n-Butyrate, a Cell Cycle Blocker, Inhibits the Replication of Polyomaviruses and Papillomaviruses but Not That of Adenoviruses and Herpesviruses

F. F. SHADAN,¹ LEX M. COWSERT,² AND LUIS P. VILLARREAL^{1*}

*Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717,¹
and ISIS Pharmaceuticals, Carlsbad Research Center, Carlsbad, California 92008²*

Received 4 February 1994/Accepted 19 April 1994

Small DNA viruses are dependent on the interaction of early proteins (such as large T antigen) with host p53 and Rb to bring about the G₁-to-S cell cycle transition. The large DNA viruses are less dependent on host regulatory genes since additional early viral proteins (such as viral DNA polymerase, DNA metabolic enzymes, and other replication proteins) are involved in DNA synthesis. A highly conserved domain of large T antigen (similar to the p53-binding region) exclusively identifies papovavirus, parvovirus, and papillomaviruses from all other larger DNA viruses and implies a conserved interaction with host regulatory genes. In this report, we show that 3 to 6 mM butyrate, a general cell cycle blocker implicated in inhibition of the G₁-to-S transition, inhibits DNA replication of polyomavirus and human papillomavirus type 11 but not the replication of larger DNA viruses such as adenovirus types 2 and 5, herpes simplex virus type 1, Epstein-Barr virus, and cytomegalovirus, which all bypass the butyrate-mediated cell cycle block. This butyrate effect on polyomavirus replication is not cell type specific, nor does it depend on the p53 or Rb gene, as inhibition was seen in fibroblasts with intact or homozygous deleted p53 or Rb, 3T6 cells, keratinocytes, C2C12 myoblasts, and 3T3-L1 adipocytes. In addition, butyrate did not inhibit expression of polyomavirus T antigen. The antiviral effect of butyrate involves a form of imprinted state, since pretreatment of cells with 3 mM butyrate inhibits human papillomavirus type 11 DNA replication for at least 96 h after its removal. Butyrate, therefore, serves as a molecular tool in dissecting the life cycle of smaller DNA viruses from that of the larger DNA viruses in relation to the cell cycle.

All DNA viruses share a common strategy of programmed gene expression and the necessity to induce DNA synthesis in host cells. Early viral proteins are thought to bring about both viral and host DNA replication. Yet various virus families may differ in mechanism, especially as larger DNA viruses have more genes to affect host regulation. Early viral proteins such as the large T antigens of simian virus 40 (SV40) and polyomavirus, E proteins of papillomaviruses, E1A and E1B of adenovirus, and the EBNA of Epstein-Barr virus (EBV) share some common features such as binding to p53 and Rb host regulatory proteins (70). These host regulatory proteins appear to regulate the induction of DNA replication, typically associated with the S phase of the cell cycle. In contrast to the role of early viral proteins in small DNA viruses, which compels utilization of host DNA replication machinery, the nonessential genes and viral DNA polymerase in herpes simplex virus (HSV) and other large DNA viruses participate enzymatically in viral DNA replication, and therefore these viruses are less dependent on the host cell (57, 71).

Entry of cells into the S phase has been the focus of studies that led to the initial proposal of a restriction point (13, 46) governing the G₁-to-S transition. More recently, Rb and p53 gene products have been implicated in regulation of the transition from G₁ to S (17, 40, 45). This view is supported by the finding that the SV40 large T antigen, possibly E1A and E1B of adenovirus, and the E6 and E7 proteins of papillomaviruses interact with and inhibit the transactivation (26, 47, 80) and polymerase-primase complex inactivation (10, 23, 24) properties of p53. Interaction with such proteins also leads to

the predominance of the inactive form of Rb (17, 21, 30, 42, 43, 75). Such similarities in interaction with host proteins among the DNA viruses suggest related strategies for replication.

An evolutionary analysis has recently identified a highly conserved domain among papovaviruses and papillomaviruses (61) (but not herpesviruses), pointing to the possible convergence of small DNA viruses at the level of interaction with host proteins that may control the G₁-to-S transition, as this conserved domain has similarity to the p53-like binding domain of SV40 large T antigen. We therefore sought to investigate whether DNA viruses can be further classified according to their replication dependence on the host cell cycle control. While much has been done to understand the mechanism of viral replication in individual virus-host systems, comparative studies have not addressed the issue of common versus divergent viral strategies. Specifically, we were interested in determining whether a butyrate-mediated cell cycle block could differentiate between the life cycles of the DNA viruses in permissive host cells. Butyrate has been an important tool for cell cycle studies, as it has been reported to cause a G₁ block and inhibit the onset of DNA synthesis in a large number of cell types, including the Chinese hamster ovary (20), HeLa (18), retinoblastoma (32), mouse kidney (72), 3T3 (14), and 3T6 (78) cells. Butyrate has the added advantage of inducing the expression of early viral proteins such as large T antigen, the EBV early antigen, and cytomegalovirus (CMV) early proteins (2, 3, 29, 34, 60, 65), a property that simplifies the considerations of how butyrate might affect virus replication.

The replication of many DNA viruses is often highly localized to individual cells and is sometimes called jackpot replication (9). It has been our experience that a very informative means of identifying DNA virus replication in individual cells

* Corresponding author. Phone: (714) 856-6074.

and determining the proportion of cells that support high-level viral DNA replication is by *in situ* hybridization which can visualize jackpot replication (4, 5, 22, 50). Using this technique, we demonstrate that butyrate dramatically inhibits a large variety of cell types from supporting high-level polyomavirus DNA replication while not inhibiting large-T-antigen expression. By contrast, we demonstrate that the proportion of cells supporting high-level DNA replication of large DNA viruses such as adenovirus, CMV, and EBV clearly does not decrease following treatment with butyrate. The butyrate-mediated cell cycle block can therefore differentiate the life cycles of papovaviruses and papillomaviruses from those of the larger DNA viruses, an observation that is consistent with implications of our evolutionary study (61).

MATERIALS AND METHODS

Viral stocks and cell culture. The wild-type polyomavirus A2 strain and SV40 stocks were grown by low-multiplicity infection of 3T6 and CV1 cells, respectively, to a titer of 10^9 PFU/ml, as determined by plaque assay. CMV (50% tissue culture infective dose of $5 \times 10^{3.5}$) strain Vr 538 was purchased from the American Type Culture Collection, Rockville, Md.; adenovirus type 2 (10^9 PFU/ml), EBV-infected ARH77 cells, and HSV-1 (17 syn⁺ strain; 10^9 PFU/ml) were gifts from Arnold Berk, Michael Samoszuk, and Ed Wagner, respectively.

Mouse embryonic fibroblasts (MEF; a gift from Ben Murray) and homozygous Rb and p53 knockout primary fibroblasts (gifts from Tyler Jacks and Larry Donehower) were passed and maintained in Dulbecco modified Eagle medium (DMEM; Irvine Scientific, Irvine, Calif.) with 10% fetal bovine serum (FBS). 3T6, Vero, HeLa, and C2C12 cells and 3T3-L1 fibroblasts (American Type Culture Collection) were passaged and maintained in DMEM containing 10% FBS. Human fibroblasts and ARH77 suspension culture cells were passaged and maintained in RPMI medium (Irvine Scientific) containing 10% FBS. SCC-4 cells (American Type Culture Collection) were passaged and maintained in DMEM F-12 (Gibco BRL, Grand Island, N.Y.) supplemented with 10% FBS (HyClone, Logan, Utah) and 0.4 μ g of hydrocortisone per ml.

