Stimulus Duration and Response Time Independently Influence the Kinetics of Lytic Cycle Reactivation of Epstein-Barr Virus[▽]

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Epstein-Barr virus (EBV) can be reactivated from latency into the lytic cycle by many stimuli believed to operate by different mechanisms. Cell lines containing EBV differ in their responses to inducing stimuli, yet all stimuli require *de novo* protein synthesis (44). A crucial step preliminary to identifying these proteins and determining when they are required is to measure the duration of stimulus and response time needed for activation of expression of EBV BRLF1 and BZLF1, the earliest viral indicators of reactivation. Here we show, with four EBV-containing cell lines that respond to different inducing agents, that stimuli that are effective at reactivating EBV can be divided into two main groups. The histone deacetylase inhibitors sodium butyrate and trichostatin A require a relatively long period of exposure, from 2 to 4 h or longer. Phorbol esters, anti-immunoglobulin G (anti-IgG), and, surprisingly, 5-aza-2'-deoxycytidine require short exposures of 15 min or less. The cell/virus background influences the response time. Expression of the EBV BZLF1 and BRLF1 genes can be detected before 2 h in Akata cells treated with anti-IgG, but both long- and short-duration stimuli required 4 or more hr to activate BZLF1 and BRLF1 expression in HH514-16, Raji, or B95-8 cells. Thus, stimulus duration and response time are independent variables. Neither stimulus duration nor response time can be predicted by the number of cells activated into the lytic cycle. These experiments shed new light on the earliest events leading to lytic cycle reactivation of EBV.

Oncogenic human herpesviruses, such as Epstein-Barr virus (EBV), manifest two distinct lifestyles: latency, a state of limited viral gene expression, and lytic replication, which ultimately leads to production of virions. The switch between latency and productive lytic infection can be manipulated in cell culture. Lymphoid cell lines are unique experimental systems with which to study physiologic and molecular mechanisms underlying the transition between the two life cycles. The switch between viral latency and lytic replication is a biologically interesting and potentially tractable example of the combinatorial control of eukaryotic gene expression. Groups of viral and cellular effector molecules, some of which are transcription factors, exert both positive and negative control on the expression and the activity of two virally encoded proteins, ZEBRA and Rta, both of which act as transcription factors and replication proteins. Epigenetic control of viral and cellular gene expression, through chromatinization and DNA methylation, may also play roles in the latency-to-lytic cycle transition. The latency-to-lytic cycle switch has obvious implications for pathogenesis. While latency may be the predominant state of the life cycle in cellular reservoirs and in virus-associated cancers, the viruses must replicate lytically in order to be transmitted between cells and among individuals. Manipulation of the latency-to-lytic cycle switch has been investigated as a potential oncolytic strategy (11, 28, 36, 43).

The latency-to-lytic switch can be envisioned to be com-

posed of two distinct complex sets of events: upstream events lead to the expression of the EBV lytic cycle activator genes BZLF1 and BRLF1, which encode ZEBRA and Rta, and downstream events involve the effects of ZEBRA and Rta and their target genes on viral and cellular gene expression, DNA replication, and viral and cellular behavior in general.

Upstream events can be initiated in cell culture by the addition of certain inducing stimuli which presumably mimic as yet poorly characterized physiologic stimuli that trigger the latency-to-lytic cycle switch *in vivo*. A partial list of stimuli that can activate the latency-to-lytic switch in cultured B-cell lines includes phorbol esters (47), which are protein kinase C (PKC) agonists; sodium butyrate (NaB) (26) and trichostatin A (TSA) (46), which are histone deacetylase inhibitors (HDACi); 5-aza-2'-deoxycytidine (AzaCdR) (4), which is a DNA methyltransferase inhibitor; and anti-immunoglobulin G (anti-IgG), which activates the B-cell antigen receptor (40).

Inspection of this list of inducing stimuli, which are thought to operate by different modes of action, leads to the conclusion that upstream events are likely to activate different pathways which lead to BZLF1 and BRLF1 expression. These pathways may or may not converge on a final common event. Further complexity in understanding the upstream events is evident from the observation that not all cell/virus systems respond to the same inducing stimuli (Table 1). For example, the EBV lytic cycle in HH514-16 cells, a clonal derivative of a Burkitt lymphoma cell line, is activated by HDACi and AzaCdR but not by phorbol-12-myristate-13-acetate (TPA) (17). TPA activates the EBV lytic cycle in B95-8 cells, a cotton-top tamarin lymphoblastoid cell line, but HDACi do not activate the lytic cycle in this cell background; they inhibit the effect of TPA (9).

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TABLE 1. Cell lines and response to inducing stimuli

Cell line	Origin ^a	Response to EBV lytic cycle-inducing stimulus:			
		HDACi	AzaCdR	TPA	Anti-IgG
HH514-16	BL	+	+	—	—
B95-8	LCL	—	—	+	—
Raji	BL	S ^b	—	+	—
Akata	BL	—	+/-	—	+

^a BL, Burkitt lymphoma; LCL, lymphoblastoid cell line (cotton-top marmoset).

^b S, in Raji cells HDACi are synergistic with TPA.

The EBV lytic cycle in Raji cells can be activated by TPA; while the HDACi are inactive by themselves, they are synergistic with TPA (9). In Akata cells anti-IgG strongly activates and AzaCdR weakly activates the EBV lytic cycle. Neither HDACi nor TPA activates the lytic cycle in the Akata cell background (see Table 1).

Even in a responsive cell line an inducing stimulus activates the lytic cycle in only a subpopulation of cells (17). The cells refractory to lytic induction are not permanently marked for the lack of lytic cycle response but can be induced after they have been returned to culture for several weeks (5).

