B and 5B) point to the role of some viral component in the determination of response to chemical activation of the lytic cycle.

ACKNOWLEDGMENTS

This work was supported by the following grants from the National Institutes of Health: CA 70036, AI22959, and CA12055 to G.M. and CA67391 to Y.C.

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