Preparation and application of butyrate. Butyrate (Aldrich Chemical Company, Inc., Madison, Wis.) was diluted in fresh tissue culture medium containing 10% serum, and the pH was adjusted to 7.4 by adding 10 N NaOH dropwise to the medium. Butyrate was administered at concentrations ranging from 0 to 20 mM given either concurrently for the duration of viral infection or as a 24- to 48-h pretreatment of cells or both. The optimal concentration range of butyrate (3 to 6 mM) was adopted since it produced maximum efficacy with less toxicity as reported previously (14, 72, 78).

Detection of large T antigen. The cells were fixed in 100% methanol for about 30 min at -20°C , air dried, and stored at -20°C . The tissue-nonspecific sites were blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) (PBS-BSA) for 20 min at room temperature. The tissue was incubated in a humid chamber for 2 h at 37°C with polyclonal rat anti-large T antibody (a gift from Walter Eckhart) diluted at 1:100 in PBS-BSA. The excess antibody was removed by washing in PBS for 2 min and incubated with PBS-BSA for 20 min at room temperature. The sections were incubated for 2 h in a humid chamber at 37°C with anti-rat immunoglobulin G-fluorescein isothiocyanate conjugate at a dilution of 1:64 in PBS-BSA. The sections were washed with PBS for 2 min and mounted with Permafluor medium for immunofluorescence.

DNA detection of polyomavirus, EBV, and CMV. Polyoma-

virus-, EBV-, and CMV-infected cells were fixed in 100% ethanol at -20°C for 20 min. The cells were treated for 2 to 20 h with RNase (100 μ g/ml) at 37°C and then fixed and processed for viral DNA detection by using the Digene kit protocol (Digene Diagnostics, Silver Spring, Md.) for *in situ* hybridization detection of CMV and EBV DNA. Polyomavirus DNA was directly conjugated to horseradish peroxidase (custom probe made by Digene) and used according to the Digene protocol for the EBV and CMV DNA detection systems. All photographs were taken at a magnification of $\times 100$. Because of butyrate-mediated morphological alterations such as spreading, thinning of cells, and reduced nuclear staining with the nuclear fast red stain, phase-contrast microscopy was often used to better visualize the butyrate-treated cells.

Detection of adenovirus replication. Adenovirus replication was studied by both plaque assay and infected cell assay systems.

(i) **Infected cell assay system.** To determine adenovirus replication on a quantal basis (i.e., infected versus uninfected, or on versus off), HeLa cells were grown in culture to about 30% subconfluency and incubated for 24 h in DMEM with 10% FBS either with or without 3 to 6 mM butyrate. The cells were infected with adenovirus at a multiplicity of infection (MOI) of 1 to 10 for 2 h, washed with PBS several times, and incubated for up to 6 days with DMEM plus 10% FBS, either with or without 3 mM butyrate. The cells were subsequently harvested and centrifuged for 10 min to obtain a pellet, which was sonicated for 1 min; 1 to 10% of the lysate was used to inoculate a fresh culture of uninfected HeLa cells on four-well chamber slides (Nunc, Naperville, Ill.) for a brief period sufficient to allow detectable E1A expression. The cells were fixed with methanol at -20°C for 20 min and air dried. The slides were then incubated for 20 min at room temperature in PBS-BSA. The cells were washed several times in PBS and incubated with a 1:300 dilution of rabbit anti-adenovirus nuclear antigen polyclonal antibody (Lee Biomolecular Research, San Diego, Calif.) in PBS-BSA at 37°C for 2 h. The slides were washed several times with PBS and then incubated for 20 min at room temperature in PBS-BSA. The cells were then incubated with a 1:20 dilution of goat anti-rabbit fluorescein isothiocyanate-conjugated immunoglobulin G (Sigma, St. Louis, Mo.) for 2 h at 37°C , washed several times with PBS, overlaid with 2 drops of Permafluor (Lipshaw Immunon, Pittsburgh, Pa.), and examined for green fluorescence under a microscope using a UV light source. The presence of fluorescent cells therefore represents on a quantal basis an on state, i.e., infection by adenovirus obtained from the lysate. The validity of this method was confirmed by plaque assay.

(ii) **Plaque assay.** To confirm the validity of the infected cell assay system and to determine whether butyrate-resistant replication of adenovirus occurs under conditions in which E1A is expressed as part of the host chromatin and not viral DNA, we observed the effect of butyrate on the replication of adenovirus type 5 mutants deleted in the E1A region in E1A-expressing 293 host cells. A plaque assay was performed as follows. 293 cells were maintained in DMEM containing 10% FBS. The cells were infected with either E1A-deleted adenovirus type 5 (deletion 309) or adenovirus type 5 (deletion 327) when 90% confluent and incubated for 1 h at 37°C while gently rocking the plate every 15 min. The virus-containing medium was then removed, an overlay was added immediately, and the medium was allowed to sit in a laminar-flow hood for 10 min at room temperature and subsequently transferred to a 37°C incubator. Three to five milliliters of overlay was added every third day in the following proportions: 4% FBS plus 2.8% agar in DMEM; 1 to 3 mM butyrate was added to the

overlay either at the time of infection or on day 2 postinfection. By days 4 to 8, plaques were usually visible and were stained with 4% FCS, 2.8% agar, and 0.1% neutral red in DMEM.

Detection of HPV-11 DNA replication. Butyrate-pretreated or concurrently treated SCC-4 cells were electroporated with human papillomavirus type 11 (HPV-11) DNA and plated directly into medium containing butyrate at time zero. Fresh growth medium with or without butyrate was added daily for 4 days. Cells were harvested, and low-molecular-weight DNA was extracted by the method of Hirt (31). The resulting DNAs were digested with both *DpnI*, to digest nonreplicated input DNA, and *HindIII*, a single-cut enzyme for HPV-11. The products were analyzed by Southern blotting using random-primed HPV-11 total genomic DNA. The blot was visualized with a PhosphorImager.

Detection of HSV-1 replication. To assess the effect of butyrate on the replication of HSV-1, we found the plaque assay to be a convenient and adequate means of producing clear results. Confluent monolayers of Vero cells were prepared on six-well plates (Corning Glass Works, Corning, N.Y.). One milliliter of various dilutions of HSV-1 (17 syn⁺ strain) was added per well, and the cells were incubated for 2 h, washed several times with PBS, incubated with DMEM–10% FBS with or without human anti-HSV immunoglobulin (0.1% immunoglobulin G), and incubated for 72 h at 37°C. The medium was aspirated off gently, and the cells were fixed with 50% methanol–50% saline for 20 min. The fixative was aspirated off, and absolute methanol was added for 10 min. Then 0.1% Giemsa–10% glycerol stain was added to the cells for up to 20 min. The cells were then rinsed with cool water, and the plaques were counted. To study the effect of butyrate, the cells were typically preincubated with up to 3 mM butyrate for 24 h prior to the addition of virus for plaque assay. We have typically observed that at cytostatic concentrations of greater than 1 mM butyrate, monolayers of Vero cells stain relatively lightly with Giemsa stain but nevertheless produce visible plaques when observed against a lightly shaded background.