Our laboratory has been attempting to understand the cell line-specific behavior of lytic cycle-inducing agents in an effort to identify events that are common or different among the lytic induction pathways. In one series of experiments, we compared the levels of PKC in cell lines that were susceptible (e.g., B95-8) and in those which were refractory (e.g., HH514-16, W91, or FF41) to lytic cycle induction by TPA, a protein kinase cell agonist (17). PKC had been assumed to play an essential role in the initiation of the lytic cascade of EBV (13). We found that TPA induced PKC activity in all cell backgrounds, a finding which suggested that the lack of a lytic response to TPA was not the result of a failure of TPA to activate PKC. The variable role of the PKC pathway in induction of the lytic cycle in responsive or refractory cell lines could not be accounted for by polymorphisms in Zp or Rp, the promoters of the BZLF1 and BRLF1 genes; by the start sites of transcription of these genes; or by differences in the nucleosomal organization of Zp or Rp (17). From those experiments we concluded that activation of PKC was not by itself a sufficient stimulus for activation of the EBV lytic cascade.

In more recent experiments we studied cell lines that were refractory or susceptible to lytic cycle induction by HDACi (9). It has been proposed that Zp and Rp are repressed by chromatin and that HDACi, by increasing hyperacetylation of histone tails, would open chromatin and allow access of positively acting factors that would lead to transcription of BZLF1 and BRLF1 (7, 18, 21). We found, using chromatin immunoprecipitation, that two HDACi, NaB and TSA, caused hyperacetylation of histones H3 and H4 on Zp and Rp in cell lines, such as Raji and B95-8, which are refractory to EBV lytic cycle induction by these agents, as well as in HH514-16 cells, which are responsive. However, valproic acid, another HDACi, induced hyperacetylation of H3 in both susceptible (HH514-16) and refractory (B95-8, Raji) cell lines but failed to induce the lytic cycle. From these studies we concluded that open chro-

matin at the EBV BZLF1 and BRLF1 promoters was not sufficient to activate EBV lytic cycle gene expression.

New protein synthesis appears to be required for EBV lytic cycle induction following application of all of the lytic cycle-inducing stimuli that we have studied and in all cell backgrounds (44). This conclusion is based on experiments with cycloheximide (CHX), an inhibitor of protein synthesis, which blocks expression of BRLF1 and BZLF1 mRNAs after application of an inducing stimulus. In HH514-16 cells, the new proteins are made by 6 h after application of HDACi and by 4 h after treatment with AzaCdR. Addition of CHX after these times does not inhibit, but enhances, BRLF1 or BZLF1 expression. In B95-8 cells, the new proteins are made by 4 h after treatment with TPA. From these experiments we can hypothesize that an EBV lytic response is dependent on one or more proteins to be newly synthesized between 4 and 6 h after application of an inducing stimulus to HH514-16 and B95-8 cells.

A current long-term goal is to identify the newly synthesized proteins, presumably cellular in origin, that play a role in EBV lytic induction by various stimuli. The experiments described in this report address an essential preliminary goal, namely, determining how long the inducing stimulus must be present and how soon after application of the inducing stimulus BZLF1 and BRLF1 mRNAs and ZEBRA and Rta proteins can be detected. The cellular proteins that play a role in lytic cycle induction should be kinetically upstream of expression of the virally encoded lytic cycle activator genes. From the experiments we describe in this report, we draw three conclusions. (i) The stimuli can be classified into two groups: those such as TPA, anti-IgG, and AzaCdR, which can act after an exposure of short duration (<15 min), and those such as the HDACi NaB and TSA, which require a longer duration of exposure (2 to 8 h or longer). (ii) The response time differs dramatically among cell lines but is independent of stimulus duration. In Akata cells the response is rapid; BZLF1 and BRLF1 mRNAs can be detected within 1.5 h. In HH514-16, B95-8, and Raji cells the response time is slow, between 4 and 6 h. (iii) Neither stimulus duration nor response time can be predicted by the number of cells induced into the EBV lytic cycle.

MATERIALS AND METHODS

Cell lines. The EBV-infected cell lines used in this study were HH514-16, a subclone of the P3J-HRIK Burkitt lymphoma cell line (20); B95-8, a lymphoblastoid cell line derived by in vitro infection of cotton-top marmoset lymphocytes with EBV (30); and the Raji (10) and Akata Burkitt lymphoma cell lines (41). Akata cells were kindly supplied by Kenzo Takada. Cell lines were cultured in RPMI 1640 supplemented with 8% fetal bovine serum. Cell cultures were treated with penicillin (50 U/ml), streptomycin (50 U/ml), and amphotericin B (1 µg/ml). Cells were grown at 37°C under 5% CO₂.

EBV lytic cycle induction. Cell lines in logarithmic-phase growth, usually at 48 h after the last subculture, were resuspended at 10⁶/ml in fresh medium and treated with lytic cycle-inducing chemicals at doses previously determined to be in the optimal range for a maximal response. The inducing chemicals were NaB (Sigma no. B5887), used at 3 mM; TSA (WAKO no. 204-11991), used at 5 µM; AzaCdR (Sigma no. 3656), used at 5 µM; TPA (Calbiochem no. 524400), used at 20 ng/ml; and rabbit anti-human IgG (anti-IgG) (Dako no. A042301-2), used at 7.5 µg/ml.

Detection of BZLF1 and BRLF1 mRNAs. The procedures for preparation of total cellular RNA, electrophoresis, transfer to nylon membranes, and preparation of a ³²P-radiolabeled probe Z(301), which detects both BRLF1 and BZLF1 mRNAs and was used for Northern blotting, have recently been described (44). Similarly, the procedures for preparation of RNA, reverse transcription, and

quantitation by real-time PCR have been described previously (44). The internal control for Northern blots was a probe for H1 RNA of RNase P (3). The primer pair which spans an intron for detection of BRLF1 mRNA has been described previously (44). The primer pair for detection of BZLF1 mRNA, which spans the second intron, is 5'TACAAGAATCGGGTGGCTTC and 5'GCACATCTGCTTCAACAGGA.

Detection of EBV lytic cycle polypeptides. The procedures used in immunoblotting for preparation of cell extracts, transfer of polypeptides to nitrocellulose, exposure to antibodies, detection of bound antibodies by 125 I-labeled protein A, and autoradiography have been described previously (9). Rabbit polyclonal antibodies to EBV Rta (35), ZEBRA (42), BLRF2 (37), and BFRF3 (23) have been described previously. A polyclonal antibody to EBV DNA polymerase (BALF5) was produced in rabbits immunized with a polypeptide fragment encompassing amino acids 331 to 663, expressed in *Escherichia coli*, and purified by Ni^{2+} affinity chromatography. EBV BMRF1 (EA-D) was detected with mouse monoclonal antibody R3.1 and a rabbit anti-mouse Ig bridge (33). Mouse antibody to β -actin (A5136; Sigma) was used to control for protein loading.

Detection of lytically induced cells by a FACS-based assay. Lytically induced HH514-16 or B95-8 cells were detected with a fluorescence-activated cell sorter (FACS)-based method using human antibodies (5). Briefly, 0.5×10^6 to 1×10^6 cells were fixed and permeabilized using BD Cytotfix/Cytoperm (BD Biosciences). Cells were incubated with a 1:10 dilution of a reference EBV-seropositive serum or a reference EBV-seronegative serum in the presence of 1 mg/ml polyclonal mouse IgG to minimize nonspecific binding of IgG antibodies in human sera via their Fc protein. Cells were washed three times. Bound human IgG antibodies were detected by incubation of cells with a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-human IgG (F3512; Sigma). A FACS-based assay was used to detect Akata cells expressing EA-D. Cells were incubated with a 1:25 dilution of R3.1 monoclonal antibody or mouse IgG2a isotype control antibody in the presence of polyclonal mouse IgG. Bound antibody was detected with a 1:300 dilution of phycoerythrin-conjugated anti-mouse Ig (550589; BD Pharmingen). Labeled cells were analyzed using a FACSCalibur instrument. Live cells were analyzed based on forward- and side-scatter profiles. The percentage of lytically induced HH514-16 or B95-8 cells was determined by pairwise comparison of cells incubated with reference EBV antibody-positive and EBV antibody-negative human sera. The percentage of lytically induced Akata cells was determined by pairwise comparison of cells incubated with R3.1 and identically treated cells incubated with isotype control antibody.

Southern blotting. The procedures for isolation of total cellular DNA, digestion with BamHI, transfer to nitrocellulose filters, preparation of ^{32}P -radiolabeled probes, hybridization, and detection of radioactive DNA fragments have been described previously (19). Probes were a 300-nucleotide subfragment of EBV XhoI.9, used to detect the EBV terminal repeats (34), and EBV BamHI W, used to detect the internal repeats (2, 16).

RESULTS

Overview of experiments. The duration of stimulus and the time required for detection of a response of EBV lytic gene expression were studied in four cell lines that differ in their susceptibility to inducing stimuli (Table 1). These cell lines were HH514-16 (see Fig. 1 to 6), B95-8 (see Fig. 7 and 8), Raji (see Fig. 9), and Akata (see Fig. 10 and 11). The duration of stimulus required for activation of BRLF1 and BZLF1 mRNA expression was measured in experiments in which the inducing stimulus was removed by washing (see, e.g., Fig. 4, 7, 9, and 10). Several parameters were used to measure the EBV lytic gene response. These included, in addition to detection of expression of BRLF1 and BZLF1 mRNAs, expression of Rta, ZEBRA, EA-D, BALF5, and BFRF3 polypeptides; lytic EBV DNA replication (see Fig. 3 and 8); and determination by FACS of the number of cells activated into the EBV lytic cycle (see Fig. 1A, 8C and D, and Fig. 11A). The response time was considered to be the time after application of an inducing stimulus when one of these parameters of EBV lytic gene expression was above the background level for untreated cells studied simultaneously.

Kinetics of EBV lytic gene expression in HH514-16 cells treated with HDACi. Figure 1 presents three different assays that were used to determine the time of appearance of lytic gene expression in HH514-16 cells treated continuously with HDACi. In one assay (Fig. 1A) the cells were assessed for lytic activation using FACS. Fewer than 1 in 1,000 HH514-16 cells spontaneously expressed lytic antigens detectable with human antibodies. About 1% of cells treated with NaB expressed the antigens by 4 h; this fraction rose gradually to about 3% at 7 h, 8% at 12 h, and a maximum level of about 30% lytically induced cells evident by 24 h. The identity of the antigens detected by the human antibodies is not known, but they do not require lytic viral DNA synthesis, since approximately the same fraction of lytic cells was detected in the absence and presence of phosphonoacetic acid.

When expression of EBV lytic polypeptides after treatment with TSA was examined by immunoblotting (Fig. 1B), the kinetics were generally similar to those seen in the FACS-based assay. It can be seen that Rta protein was about 2- to 3-fold above background at 4 and 8 h; this rose gradually to 8- and 10-fold above background at 8 and 10 h and 30-fold above background at 12 h. ZEBRA was first detected above background levels at 6 and 8 h and then was more significantly induced at 10 h and 12 h.

In Fig. 1C the readout of EBV lytic gene expression was the appearance of EBV BRLF1 mRNA, as detected by quantitative reverse transcription-PCR (qRT-PCR). A signal approximately threefold that for untreated cells was first detected at 6.5 h after treatment with TSA, and a signal fourfold that of untreated cells was detected at 8 h after TSA treatment. Although there are obvious differences in the sensitivities of these three different assays, they all demonstrate that HDACi induce EBV lytic gene expression relatively slowly and gradually in HH514-16 cells.