RESULTS

Butyrate efficiently inhibits high-level polyomavirus DNA replication in both normal and immortal cells without inhibition of large T-antigen expression. To characterize the butyrate-mediated cell cycle block, using polyomavirus DNA replication as an index of S phase, we sought to determine whether the butyrate effect discriminates between normal and immortal cells and whether the effect involves inhibition of expression of large T antigen. Normal MEF were either pretreated with 3 to 6 mM butyrate for 24 to 48 h and subsequently infected with polyomavirus for 48 h in the presence of butyrate or given butyrate and polyomavirus concurrently for 48 h. The slides were then fixed and examined for polyomavirus DNA replication and large-T-antigen expression by in situ hybridization (using peroxidase-labelled polyomavirus DNA as a probe) and immunofluorescence techniques. A parallel experiment was carried out with the immortal 3T6 cell line. As expected, positive controls consisting of polyomavirus-infected MEF and 3T6 cells replicated high-level viral DNA and expressed nuclear T antigen (Fig. 1A, B, and F). In these photographs, cells supporting high-level replication of viral DNA as observed by in situ hybridization appear with black nuclei, while large T-antigen-expressing cells as detected by immunofluorescence appear with white nuclei. By contrast, butyrate given either concurrently (Fig. 1C) or as a pretreatment followed by concurrent treatment (Fig. 1D and G) prevented an increase in cell number and completely

inhibited high-level viral DNA replication without inhibition of large-T-antigen expression (Fig. 1E and H) in both MEF and 3T6 cells. In one experiment, for example, upon counting about 600 nuclei per field (designated *n*), we found that on average 77.9% of MEF nuclei stained positive for high-level polyomavirus DNA (standard deviation [SD] = 1.64%, *n* = 2), while 6.4 mM butyrate treatment reduced this value to 7.1% (SD = 4.47%, *n* = 6). In another experiment, after counting about 800 nuclei, we found that on average 56.65% of 3T6 nuclei stained positive for high-level polyomavirus DNA (SD = 6.24%, *n* = 8), while 6 mM butyrate reduced the proportion of positive nuclei to 0.7% (SD = 1.6%, *n* = 8). These observations are in agreement with results obtained by others and us on the basis of cellular proliferation and related tritiated thymidine incorporation studies to measure DNA synthesis. Briefly, butyrate (3 to 6 mM) was found to inhibit cellular proliferation and related [³H]thymidine incorporation in a variety of cell types, including mouse 3T6 (uninfected or polyomavirus infected), HeLa (uninfected or adenovirus type 2 infected), Vero (uninfected or HSV-1 17 syn⁺ infected), and SCC-4 and CV1 (unpublished data) cells. Butyrate-mediated inhibition of viral and host DNA synthesis in polyomavirus-infected 3T6 cells and other cell types has also been reported by Wintersberger et al. (78) and others (14, 38, 72). Inhibition of adenovirus type 5 DNA synthesis without detectable cellular DNA replication in butyrate-treated HeLa cells has been described earlier (18). Butyrate thus appears to inhibit polyomavirus DNA replication as well as host DNA synthesis.

The intensity of large-T-antigen immunofluorescence along with inhibition of high-level polyomavirus DNA replication under low-MOI conditions (MOI of 1 to 10) suggests that T-antigen expression is not inhibited by butyrate and that the butyrate-treated cells which do not support high-level viral DNA replication contain low copy numbers of viral DNA. It has been reported by others that early viral protein expression is in fact increased by butyrate (for studies of T antigen and other early viral proteins, see references 2, 29, and 60). Our in situ hybridization and immunofluorescence results suggest that butyrate is effective in both normal and immortal cells, and the inhibition of high-level polyomavirus DNA replication is uncoupled from T-antigen expression in these cells.

Butyrate is not cell type specific, affecting cells that are capable of differentiation as well as those with genetic defects in checkpoint control. To examine the generality of the butyrate effect, we studied the response of primary cultures of mouse keratinocytes, C2C12 myoblasts (myotube precursor cells), and 3T3-L1 adipocyte precursor cells (all of which are untransformed mouse cells that under appropriate conditions are capable of differentiation into keratinized anucleate cells, myotubes, and adipocytes, respectively [27, 63, 67, 79]) as well as primary mouse fibroblasts that are homozygous knockout mutants for either the p53 or Rb gene. In addition to broadening the spectrum of cells ranging from the immortal state (3T6 fibroblasts) to the normal differentiating cell types (3T3-L1, C2C12, and mouse keratinocytes), the p53 and Rb knockout cells also served as checkpoint controls for the G₁-to-S transition. Mouse keratinocytes, 3T3-L1 cells, C2C12 cells, and p53 and Rb knockout cells were seeded at a low density, treated with 3 to 6 mM butyrate for 48 h, infected with polyomavirus at a low MOI (1 to 10) while being concurrently treated with 3 to 6 mM butyrate for 48 h, and compared with polyomavirus-infected untreated controls for viral DNA synthesis by in situ hybridization, using peroxidase-labelled polyomavirus DNA as a probe (Fig. 2). In all cell types, butyrate prevented an increase in cell number and resulted in the inhibition of high-level polyomavirus DNA replication (Fig.

FIG. 1. Butyrate inhibition of polyomavirus DNA replication but not T-antigen expression in normal MEF and immortal 3T6 fibroblasts. MEF were infected with polyomavirus (48 h), fixed with ethanol, and examined for polyomavirus DNA replication by in situ hybridization (using peroxidase-labelled polyomavirus DNA as a probe) (A) and for T-antigen expression (B) by using the immunofluorescence technique. Concurrent administration of 3 to 6 mM butyrate and polyomavirus dramatically reduces polyomavirus DNA replication to a small subset of cells (C), while 48-h pretreatment of the MEF with butyrate almost completely inhibits polyomavirus DNA replication (D) without inhibition of T-antigen expression (E). 3T6 fibroblasts were infected with polyomavirus (for 48 h), fixed, and examined by in situ hybridization in the absence (F) and presence (G) of 6 mM butyrate treatment. (H) Immunofluorescence analysis of T-antigen expression in butyrate-treated 3T6 fibroblasts.

FIG. 2. Butyrate is not cell type specific, affecting cells capable of normal differentiation and cells with genetic defects in checkpoint control. Polyomavirus DNA replication is shown by in situ hybridization (using peroxidase-labelled polyomavirus DNA as a probe) in untreated 3T3-L1 lipocyte precursor cells (A) and C2C12 myoblasts (C). Butyrate at 3 to 6 mM inhibits polyomavirus DNA replication in 3T3-L1 cells (B) and C2C12 myoblasts (D). Homozygous p53 knockout mouse fibroblasts were infected with polyomavirus and examined for viral DNA replication by in situ hybridization in the absence (E) and presence (F) of butyrate treatment. Similarly, homozygous Rb knockout mouse fibroblasts were infected with polyomavirus and examined for viral DNA replication in the absence (G) and presence (H) of butyrate treatment.

2B, D, F, and H) relative to the untreated polyomavirus-infected controls (Fig. 2A, C, E, and G). In these photographs, polyomavirus-infected cells appear with black nuclei following in situ hybridization (Fig. 2A, C, E, and G), while cells in which high-level polyomavirus DNA replication has been inhibited by butyrate lack the dark nuclei (Fig. 2B, D, F, and H). For example, a 10-fold reduction in the number of acutely infected keratinocytes supporting high-level viral DNA replication was observed following treatment with 3 to 6 mM butyrate for 24 to 48 h (not shown). Similarly, 3 mM butyrate treatment reduced the proportion of C2C12 nuclei supporting high-level viral DNA replication from 40.4% (SD = 6.4%, $n = 8$) in the untreated cells to 1.2% (SD = 2.1%, $n = 8$). In 3T3-L1 cells, butyrate decreased the proportion of DNA-replicating nuclei from 46.4% (SD = 7.83%, $n = 4$) in the untreated cells to almost 0%; 58.1% of untreated p53 $-/-$ knockout fibroblasts supported polyomavirus DNA replication (SD = 5.3%, $n = 4$), while in the butyrate-treated state, this proportion was reduced to 2.5% (SD = 2.5%, $n = 4$). This result is not substantially different from that observed with normal MEF that are wild type for the p53 gene (see above). Two examples of the rare butyrate-treated p53 $-/-$ cells that are positive for polyomavirus DNA are shown in Fig. 2F. Similarly, 44.2% of untreated Rb $-/-$ knockout fibroblasts (SD = 11.53%, $n = 4$) supported polyomavirus DNA replication, in contrast to almost 0% of nuclei treated with 6 mM butyrate, again a result that is not substantially different from that for MEF that are wild type for Rb. The rare butyrate-resistant cells that replicate viral DNA represent a curious phenomenon that we suspect involves a form of high-level viral DNA replication related to terminal differentiation (22, 69). We have obtained similar results regarding the inhibitory effect of butyrate on the replication of SV40 (unpublished data), thus extending the inhibitory effect of butyrate to other members of the papovavirus family. The results therefore suggest that butyrate inhibits the replication of papovaviruses in a wide spectrum of host cells, ranging from normal to immortal, and without overt cell type specificity, including those with genetic defects in checkpoint control.