Both duration of stimulus and response time influence the kinetics of EBV lytic antigen expression in HH514-16 cells treated with NaB. The kinetics of the lytic viral gene response illustrated in Fig. 1 could be influenced by at least four factors: the cell/virus background, the nature of the inducing stimulus, the duration of exposure to the inducing stimulus, and the time required for a detectable response to the inducing agent. Figure 2 illustrates two early experiments that attempted to differentiate stimulus duration and response time after treatment with NaB. Figure 2A compares Rta and ZEBRA expression following exposure to NaB for four durations, i.e., 4, 8, 18, and 24 h. At each time the cells were washed and returned to culture. If one examines the response when the cells were harvested at 24 h (lanes 6, 8, 10, and 11), it can be seen that a 4-h exposure to NaB leads to low levels of expression of Rta and ZEBRA (lane 6), whereas an 8-h exposure leads to much higher levels of expression of Rta and ZEBRA (lane 8). Increasing the duration of exposure to NaB to more than 8 h did not increase the response at 24 h (lanes 10 and 11). The experiment demonstrates that a 4-h duration of exposure to NaB does not produce a maximal response. The experiment also demonstrates that while an 8-h exposure to NaB is adequate to produce a marked response at 24 h, it is not sufficient to lead to a detectable response when the cells are harvested at 8 h (compare lanes 7 and 8). This experiment was an early

FIG. 1. Kinetics of EBV lytic gene expression in HH514-16 cells treated with HDACi. (A) FACS analysis of lytic antigen appearance in HH514-16 cells following treatment with 3 mM NaB either without or with phosphonoacetic acid (PAA) for the indicated lengths of time. Cells at each time point were fixed, permeabilized, and incubated with EBV antibody-positive or -negative human reference sera, followed by a subsequent incubation with fluorescein isothiocyanate-conjugated goat anti-human IgG. The percentage of lytic cells was obtained from the number of cells detected by the EBV-positive serum minus the number detected by the EBV-negative serum (5). (B) Western Blot of HH514-16 cells treated with TSA for the indicated lengths of time. The immunoblot was probed sequentially with rabbit polyclonal antisera generated against ZEBRA and Rta and with a mouse monoclonal antibody to β -actin as an internal loading control. S.I., stimulation index (densitometer units of TSA-treated cells/densitometer units of untreated cells) at the same time point, corrected for β -actin. (C) Kinetics of appearance of EBV BRLF1 mRNA. TSA-treated and control cells were harvested at the indicated times, RNA was prepared, and BRLF1 mRNA was measured by qRT-PCR. The stimulation index is the relative amount of BRLF1 mRNA present in TSA-treated cells compared to that in untreated cells at the same time point.

indication that stimulus duration and response time were independent variables.

The experiment illustrated in Fig. 2B measures the response time when the duration of stimulus with NaB was held constant

at 8 h. Both Rta and ZEBRA were weakly induced at 8 h, but higher levels of Rta and ZEBRA were present when the cells were assessed at 12 to 24 h. The experiment illustrates two other features of the lytic response after an 8-h exposure to

FIG. 2. Duration of exposure to NaB required for detection of a lytic antigen response in HH514-16 cells. (A) HH514-16 cells were untreated (lanes 1 to 4) or treated for 4, 8, 18, or 24 h with NaB (lanes 5 to 11), after which the cells were washed, further incubated in the absence of NaB, and harvested at the indicated times. The boxed area (lanes 6 through 8) compares the response at 24 h for stimulus durations of 4 and 8 h; it also compares the response times of 8 h and 24 h after a stimulus of 8 h. (B) HH514-16 cells were untreated (lanes 1 and 2) or treated with NaB for 8 h (lanes 3 to 9), after which the cells were washed, incubated in the absence of NaB, and harvested at the indicated times. A total of 1×10^6 cells were loaded in each lane of a 10% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed, and transferred to nitrocellulose. Immunoblots were probed sequentially with polyclonal rabbit sera against ZEBRA, Rta, LR2, and a mouse monoclonal antibody directed against β -actin.

NaB. (i) Between 12 and 24 h there was a significant decrease in the electrophoretic mobility of Rta (Fig. 2B, lanes 7 to 9), which suggests that Rta was being progressively modified. (ii) While there was a notable increase in the amounts of Rta and ZEBRA observed between 10 and 12 h after the start of the experiment, a late gene, BLRF2, was not detectable until 16 h and was at the maximum level at 20 h. This result shows that the response time is influenced by the kinetic class of the lytic protein being examined.

Duration of exposure to NaB and response time for detection of lytic EBV DNA synthesis and late gene expression in HH514-16 cells. The data in Fig. 2 can be interpreted to show that an 8-h exposure to NaB gives rise to a near-maximal

response of ZEBRA, when assessed at 20 to 24 h. The experiment in Fig. 3 shows that an NaB exposure time of 8 h and response time of 24 h were not maximal for detecting lytic EBV DNA replication. Whether the NaB stimulus was present continuously (lanes 3 to 5), or present for 8, 16, or 24 h, near-maximal lytic DNA replication was not observed until 48 h or later. Moreover, an 8-h stimulus with NaB was not as potent as a 16-h stimulus for detecting lytic DNA replication (Fig. 3, compare lanes 7 and 8 and lanes 10 and 11). In cells treated with NaB, only minimal expression of BFRF3 polypeptide was evident at 24 h, and maximal levels of BFRF3 polypeptide were present at 48 h and 72 h. This was true whether or not NaB was removed at 16 h or 24 h (data not

FIG. 3. Duration of exposure and response time required for detection of EBV lytic DNA replication following treatment of HH514-16 cells with NaB. Southern blot analysis of total cellular DNA isolated following treatment with NaB is shown. Cells were not washed (lanes 1 to 5) or washed (lanes 6 to 14), and DNA was harvested at times indicated after exposure to NaB. Ten micrograms of DNA was digested with BamHI, loaded onto a 0.8% agarose gel, and electrophoresed in Tris-borate-EDTA. Following transfer to nitrocellulose, the Southern blot filter was probed with radiolabeled Xho300 fragment, which detects the terminal repeat region, and with an excised BamHI W fragment to assess the level of DNA replication. FT, fused termini; TR, terminal repeat fragments; W, BamHI W fragment.

shown). Therefore, among the variables that affect stimulus duration and response time is the nature of the events being measured. A longer exposure to NaB was required for detection of DNA replication (Fig. 3) than for expression of the ZEBRA and Rta proteins (Fig. 2).

Comparison of the durations of exposure to HDACi and AzaCdR required to activate BRLF1 and BZLF1 mRNAs in HH514-16 cells. Preliminary experiments using Northern blotting (9) or qRT-PCR, (Fig. 1C) showed that following continuous exposure to HDACi, the EBV BZLF1 and BRLF1 mRNAs could be detected at between 6 and 8 h. We asked whether the duration of exposure required for detection of BZLF1 and BRLF1 mRNAs differed for inducing stimuli that were thought to operate via distinct mechanisms. A stimulus of NaB or TSA for 4 h or more was required to activate the mRNAs when mRNAs were measured at 8 h. For TSA, in particular, increasing the duration of the stimulus from 4 to 6 h increased the response two- to threefold (Fig. 4A, compare lanes 3 and 4). In striking contrast, a 1-hour exposure to AzaCdR induced significant expression of the BRLF1 and BZLF1 mRNAs (Fig. 4A, lane 6); a 4-h exposure was maximal (Fig. 4B and C). These experiments were the first to suggest that in the same cell background, the duration of stimulus exposure required to activate BRLF1 and BZLF1 was dependent on the nature of the stimulus itself.

For lytic activation in HH514-16 cells, a brief exposure to AzaCdR suffices. In an experiment in which BRLF1 and BZLF1 mRNAs were measured at 8 h, AzaCdR was competent to activate expression of BRLF1 and BZLF1 mRNAs after exposures as short as 15 min (Fig. 5A, lane 2), although the extent of activation was approximately twofold greater when the AzaCdR was present for 1 h (Fig. 5A, lane 5) and was threefold greater when it was present for 2 h (lane 6). To determine whether these short exposure times were accompanied by short response times, the cells were treated with AzaCdR for 1 h and RNA harvested at intervals thereafter (Fig. 5B). BRLF1 and BZLF1 mRNAs were 8- and 4-fold, respectively, above background at 6 h and reached 36- and 21-fold above background at 8 h. In the experiment illustrated in Fig. 5C, AzaCdR was present

continuously and the cells were harvested periodically and assessed for Rta and ZEBRA protein by immunoblotting. ZEBRA was first detected at 8 h and Rta at 10 h. Thus, AzaCdR requires a much shorter stimulus duration than HDACi, but in HH514-16 cells the response times to AzaCdR and HDACi, as measured by the time of appearance of ZEBRA and Rta proteins, appear to be similar (see Fig. 1B).

Simultaneous comparison of a short-duration stimulus (AzaCdR) and a long-duration stimulus (TSA) in the same cells. HH514-16 cells were continuously exposed to AzaCdR or TSA, and viral mRNA was analyzed at hourly intervals (Fig. 6). In cells treated with AzaCdR, the BRLF1 and BZLF1 mRNAs were first detected above background at 6 h and increased thereafter. In cells treated with TSA, the BRLF1 mRNA was above background at 6 h and BZLF1 mRNA at 7 h. At 8 h, when the experiment was terminated, AzaCdR stimulated the 1.0-kb BZLF1 mRNA about 1.8-fold more than the BRLF1 mRNA, whereas TSA stimulated the BRLF1 mRNA 1.9-fold more than the BZLF1 mRNA. Although the two agents differentially activated the different promoters, the kinetics of response were similar when directly compared. Since the durations of stimulation required for the two agents differ markedly (Fig. 4 and 5), the experiment clearly shows that stimulus duration and response time are independent variables in the same cell background.

Stimulus duration and response time for TPA in B95-8 cells. The finding that short exposures to AzaCdR were sufficient to activate the EBV lytic cycle prompted us to examine other well-characterized lytic cycle-inducing stimuli, such as TPA. A 1-h exposure of B95-8 cells to TPA activated maximal levels of BRLF1 and BZLF1 mRNAs (Fig. 7A). Exposures to TPA of longer than 1 h served to decrease the response. Very short exposures to TPA (15 min) (Fig. 7B) were able to activate BRLF1 and BZLF1 mRNAs in B95-8 cells. Prolonging the exposure time to 1 h increased BRLF1 by 2-fold and BZLF1 by 1.5-fold. The time for expression of Rta and ZEBRA proteins above the background level after a 15-min exposure to TPA was approximately 8 h (Fig. 7C). At later times, 12 and 15 h after the 15-min exposure to TPA, the levels of Rta and

FIG. 4. Duration of stimulus required for detection of BZLF1 and BRLF1 transcripts following treatment of HH514-16 cells with TSA, AzaCdR (AZC), and NaB. HH514-16 cells were untreated (lane 16) or exposed to TSA, AZC, or NaB for various lengths of time (lanes 1 to 15). At each indicated treatment time the cells were washed and returned to culture. RNA was isolated from all cultures at 8 h. (A) The Northern blot was probed with Z(301), which detects both the 1.0-kb BZLF1 mRNA and the 3.0-kb BRLF1 mRNA. RNase P was used as a loading control. (B and C) Densitometer analysis of the BRLF1 and BZLF1 mRNAs.

ZEBRA proteins continued to increase. These experiments with TPA in B95-8 cells produced a result that was qualitatively similar to that for AzaCdR in HH514-16 cells. Both stimuli are short acting, but the response time is delayed. Therefore, the experiment confirmed, with a different inducing agent in a different cell background, the conclusion that stimulus duration and response time are independent variables.

Short times of exposure to TPA are sufficient to activate expression of lytic DNA synthesis and late lytic antigen expression. A 1-hour exposure to TPA produced amplification of EBV DNA and the pattern of terminal EBV DNA fragments characteristic of lytic viral DNA synthesis (Fig. 8A). Continuous exposure to TPA did not significantly increase the level of lytic DNA above that detected when TPA was present for 1 h and removed (Fig. 8A, compare lane 6 and lane 9). Near-maximal amplification of EBV DNA was observed at 48 h,

regardless of the duration of exposure to TPA. However, unlike lytic DNA amplification in HH514-16 cells (Fig. 3), where almost no lytic viral DNA was detected at 24 h, in B95-8 cells there was significant lytic DNA synthesis present at 24 h. Thus, the response time for the same readout, namely, lytic DNA replication, is influenced by the virus/cell background.