Inhibition of HPV-11 DNA replication by butyrate. The effect of butyrate on the replication of papillomaviruses has not been studied so far. To examine whether the inhibitory effect of butyrate extends beyond papovaviruses to include papillomaviruses, we studied the effect of butyrate on HPV-11 DNA replication. SCC-4 cells were electroporated with HPV-11 DNA and plated into media containing different concentrations of butyrate at time zero. Cells were harvested, DNA was prepared, and the products were analyzed by Southern blotting using random-primed HPV-11 total genomic DNA (Fig. 3). In accordance with our observation of other cell types, 3 and 9 mM butyrate resulted in a dramatic inhibition in cellular growth over the 4-day period, and the effect was more pronounced at 9 mM than at 3 mM. At 3 and 9 mM butyrate, we observed an overt cytostatic effect which accompanied a substantial reduction in both cellular and viral DNA replication (Fig. 3). The signal reduction between the untreated control and 1 mM butyrate-treated sample (Fig. 3) is significant when quantitated by a PhosphorImager (not shown), and there were no overt cytostatic effects at this concentration, as judged by visual inspection. These results indicate that like papovaviruses, papillomaviruses are inhibited by the block mediated by butyrate.

The antiviral effect of butyrate involves a form of imprinted memory. To examine the reversibility of the antiviral effect of butyrate, cells were incubated in the presence of either 0, 1, 3, or 9 mM sodium butyrate for 24 h. After butyrate treatment, the cells were trypsinized and electroporated with HPV-11.

FIG. 3. HPV-11 DNA replication is inhibited by butyrate. SCC-4 cells were electroporated with HPV-11 DNA and plated directly into medium containing butyrate at time zero. The cells were harvested, low-molecular-weight DNA was prepared, and the resulting DNAs were digested with both *Dpn*I, to digest nonreplicated input DNA, and *Hind*III, a single-cut enzyme for HPV-11. The products were analyzed by the Southern blot technique, using random-primed HPV-11 total genomic DNA.

Following electroporation, cells were plated in standard growth medium, without sodium butyrate, and incubated for 96 h, at which time the cells were harvested and analyzed for replicated HPV-11 DNA. The results clearly demonstrated that a single pretreatment with either 3 or 9 mM butyrate inhibited viral DNA replication (Fig. 4). Quantitation of the blot (not shown) did not indicate significant reduction in signal for the 1 mM point but complete absence of signal for 3 or 9 mM butyrate (Fig. 4). The irreversibility of the antiviral effect of butyrate following 96 h of incubation with butyrate-free medium and continued cellular proliferation (6, 14, 72, 78) as determined by [3 H]thymidine incorporation (unpublished data) indicates that the cells remained imprinted in an antiviral mode.

Butyrate-resistant replication of wild-type and E1A-deleted adenovirus variants in HeLa and E1A-expressing 293 cells. To conveniently and quantitatively determine whether butyrate produces the kind of quantal inhibition (on versus off) of high-level viral replication observed in papovaviruses and papillomaviruses, a plaque assay was used to test the effectiveness of butyrate on the replication of adenovirus type 5 in 293 cells. A plaque assay of E1A-expressing 293 cells along with

FIG. 4. The antiviral effect of butyrate involves a form of imprinted memory. SCC-4 cells were incubated in the presence of either 0, 1, 3, or 9 mM butyrate for 24 h. The cells were trypsinized, electroporated with HPV-11, and incubated for up to 96 h in standard growth medium without butyrate. The cells were harvested and analyzed for replicated HPV-11 DNA by the Southern blot technique.

FIG. 5. Butyrate-resistant replication of adenovirus assessed by plaque assay of four different dilutions of adenovirus type 5 (Ad5) (deletion 309) in the presence or absence of 3 mM butyrate treatment of E1A-expressing 293 cells. Error bars represent SDs ($n = 2$).

FIG. 6. Butyrate-resistant replication of HSV1 (17 syn⁺) assessed by plaque assay performed with Vero cells either with or without treatment with 3 mM butyrate. Error bars represent SDs ($n = 2$).

two independent adenovirus type 5 mutants with deletions of the E1A region (referred to as deletions 309 and 327) was performed. No significant difference between the number of plaques was observed either in the presence or in the absence of butyrate with either deletion 327 (not shown) or deletion 309 (Fig. 5). As shown in Fig. 5, a comparison of four different dilutions of adenovirus type 5 (deletion 309) yielded mean PFU per well that were comparable between the butyrate-treated and untreated 293 cells, spanned in each case by overlapping SDs, suggesting a lack of significant difference between the butyrate-treated and the untreated cells in supporting viral replication. Similar results were obtained when we tested the effect of butyrate treatment on the replication of adenovirus type 2 in HeLa cells (not shown) in an infected cell assay (see Materials and Methods). The clear observation of high-level butyrate-resistant adenovirus replication by two different methods made unnecessary detection of low-copy-number viral DNA by Southern blot analysis or immunostaining for early viral proteins. Therefore, butyrate was found to be clearly ineffective in shutting off the replication of adenoviruses in permissive host cells.

Butyrate-resistant replication of large DNA viruses in permissive host cells. To determine the result of a butyrate-mediated block on replication of the larger DNA viruses, we examined the effect of butyrate on the replication of HSV-1 (17 syn⁺ strain) in monkey kidney Vero cells conveniently and quantitatively in a plaque assay (Fig. 6). The proportion of cells supporting high-level viral DNA replication was examined by in situ hybridization in cells infected with related herpesviruses such as CMV-infected human fibroblasts and EBV-infected ARH77 cells (Fig. 7). Butyrate treatment did not reduce the level of viral replication in any of these permissive virus-host systems (Fig. 6 and 7). The plaque assay technique for HSV replication did not identify butyrate as a prominent inhibitor of viral replication, as we did not find any difference between the mean number of plaques observed (PFU per well) in the presence of 0 to 3 mM butyrate (Fig. 6). The presence of equal numbers of clear plaques in the presence of butyrate relative to the untreated control cells was indicative of equally high level viral replication and rendered unnecessary either Southern blot analysis or immunostaining for early viral proteins to demonstrate the presence of low-copy-number viral DNA. The ineffectiveness of butyrate on the replication of HSV-1 was

confirmed, however, in other herpesviruses. In contrast to the dramatic inhibition observed in polyomavirus-infected cells (Fig. 1 and 2), in situ hybridization of human fibroblasts acutely infected with CMV and the EBV-infected ARH77 cell line (Fig. 7) did not show a reduction in the proportion of darkly staining nuclei (supporting high-level viral DNA replication) in response to butyrate (compare Fig. 7A and B and Fig. 7C and D), although butyrate prevented an increase in the total number of fibroblasts. For example, on average, 79.18% of untreated fibroblasts supported high-level CMV DNA replication (SD = 18.79%, $n = 9$, when about 300 to 500 cells were counted per n), while treatment with butyrate resulted in 69.69% of nuclei supporting high-level CMV DNA replication (SD = 18.27, $n = 14$), a figure that is not substantially different from the untreated control value as a result the overlapping SDs. Similarly, we did not observe a shutoff of high-level EBV DNA replication in response to butyrate (note the presence of frequent darkly stained nuclei in Fig. 7C and D). The ineffectiveness of butyrate in reducing the proportion of cells that support high-level CMV or EBV DNA replication rendered unnecessary further immunostaining for early viral proteins or Southern blot analysis to demonstrate the presence of low-copy-number viral DNA.

Inhibition of cellular proliferation and related [³H]thymidine incorporation by a variety of cell types, including adenovirus-infected HeLa cells or HSV-infected Vero cells (unpublished data), consistent with previous reports (14, 18, 38, 72, 78), indicate that a potential cellular difference in response to butyrate is not likely to account for the inability of butyrate to inhibit viral DNA synthesis in cells infected with the larger DNA viruses. To further examine this point, 3T6 cells were treated for 48 h with 6 mM butyrate and then infected with HSV-1 (17 syn⁺). Cytopathic effect and plaque formation were observed in the HSV-1-infected butyrate-treated 3T6 cells (unpublished data). These results, therefore, argue against the possibility of cellular differences accounting for the ability of larger DNA viruses to replicate in butyrate-treated cells. Thus, butyrate-resistant replication by the larger DNA viruses distinguishes the life cycles of herpesviruses from those of polyomaviruses and papillomaviruses, in accordance with the evolutionary classification of small DNA viruses (61).

FIG. 7. Butyrate-resistant DNA replication of two related herpesviruses, CMV and EBV. Acute CMV infection in human fibroblasts was followed by in situ hybridization detection of high-level CMV DNA replication in the absence (A) and presence (B) of 3 mM butyrate treatment. ARH77 cells infected with EBV were treated with butyrate for 48 h (D) and compared with the untreated control (C) in an in situ hybridization detection system. Butyrate did not significantly reduce the proportion of cells supporting high-level DNA replication of either CMV or EBV.

DISCUSSION

The early proteins of DNA viruses induce viral DNA replication as well as cellular DNA synthesis by initiating the process of G_1 -to-S transition of the cell cycle (for a review, see reference 76). More recently, p53 (45) and the Rb host gene products have been implicated in the regulatory mechanism of the G_1 -to-S transition (for reviews, see references 51 and 17). With SV40 large T antigen, the mechanism affecting the cellular G_1 -to-S transition has been extensively studied. Early reports suggested that the SV40 large T antigen binds and inhibits the p53-mediated inhibition of the polymerase alpha-prime complex (10, 28). More recently, it has been shown that p53 is a negative transcription factor of genes responsible for the G_1 -to-S transition and is inhibited through binding of the SV40 large T antigen (26). Similarly, the genes encoding cellular protein p90 (*mdm2*) (25, 49), E6 of HPV-16 (47), and adenovirus E1B are thought to inhibit p53 activity (80). E1A appears to cooperate with E1B to transform cells by binding the Rb gene product and inhibiting the Rb transactivation of E2F (30). Thus, these early viral genes appear to have common mechanisms to affect host cell cycle control by interacting with these cellular regulatory proteins.

Recently, we examined the conservation of sequences among the small DNA viruses (61) and identified a domain within the p53-like binding/ATPase region of large T antigen that has been most conserved. This conserved domain was identified in early viral proteins of members of the polyomavirus, papillomavirus, and parvovirus families (61) but not the herpesviruses. Because the observed pattern of conservation of the p53-like binding region is specific to the small (61) but not

larger DNA viruses, and because both groups of viruses (including adenovirus) appear to affect the G_1 transition of the host cell cycle, we compared the life cycles of small DNA viruses with those of other DNA viruses in the context of a cell cycle block.

Butyrate (3 to 6 mM) inhibits cellular proliferation and related [3 H]thymidine incorporation in polyomavirus-infected and uninfected 3T6 fibroblasts and in a variety of cell types with or without infection by other DNA viruses (14, 18, 72, 78). Butyrate has been shown to inhibit entry into S phase by inhibition of thymidine incorporation into genomic DNA in polyomavirus-infected and uninfected 3T6 cells (78) as well as to arrest the growth of Swiss 3T3 cells (67), normal and SV40-transformed human keratinocytes (63), BALB/c 3T3 mouse fibroblasts (14), adenovirus-infected HeLa cells (18), retinoblastoma cells (32), and normal and Rous sarcoma virus-transformed chicken heart mesenchymal cells (6). All of these reports are consistent with the butyrate-mediated cell cycle block, thought to be at the G_1 phase of the cell cycle (13, 46, 62, 77). That butyrate also inhibited polyomavirus and SV40 replication with either comparable or lower sensitivity (19, 38, 48, 78) indicates that viral early genes were not able to fully override the block induced by butyrate and, perhaps, butyrate-mediated inhibition of viral replication is characteristic of papovaviruses.

In addition, butyrate inhibition of polyomavirus DNA replication is not cell type specific; a large variety of cell types, such as MEF, the immortal 3T6 cell line (Fig. 1), primary mouse keratinocytes, C2C12 myoblasts (myotube-forming precursor cells), 3T3-L1 adipocyte precursor cells, and primary

fibroblasts with homozygous null mutations for the p53 or Rb gene product all showed inhibition with comparable concentrations of butyrate (3 to 6 mM) (Fig. 2). The antiviral effect of butyrate has also been reported in primary culture of polyomavirus-infected mouse kidney cells (72). Butyrate is a generally effective cell cycle blocker, associated with a putative restriction point, related to termination of expression of a labile protein (13, 46, 77). Decreased p53 levels in correlation with butyrate treatment (29, 62, 77) and the inhibition of polyomavirus DNA replication in p53 and Rb knockout primary mouse fibroblasts in response to butyrate appear to rule out a direct involvement of these gene products individually in the mechanism of butyrate and imply that a putative control point perhaps lies at a later step in the cell cycle. It therefore appears that the butyrate effect is likely to involve a mechanism fundamentally conserved among cell types, but it does not appear to be exerted directly via the Rb or p53 gene product.

Our immunofluorescence analysis indicated that large-T-antigen expression was uncoupled from high-level polyomavirus DNA replication in butyrate-treated MEF and 3T6 host cells. This result confirms that T-antigen expression is not inhibited as a result of treatment with butyrate. Indeed, a comparison of the intensity of fluorescence relative to the low MOI suggests indirectly that T-antigen expression has increased. Similar observations have been reported for SV40-transformed WI-38 fibroblasts (29) and polyomavirus-infected mouse kidney cells (72). Butyrate treatment has been reported to result in the induction of the human CMV major immediate-early protein (IE1) by activating the IE1 promoter via cellular factors (65) in a human epithelial thyroid papillary carcinoma cell line. Similarly, EBV early antigen is induced by butyrate in the P3HR-1 cell line (3, 34, 37, 60, 64) as well as in Raji (2, 3, 34, 37) and NC37 (2, 37) cell lines. In addition, butyrate activates the long terminal repeat-directed expression of human immunodeficiency virus (8) and induces the murine sarcoma virus enhancer-promoter element (66). Therefore, butyrate appears associated with a general induction of early viral proteins.