A 1-h exposure to TPA was sufficient to detect the small capsid late antigen (BFRF3) at 15 h and 24 h. Prolonging the time of exposure to TPA to 15, 24, 48, or 72 h did not increase the levels of FR3 (Fig. 8B and data not shown). Therefore, a short exposure to TPA is sufficient to activate the entire lytic cascade many hours later.

The number of B95-8 cells responding to short exposures to TPA with expression of lytic antigens was determined by the FACS-based assay. A 15-min exposure to TPA caused 2.5% of B95-8 cells to express lytic antigens when assessed at 24 h (Fig.

FIG. 5. Duration of stimulus and response time for detection of BZLF1 and BRLF1 mRNAs in HH514-16 cells following treatment with AzaCdR. (A) Northern blot of HH514-16 cells treated with AzaCdR (AZC) for the indicated length of time and then washed. RNA was harvested after a total incubation time of 8 h. Twenty-five micrograms of RNA was loaded on an agarose gel, transferred to a Nitran filter, and probed with Z(301), which detects both the 1.0-kb BZLF1 mRNA and the 3.0-kb BRLF1 mRNA. RNase P serves as a loading control. The relative mRNA level was calculated by dividing densitometer units for induced cells at a given time by densitometer units for untreated cells (lane 1). (B) Northern blot of HH514-16 cells untreated (lane 1) or treated with AZC for 1 hour, washed, and incubated without additional drug for the indicated lengths of time. RNA was harvested, loaded onto an agarose gel, transferred to a Nitran membrane, and probed as for panel A. (C) Western immunoblot of HH514-16 cells treated with AZC for 1 hour, after which the cells were washed to remove drug and incubated in the absence of drug for the indicated lengths of time. Immunoblots were probed sequentially with polyclonal rabbit sera against ZEBRA and Rta and a mouse monoclonal antibody against β -actin.

FIG. 6. Comparison of times of response to AzaCdR and TSA in HH514-16 cells. Cells from the same culture flask were treated continuously with either AzaCdR (AZC) (lanes 2 to 9) or TSA (lanes 11 to 18). RNA, harvested at hourly intervals from 1 to 8 h, was assessed for BRLF1 and BZLF1 mRNAs by Northern blotting. The relative levels of mRNA in the treated cells was compared with those in untreated cells harvested at 0 h (lane 1) or 8 h (lanes 10 and 19). The H1 component of RNase P controlled for loading of RNA.

8C) and 4% of cells to express those antigens when examined at 48 h (Fig. 8D). Longer durations of stimulation by TPA, up to 1 h, 24 h, or 48 h, did not increase the number of responding cells. This experiment showed that a short stimulus duration is not predicated on a large number of responding cells. HDACi, which require a long stimulus duration, induce a 10-fold-greater number of responding HH514-16 cells (Fig. 1A).

Stimulus duration and response time for synergistic activation of the EBV lytic cycle in Raji cells by TPA and NaB. The results (Fig. 4 and 5) showing that HDACi require prolonged exposure and AzaCdR needs only a short exposure in HH514-16 cells indicated that stimulus duration is a property intrinsic to the stimulus itself. Raji cells offered another experimental system in which to compare two different stimuli in the same cell background. In Raji cells, TPA activated expression of the BRLF1 and BZLF1 mRNAs (Fig. 9A, lanes 5, 6). NaB by itself did not activate these lytic mRNAs (Fig. 9A, lanes 3 and 4); however, when NaB was present together with TPA there was synergy. This synergy was evident at 12 h (Fig. 9A, lane 8).

The action of TPA in stimulating BRLF1 and BZLF1 transcription in Raji cells was maximal after an exposure time of 0.5 h or less (Fig. 9B). Longer exposure times to TPA decreased the response, an effect similar to that observed in B95-8 cells (Fig. 8C). For example, an 8-h (lane 8) or 12-h (lane 10) exposure to TPA elicited a response that was about one-third of the magnitude of the response observed with a 0.5-h exposure. However, longer exposures to NaB were required to produce synergy. A 0.5-h exposure to TPA and NaB did not differ from exposure to TPA alone (Fig. 9B, lanes 2 and 3). Exposures to TPA and NaB of 2, 4, 8, or 12 h produced synergistic activation, increasing the level of BRLF1 mRNA an average 3.9-fold and that of BZLF1 mRNA by 3.4-fold, compared to that measured with TPA alone.

To measure the duration of exposure to NaB required for a maximal synergistic effect, the time of exposure to TPA was

held constant at 0.5 h, and NaB was added and left for longer periods of time. Figure 9C shows that there was progressively greater synergy when NaB was present for longer durations; maximal synergy was observed following a 6-h exposure to NaB. These experiments confirm the conclusion that stimulation duration is a feature of the stimulus itself. TPA requires a short exposure and NaB requires a longer exposure time for the maximal effect in the same cell background.

Induction of the EBV lytic cycle in Akata cells by anti-IgG is characterized by short stimulus exposure duration and rapid response. Figure 10A illustrates an experiment in which Akata cells were continuously exposed to anti-IgG. Cell extracts harvested at hourly intervals were examined for BRLF1 and BZLF1 mRNAs by Northern blotting. These very early viral mRNAs were first detected after 2 h (lane 5) and were maximal at 3 h (lane 7) after anti-IgG treatment. Thus, the response time in Akata cells is 4 h more rapid than that in the three other cell/virus systems that we studied.