Our results now extend the butyrate inhibition of viral DNA replication to the papillomavirus family (Fig. 3) and further demonstrate a persistent nature of this antiviral state. Papillomaviruses, like papovaviruses, rely heavily on the host DNA synthesis machinery for replication. They also lack viral DNA polymerase and chromatin proteins and utilize early viral proteins such as E6 (of HPV-16) to bind to and inhibit the negative transcriptional activity of p53 (47). That a butyrate-mediated cell cycle block also interfered with the acute papillomavirus DNA replication suggests that papillomaviruses and polyomaviruses have similar requirements with respect to cell cycle control. It was interesting that cells pretreated with either 3 or 9 mM butyrate demonstrated a stable form of antiviral state which lasted at least 96 h following the removal of butyrate. This observation seems similar to that reported for the butyrate induction of the Moloney murine sarcoma virus enhancer-promoter element; in this case, induced transcription was shown to be propagated from mother to daughter cells after withdrawal of the inducer (66). Although the mechanism of this stable butyrate effect is unknown, the inheritance of active transcription complexes or a modified chromatin structure (11, 74), possibly via butyrate-mediated acetylation of cellular histones (58), has been suggested (66). A persisting antiviral effect of butyrate could lead to novel therapeutic approaches against papillomavirus infections.

The replication of all of the larger DNA viruses that we examined (adenovirus and the HSV, EBV, and CMV herpesvirus family members) was not inhibited by butyrate. It there-

fore appears that these larger DNA viruses have the requisite biochemical machinery to bypass the cell cycle block and replicate in a butyrate-resistant manner. The genomes of these larger DNA viruses (including adenovirus) contain genes encoding a greater number of early proteins with roles in DNA replication, such as viral DNA polymerase, DNA-binding proteins, and helicases, and other nonessential or so-called redundant genes such as those encoding adenovirus E3 and E4 proteins. It is possible that these nonessential genes are important for virus replication in quiescent or differentiated cells and can compel infected cells to synthesize DNA outside the normal cell cycle control systems. Cellular DNA polymerases alpha and gamma, however, have been shown to be involved in the replication of adenovirus DNA (1, 39, 68). Yet our results indicate that in the permissive HeLa cells that have undergone butyrate treatment, adenovirus type 2 can replicate efficiently. In this case, the adenovirus early proteins and adenovirus DNA polymerase are clearly not redundant with host proteins and appear to be active in an alternate mode of initiation of DNA synthesis. However, others report that butyrate treatment did inhibit adenovirus replication in semipermissive 3T3 cells (33), suggesting the possible involvement of additional host factors in butyrate sensitivity. Our butyrate-treated E1A-expressing 293 cells also supported the replication of adenovirus type 5 mutants which lack the E1A region (deletions 309 and 327), so at least virus-encoded E1A is not required for butyrate-resistant replication of adenovirus.

The herpesvirus family members were also capable of bypassing the butyrate-mediated block, which is probably due to the role of viral early genes in DNA synthesis, such as the viral DNA polymerase, DNA-binding protein, and helicase genes. Herpesviruses also code for viral enzymes that contribute to the synthesis of DNA metabolites (56, 71). With HSV-1, these early genes are often not required for growth in cultured cells and include the viral gamma 1 34.5 (15), US2, US4, US5 (73), dUTPase, UL51, UL46, and UL47 (7) genes. Because adenovirus does not code for genes involved in the metabolism of DNA (such as the thymidine kinase gene) but was able to replicate in the presence of butyrate, we conclude that these virus-encoded metabolic enzymes are probably not involved in bypassing the butyrate block. Others have observed that the restriction of neuroblastoma cells to HSV replication can be reversed by butyrate treatment, implying an active role for the butyrate block in HSV replication (69). It now seems likely that a genetic analysis of the early HSV and adenovirus genes (particularly mutants of E3 or E4) could identify the specific viral genes needed to overcome a butyrate-blocked cell cycle. A similar analysis with HSV, EBV, and CMV, such as with the LMP2A (41) and CMV US10 and US11 (35) gene products, could identify the analogous gene for these viruses as well. Such an analysis might offer insights into the reports that butyrate can induce EBV to replicate in P3HR-1 (16, 36, 44, 59), Raji, Daudi, and B95-8 (44) cell lines as well as induce CMV to replicate in human epithelial papillary carcinoma cells (65) and in cultured endothelial cells (54) under conditions that are conducive to terminal differentiation (69). Butyrate has been reported to exert additional cytostatic effects such as G₂/M blockage and antiviral activity against RNA viruses (12, 20, 52, 53, 63). We have not examined the possible differences in chromatin structure between large and small DNA viruses in the presence and absence of butyrate to study the potential butyrate-mediated alterations of chromatin structure (33, 58) that might contribute to the observed differences in replication patterns among DNA viruses.

In summary, we have presented a comparative analysis of the replication sensitivities of various DNA viruses to a

butyrate-induced cell cycle block. Using simple yet clear methods, we have established viral replication cycle sensitivity to a cell cycle blocker. We have demonstrated that even though all of these DNA viruses must induce DNA synthesis in infected host cells, they do so in a manner that can be distinguished by a butyrate-induced block. The block by butyrate, therefore, appears to distinguish the life cycles of the small versus larger DNA virus families. The polyomaviruses and papillomaviruses, which have a distinct evolutionary conservation within their early regulatory genes (a p53-like binding/ATPase domain) and which seem to have coevolved with host genes (61), are sensitive to a butyrate-induced cell cycle block. We propose that such a dependence on cell cycle control may be related to the ability of these smaller viruses to establish low-level episodically maintained persistent infections of animal tissues (5, 50, 55).

ACKNOWLEDGMENTS

We thank Siddiqua Hirst for technical assistance in setting up the plaque assays, Isabella A. Atencio and Mariana del Vas for help in growing primary keratinocytes and C2C12 cells, respectively, and for comments on this work, and Ed Wagner and Zhiyong You for helpful suggestions regarding the manuscript.

This work was supported by National Institutes of Health grant GM36605, the Organized Research Unit on the Molecular Biology of Animal Viruses at the University of California, Irvine, the University of California Cancer Research Institute, and the Medical Scientist Program.