In the experiment shown in Fig. 10B, the response was measured at 2.5 h and the duration of exposure to anti-IgG was varied, from 5 min to 45 min, by washing off the anti-IgG. Another culture was continuously exposed to anti-IgG for 2.5 h. This experiment showed that a 5-min exposure to anti-IgG produced a maximal response. In experiments with Akata cells treated with anti-IgG (Fig. 10A and B), the 1.0-kb BZLF1 mRNA was always 2 to 4 times more abundant than the 3.0-kb BRLF1 mRNA. This experiment was repeated using qRT-PCR (Fig. 10C and D). A 5-min exposure to anti-IgG produced a near-maximal response. While there was some fluctuation of the amount of BZLF1 mRNA, the relative level of stimulation of the 1.0-kb BZLF1 mRNA was always greater than that of the 3.0-kb BRLF1 mRNA.

The short exposure times required for anti-IgG do not result from a residual induction stimulus. We carried out two types of experiments to test whether the short exposure times required for

FIG. 7. Duration of stimulus and response time required for lytic activation of BRLF1 and BZLF1 expression in B95-8 cells treated with a phorbol ester, TPA. (A and B) B95-8 cells were untreated (-) or were treated with TPA for the indicated times (+), after which the cells were washed and returned to culture. Total RNA harvested at 8 h was examined for BRLF1 and BZLF1 mRNAs by Northern blotting. (C) B95-8 cells were treated with TPA for 15 min, washed, and returned to culture. At the indicated times after treatment, cells were harvested and analyzed for Rta and ZEBRA by immunoblotting. X, a protein detected in B95-8 cells with the antibody to Rta, controlled for protein loading.

anti-IgG could be accounted for by the presence of a residual stimulus that was not eliminated by removing the medium, washing, and adding fresh medium to the cells. We estimate that this washing procedure resulted in a dilution of anti-IgG of 1:1,000 or greater. A dose-response experiment for the capacity of anti-IgG to activate BZLF1 and BRLF1 mRNAs showed that a concentration of 40 ng/ml, a 1:188 dilution of the usual stimulation dose of 7.5 μ g/ml, eliminated more than 90% of the activity. In the second experiment, we tested the medium from Akata cells which had been treated with anti-IgG for 30 min, washed, and fresh medium replaced. When the replenished medium was placed on previously untreated Akata cells, it contained no activity that was able to activate expression of BRLF1 or BZLF1 mRNA after 2.5 h (data not shown).

Response time of EBV lytic antigens and polypeptides following anti-IgG treatment of Akata cells. Using a FACS-based assay, Akata cells in the lytic cycle were detectable above background at 3 h and rose progressively thereafter to approximately 14% of cells by 24 h (Fig. 11A). The ZEBRA protein was 15-fold above background at 3 h after anti-IgG treatment and reached a maximal level at 4 h (Fig. 11B). Rta protein was approximately

sevenfold above background at 3 h but did not reach a maximal level until 6 h (Fig. 11C). The response of BALF5, the DNA polymerase (Fig. 11C), was minimally above background at 3 h and 4 h and maximally induced at 6 and 23 h. A similar pattern was observed for BMRF1, the DNA polymerase processivity factor. Thus, whether assessed by BZLF1 or BRLF1 mRNA, ZEBRA or Rta protein, early viral lytic cycle proteins, or the number of cells expressing early antigens, a significant EBV lytic response was evident in Akata cells by 3 h. Thus, Akata cells differ from the three other cell lines in being characterized by both a short stimulus duration and a rapid response.

DISCUSSION

Replication of viral DNA that can be packaged into an infectious particle is critical for survival of EBV in the human population. Yet, our understanding of the process that triggers viral lytic replication within a host is woefully incomplete. To gain further insight into this process, we have studied the time required for a wide range of stimuli, with potentially different modes of action, to trigger lytic replication in four well-

FIG. 8. A 1-hour exposure to TPA is sufficient to induce EBV lytic DNA replication and late gene expression in B95-8 cells. (A) B95-8 cells were untreated or treated with TPA. The TPA-treated cells were either not washed (none) or washed at 1 h or 24 h after treatment. At the indicated times, total cellular DNA was harvested and a Southern blot was probed for the EBV terminal repeat and for BamHI W. (B) B95-8 cells were untreated (–) or treated with TPA (+). The TPA-treated cultures were washed at 1 or 15 h after treatment or not washed. Cells were harvested after 15 or 24 h and analyzed for ZEBRA or BFRF3 (FR3) late protein and β -actin by immunoblotting. (C and D) The number of B95-8 cells induced into the lytic cycle following short exposures to TPA, as determined by FACS analysis with human antisera. Cells were treated for 15, 30, or 45 min or 1 h and harvested at 24 h (C) or 48 h (D). Cells harvested immediately after treatment (0 h) served as a negative control.

characterized EBV-infected cell lines. One cell line consisted of lymphoblastoid cells generated with virus from a patient with infectious mononucleosis, while three others were derived from patients with Burkitt lymphoma. We used

a range of readouts to measure qualitative and quantitative differences in the temporal patterns of lytic induction. A central theme that emerges from these studies is that lytic induction is “not all created equal.” Duration of stimulus and response time are two independent variables which determine the specific outcome being measured. Stimulus duration is dependent on the stimulus itself, while response time is influenced by a combination of factors, including the nature of the stimulus, the virus/cell background, and the specific readout for lytic induction.

Some conclusions about stimulus duration. Table 2 summarizes the duration of stimulus required for detection of BRLF1 and BZLF1 mRNAs in the different systems we studied. We find that the duration of exposure required for EBV lytic cycle activation differs among stimuli. A short exposure time of 15 min or less is adequate for TPA, anti-IgG, and AzaCdR to activate the lytic cycle, whereas exposure times of 2 h or more are required for lytic induction by HDACi, NaB, and TSA. Short-duration stimuli, such as anti-IgG, may rapidly become irreversibly bound and thereafter cannot be removed by washing. It is unclear whether such stimuli can be internalized and recycled.