REFERENCES

- Abboud, M. M., and M. S. Horwitz. 1979. The DNA polymerases associated with the adenovirus type 2 replication complex: effect of 2'-3'-dideoxythymidine-5'-triphosphate on viral DNA synthesis. *Nucleic Acids Res.* **6**:1025-1039.
- Anisimova, E., K. Prachova, J. Roubal, and V. Vonka. 1984. Effects of n-butyrate and phorbol ester (TPA) on induction of Epstein-Barr virus antigens and cell differentiation. *Arch. Virol.* **81**:223-237.
- Anisimova, E., A. K. Saemundsen, J. Roubal, V. Vonka, and G. Klein. 1982. Effects of n-butyrate on Epstein-Barr virus-carrying lymphoma lines. *J. Gen. Virol.* **58**:163-171.
- Atencio, I. A. 1993. Polyomavirus replication in vivo and in tissue culture in response to host cell differentiation. Ph.D. thesis. University of California, Irvine.
- Atencio, I. A., F. F. Shadan, X. J. Zhou, N. D. Vaziri, and Luis P. Villarreal. 1993. Adult mouse kidneys become permissive to acute polyomavirus infection and reactivate persistent infections in response to cellular damage and regeneration. *J. Virol.* **67**:1424-1432.
- Balk, S., H. Gunther, and A. Morisi. 1984. Butyrate reversibly arrests the proliferation of normal and Rous sarcoma virus-infected chicken heart mesenchymal cells. *Life Sci.* **34**:803-808.
- Barker, D. E., and B. Roizman. 1990. Identification of three genes nonessential for growth in cell culture near the right terminus of the unique sequences of long component of herpes simplex virus 1. *Virology* **177**:684-691.
- Bohan, C., D. York, and A. Srinivasan. 1987. Sodium butyrate activates human immunodeficiency virus long terminal repeat-directed expression. *Biochem. Biophys. Res. Commun.* **148**:899-905.
- Botchan, M., L. Berg, J. Reynolds, and M. Lusky. 1986. The bovine papillomavirus replicon. *CIBA Found. Symp.* **120**:53-67.
- Braithwaite, A. W., H. W. Sturzbecher, C. Addison, C. Palmer, K. Rudge, and J. R. Jenkins. 1987. Mouse p53 inhibits SV40 origin-dependent DNA replication. *Nature (London)* **329**:458-460.
- Brown, D. D. 1984. The role of stable complexes that repress and activate eucaryotic genes. *Cell* **37**:359-365.
- Bruce, J. H., A. Ramirez, L. Lin, and R. P. Agarwal. 1992. Effects of cyclic AMP and butyrate on cell cycle, DNA, RNA, and purine synthesis of cultured astrocytes. *Neurochem. Res.* **17**:315-320.
- Campisi, J., E. E. Medrano, G. Morreo, and A. B. Pardee. 1982. Restriction point control of cell growth by a labile protein: evidence for increased stability in transformed cells. *Proc. Natl. Acad. Sci. USA* **79**:436-440.
- Charollais, R. H., C. Buquet, and J. Mester. 1990. Butyrate blocks the accumulation of CDC2 mRNA in late G1 phase but inhibits both the early and late G1 progression in chemically transformed mouse fibroblasts BP-A31. *J. Cell. Physiol.* **145**:46-52.
- Chou, J., E. R. Kern, R. J. Whitley, and B. Roizman. 1990. Mapping of herpes simplex virus-1 neurovirulence to γ_1 34.5, a gene nonessential for growth in culture. *Science* **250**:1262-1266.
- Contreras-Salazar, B., B. Ehlin-Henriksson, G. Klein, and M. G. Masucci. 1990. Up regulation of the Epstein-Barr virus (EBV)-encoded membrane protein LMP in the Burkitt's lymphoma line Daudi after exposure to n-butyrate and after EBV superinfection. *J. Virol.* **64**:5441-5447.
- Cooper, J. A., and P. Whyte. 1989. RB and the cell cycle: entrance or exit? *Cell* **58**:1009-1011.
- Daniell, E. 1980. Cells inhibited by n-butyrate support adenovirus replication. *Virology* **107**:514-519.
- Daniell, E., J. L. Burg, and M. J. Fedor. 1982. DNA and histone synthesis of butyrate-inhibited BSC-1 cells infected with SV40. *Virology* **116**:196-206.
- D'Anna, J. A., R. A. Tobey, and L. R. Gurley. 1980. Concentration-dependent effects of sodium butyrate in Chinese hamster cells: cell-cycle progression, inner-histone acetylation, histone H1 dephosphorylation, and induction of an H1-like protein. *Biochemistry* **19**:2656-2671.
- DeCaprio, J. A., J. W. Ludlow, J. Figge, J. Y. Shew, C. M. Huang, W. H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston. 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54**:275-283.
- DePolo, N. J., and L. P. Villarreal. 1993. Aphidicolin-resistant polyomavirus and subgenomic cellular DNA synthesis occur early in the differentiation of cultured myoblasts to myotubes. *J. Virol.* **67**:4169-4181.
- Deppert, W., and T. Steinmayer. 1989. Metabolic stabilization of p53 in SV40-transformed cells correlates with expression of the transformed phenotype but is independent from complex formation with SV40 large T antigen. *Curr. Top. Microbiol. Immunol.* **144**:77-83.
- Deppert, W., T. Steinmayer, and W. Richter. 1989. Cooperation of SV40 large T antigen and the cellular protein p53 in maintenance of cell transformation. *Oncogene* **4**:1103-1110.
- Fakhrazadeh, S. S., S. P. Trusko, and D. L. George. 1991. Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. *EMBO J.* **10**:1565-1569.
- Farmer, G., J. Bargonetti, H. Zhu, P. Friedman, R. Prywes, and C. Prives. 1992. Wild-type p53 activates transcription in vitro. *Nature (London)* **358**:83-86. (Comment.)
- Felsani, A., R. Maione, L. Ricci, and P. Amati. 1985. Coordinate expression of myogenic functions and polyoma virus replication. *Cold Spring Harbor Symp. Quant. Biol.* **50**:753-757.
- Gannon, J. V., and D. P. Lane. 1987. p53 and DNA polymerase alpha compete for binding to SV40 T antigen. *Nature (London)* **329**:456-458.
- Goldberg, Y. P., V. D. Leaner, and M. I. Parker. 1992. Elevation of large-T antigen production by sodium butyrate treatment of SV40-transformed WI-38 fibroblasts. *J. Cell. Biochem.* **49**:74-81.
- Hiebert, S. W., S. P. Chellappan, J. M. Horowitz, and J. R. Nevins. 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. *Genes Dev.* **6**:177-185.
- Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.
- Howard, M. A., S. Wardwell, and D. M. Albert. 1991. Effect of butyrate and corticosteroids on retinoblastoma in vitro and in vivo. *Invest. Ophthalmol. Visual Sci.* **32**:1711-1713.
- Iseki, S., and R. Baserga. 1983. Effect of butyrate on adenovirus infection in semipermissive cells. *Virology* **124**:188-191.
- Ito, Y., M. Kawanishi, T. Harayama, and S. Takabayashi. 1981. Combined effect of the extracts from Croton tiglium, Euphorbia

- lathyrus or Euphorbia tirucalli and *n*-butyrate on Epstein-Barr virus expression in human lymphoblastoid P3HR-1 and Raji cells. *Cancer Lett.* **12**:175–180.
35. Jones, T. R., V. P. Muzithras, and Y. Gluzman. 1991. Replacement mutagenesis of the human cytomegalovirus genome: US10 and US11 gene products are nonessential. *J. Virol.* **65**:5860–5872.
 36. Kallin, B., J. Luka, and G. Klein. 1979. Immunochemical characterization of Epstein-Barr virus-associated early and late antigens in *n*-butyrate-treated P3HR-1 cells. *J. Virol.* **32**:710–716.
 37. Kawanishi, M., K. Sugawara, and Y. Ito. 1981. Epstein-Barr virus-induced early polypeptides in Raji and NC37 cells activated by diterpene ester TPA in combination with *N*-butyrate. *Virology* **115**:406–409.
 38. Kawasaki, S., L. Diamond, and R. Baserga. 1981. Induction of cellular deoxyribonucleic acid synthesis in butyrate-treated cells by simian virus 40 deoxyribonucleic acid. *Mol. Cell. Biol.* **1**:1038–1047.
 39. Krokan, H., P. Schaffer, and M. L. DePamphilis. 1979. Involvement of eucaryotic deoxyribonucleic acid polymerases α and γ in the replication of cellular and viral deoxyribonucleic acid. *Biochemistry* **18**:4431–4443.
 40. Levine, A. J., J. Momand, and C. A. Finlay. 1991. The p53 tumour suppressor gene. *Nature (London)* **351**:453–456.
 41. Longnecker, R., C. L. Miller, X. Q. Miao, A. Marchini, and E. Kieff. 1992. The only domain which distinguishes Epstein-Barr virus latent membrane protein 2A (LMP2A) from LMP2B is dispensable for lymphocyte infection and growth transformation in vitro; LMP2A is therefore nonessential. *J. Virol.* **66**:6461–6469.
 42. Ludlow, J. W., J. A. DeCaprio, C. M. Huang, W. H. Lee, E. Paucha, and D. M. Livingston. 1989. SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. *Cell* **56**:57–65.
 43. Ludlow, J. W., J. Shon, J. M. Pipas, D. M. Livingston, and J. A. DeCaprio. 1990. The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T. *Cell* **60**:387–396.
 44. Luka, J., B. Kallin, and G. Klein. 1979. Induction of the Epstein-Barr virus (EBV) cycle in latently infected cells by *n*-butyrate. *Virology* **94**:228–231.
 45. Martinez, J., I. Georgoff, J. Martinez, and A. J. Levine. 1991. Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. *Genes Dev.* **5**:151–159.
 46. Medrano, E. E., and A. B. Pardee. 1980. Prevalent deficiency in tumor cells of cycloheximide-induced cycle arrest. *Proc. Natl. Acad. Sci. USA* **77**:4123–4126.
 47. Mietz, J. A., T. Unger, J. M. Huibregtse, and P. M. Howley. 1992. The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. *EMBO J.* **11**:5013–5020.
 48. Mitsudomi, T., and G. Kimura. 1985. Effects of sodium *n*-butyrate on entry into S phase in resting rat 3Y1 cells infected with simian virus 40. *J. Virol.* **56**:951–957.
 49. Momand, J., G. P. Zambetti, D. C. Olson, D. George, and A. J. Levine. 1992. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69**:1237–1245.
 50. Moreno, J. P., and L. Villarreal. 1992. Analysis of cellular DNA synthesis during polyoma virus infection of mice: acute infection fails to induce cellular DNA synthesis. *Virology* **186**:463–474.
 51. Perry, M. E., and A. J. Levine. 1993. Tumor-suppressor p53 and the cell cycle. *Curr. Opin. Genet. Dev.* **3**:50–54.
 52. Pouillart, P., I. Cerutti, G. Ronco, P. Villa, and C. Chany. 1992. Enhancement by stable butyrate derivatives of antitumor and antiviral actions of interferon. *Int. J. Cancer* **51**:596–601.
 53. Pouillart, P., I. Cerutti, G. Ronco, P. Villa, and C. Chany. 1992. Protective activity of monoacetone glucose 3-butyrate, a prodrug of *n*-butyric acid, against the fatal effect of encephalomyocarditis virus infection in mice. *C. R. Acad. Sci. III* **314**:49–54. (In French.)
 54. Radsak, K., R. Fuhrmann, R. P. Franke, D. Schneider, A. Kollert, K. H. Brucher, and D. Drenckhahn. 1989. Induction by sodium butyrate of cytomegalovirus replication in human endothelial cells. *Arch. Virol.* **107**:151–158.
 55. Rochford, R., J. P. Moreno, M. L. Peake, and L. P. Villarreal. 1992. Enhancer dependence of polyomavirus persistence in mouse kidneys. *J. Virol.* **66**:3287–3297.
 56. Roizman, B. 1990. Herpesviridae: a brief introduction, p. 1787–1794. In B. N. Fields and D. M. Knipe (ed.), *Fields virology*, vol. 2. Raven Press, New York.
 57. Roizman, B., and A. E. Sears. 1990. Herpes simplex viruses and their replication, p. 1795–1842. In B. N. Fields and D. M. Knipe (ed.), *Fields virology*, vol. 2. Raven Press, New York.
 58. Roman, A. 1982. Alteration in the simian virus 40 maturation pathway after butyrate-induced hyperacetylation of histones. *J. Virol.* **44**:958–962.
 59. Roubal, J., J. Luka, and G. Klein. 1980. Effect of retinoic acid (RA) on the Epstein-Barr virus (EBV)-inducing effect of sodium butyrate. *Cancer Lett.* **8**:209–212.
 60. Saemundsen, A. K., B. Kallin, and G. Klein. 1980. Effect of *n*-butyrate on cellular and viral DNA synthesis in cells latently infected with Epstein-Barr virus. *Virology* **107**:557–561.
 61. Shadan, F. F., and L. P. Villarreal. 1993. Coevolution of persistently infecting small DNA viruses and their hosts linked to host-interactive regulatory domains. *Proc. Natl. Acad. Sci. USA* **90**:4117–4121.
 62. Shen, D. W., F. X. Real, A. B. DeLeo, L. J. Old, P. A. Marks, and R. A. Rifkind. 1983. Protein p53 and inducer-mediated erythroleukemia cell commitment to terminal cell division. *Proc. Natl. Acad. Sci. USA* **80**:5919–5922.
 63. Staiano-Coico, L., M. Steinberg, and P. J. Higgins. 1990. Epidermal cell-shape regulation and subpopulation kinetics during butyrate-induced terminal maturation of normal and SV40-transformed human keratinocytes: epithelial models of differentiation therapy. *Int. J. Cancer* **46**:733–738.
 64. Sugawara, K., M. Kawanishi, and Y. Ito. 1982. Epstein-Barr virus-related DNA-binding proteins induced by *n*-butyrate in P3HR-1 cells. *Virology* **116**:354–358.
 65. Tanaka, J., H. Sadanari, H. Sato, and S. Fukuda. 1991. Sodium butyrate-inducible replication of human cytomegalovirus in a human epithelial cell line. *Virology* **185**:271–280.
 66. Tang, D. C., and M. W. Taylor. 1992. Memory of butyrate induction by the Moloney murine sarcoma virus enhancer-promoter element. *Biochem. Biophys. Res. Commun.* **189**:141–147.
 67. Toscani, A., D. R. Soprano, and K. J. Soprano. 1990. Sodium butyrate in combination with insulin or dexamethasone can terminally differentiate actively proliferating Swiss 3T3 cells into adipocytes. *J. Biol. Chem.* **265**:5722–5730.
 68. van der Vliet, P. C., and M. M. Kwant. 1978. Role of DNA polymerase γ in adenovirus DNA replication. *Nature (London)* **276**:532–534.
 69. Villarreal, L. P. 1991. Relationship of eukaryotic DNA replication to committed gene expression: general theory for gene control. *Microbiol. Rev.* **55**:512–542.
 70. Villarreal, L. P., and H. Fan. 1989. History and biological strategy of polyomavirus, adenovirus, and papillomavirus, p. 1–17. In L. P. Villarreal (ed.), *Common mechanisms of transformation by small DNA tumor viruses*. American Society for Microbiology, Washington, D.C.
 71. Wagner, E. K. 1991. Herpesvirus transcription—general aspects, p. 1–15. In E. K. Wagner (ed.), *Herpesvirus transcription and its regulation*. CRC Press, Boca Raton, Fla.
 72. Wawra, E., E. Pockl, E. Müllner, and E. Wintersberger. 1981. Effect of sodium butyrate on induction of cellular and viral DNA syntheses in polyoma virus-infected mouse kidney cells. *J. Virol.* **38**:973–981.
 73. Weber, P. C., M. Levine, and J. C. Glorioso. 1987. Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn5 mutagenesis. *Science* **236**:576–579.
 74. Weintraub, H. 1985. Assembly and propagation of repressed and depressed chromosomal states. *Cell* **42**:705–711.
 75. Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow. 1988. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature (London)* **334**:124–129.
 76. Wintersberger, E. 1991. Biochemical events controlling initiation

- and propagation of the S phase of the cell cycle. *Rev. Physiol. Biochem. Pharmacol.* **118**:49–95.
77. **Wintersberger, E., and I. Mudrak.** 1984. Sodium butyrate inhibits the synthesis of the transformation related protein p53 in 3T6 mouse fibroblasts. *FEBS Lett.* **166**:326–330.
78. **Wintersberger, E., I. Mudrak, and U. Wintersberger.** 1983. Butyrate inhibits mouse fibroblasts at a control point in the G1 phase. *J. Cell. Biochem.* **21**:239–247.
79. **Yaffe, D., and O. Saxel.** 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature (London)* **270**:725–727.
80. **Yew, P. R., and A. J. Berk.** 1992. Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature (London)* **357**:82–85.