In two different cell/virus systems we showed that the required duration of exposure to the stimulus is a feature of the stimulus itself and independent of the cell/virus background. In HH514-16 cells, AzaCdR is a short-duration stimulus, whereas the HDACi are long-duration stimuli in the same cells. In Raji cells, an exposure time of 0.5 h is maximal for lytic induction by TPA, but longer exposure times, of up to 6 h, are required for maximal synergy between TPA and NaB.

For certain short-acting stimuli such as TPA and anti-IgG, prolonging the exposure time does not enhance the response and, in fact, may diminish it. This is evident in the response to TPA in Raji cells (Fig. 9B) and in the number of B95-8 cells induced into the lytic cycle by TPA (Fig. 8C and D). For other short-exposure stimuli such as AzaCdR, prolonging the exposure time from 15 min to 4 h does increase the response by approximately two- to threefold (Fig. 4 and 5A). Prolonging the exposure time to TSA from 4 to 6 h appears to produce a cumulative effect (Fig. 4). However, exposure times of greater than 6 h diminish the response.

The stimulus duration is independent of the response time. For example, in HH514-16 cells, the short-duration stimulus AzaCdR and long-duration stimuli such as NaB and TSA have similar response times (Fig. 1C, 5B, and 6). Moreover, a short stimulus duration does not predict that a large number of cells will be activated into the lytic cycle. Relatively few B95-8 cells are activated even though a 15-min exposure to TPA is an adequate stimulus.

Some conclusions about the response time. The response time is strongly influenced by the virus/cell background. A very rapid response was observed in Akata cells (Fig. 10). This rapid response in Akata cells may be influenced by the cell line itself and a very-short-acting stimulus. However, a slow response was seen in HH514-16 cells (Fig. 1C and 5B). The response time in HH514-16 cells appears to be independent of the stimulus duration. A relatively slow response of about 6 h is observed with both AzaCdR and TSA in HH514-16 cells (Fig. 6) (44). However, the selectivity of the response does seem to be dependent on the stimulus itself. For example, TSA activates

FIG. 9. Response time and duration of stimulus exposure required for detection of synergistic activation of BRLF1 and BZLF1 expression by TPA and NaB in Raji cells. (A) Raji cells were untreated (–) or were continuously treated with NaB, TPA, or TPA plus NaB. RNA harvested at 8 and 12 h after treatment was analyzed for BRLF1 and BZLF1 mRNA expression. (B) Raji cells were treated for various lengths of time with TPA alone or TPA plus NaB, after which the cells were washed. One group of cultures was untreated (lane 1); another culture was treated with NaB alone (lane 12). Total RNA was harvested after 12 h and analyzed for BRLF1 and BZLF1 mRNAs. (C) Raji cells were untreated (lane 1) or treated for 0.5 h with TPA alone (lane 2) or TPA for 0.5 h plus NaB for various lengths of time (lanes 3 to 7). One culture received NaB alone (lane 8). RNA was harvested after 12 h. In all experiments Northern blots were probed for BRLF1 and BZLF1 mRNAs. RNase P was used to control for loading RNA and to calculate relative levels of mRNA.

higher levels of expression of the 3.0-kb BRLF1 mRNA than of the 1.0-kb BZLF1 mRNA (Fig. 4A), while AzaCdR and anti-IgG preferentially stimulate the 1.0-kb BLZF1 mRNA (Fig. 5C and 10). These observations raise the question whether certain stimuli preferentially target Zp whereas other stimuli preferentially target Rp.

Some of these stimulus-dependent effects were also observed in the relative kinetics of response of the Rta and ZEBRA proteins. In HH514-16 cells, TSA induced Rta protein earlier than ZEBRA protein (Fig. 1B), while AzaCdR induced an earlier appearance of ZEBRA protein than of Rta protein (Fig. 5C). Anti-IgG induced high levels of ZEBRA protein (15- to 20-fold above background) at 3 to 4 h, while comparable levels of Rta protein were not observed until 6 h

(Fig. 11). Autostimulation of BZLF1 and cross-stimulation of BRLF1 by ZEBRA may account for some of these differences in kinetics and magnitude (1, 12, 14, 24).

The response time is dependent on the specific readout for lytic induction. Two features of the readout will influence the response time. One is the sensitivity of the assay. The highly sensitive FACS-based assay using human antibodies can detect about a 10-fold increase of lytically induced cells at 4 h after treatment of HH514-16 cells with NaB, but immunoblotting cannot detect this level of stimulation of viral lytic polypeptide expression until 8 to 10 h after lytic induction (Fig. 1B and 5C). Response time is also obviously influenced by the temporal position of the event being measured in the viral life cycle. Lytic viral DNA replication, a relatively late event, is not sig-

FIG. 10. Response time and duration of stimulus required for detection of BRLF1 and BZLF1 mRNAs in Akata cells treated with anti-IgG. (A) Akata cells were untreated (–) or treated continuously with anti-IgG (+). Total cellular RNA was harvested at hourly intervals and examined for BRLF1 and BZLF1 mRNAs by Northern blotting. (B) Akata cells were treated for various lengths of time with anti-IgG and then washed and returned to culture. Total RNA was harvested from all cultures after 2.5 h and analyzed by Northern blotting. (C and D) In a separate experiment, Akata cells were untreated or exposed to anti-IgG for the indicated times, washed, and returned to culture. RNA harvested at 2.5 h was analyzed by qRT-PCR for BZLF1 (C) or BRLF1 (D) mRNA. The fold increase is relative to untreated cells handled in parallel.

nificantly above background in HH514-16 cells until sometime between 24 and 48 h after treatment with NaB (Fig. 3). Measuring lytic DNA replication as a response also illustrates that the stimulus duration itself may be affected by the readout. While an 8-h exposure to NaB leads to near-maximal levels of ZEBRA protein at 24 h (Fig. 2A), an 8-h exposure to NaB does not produce a maximal level of lytic viral DNA amplification (Fig. 3). This observation raises the question

whether lytic cycle-inducing stimuli might play several roles during different phases of the viral life cycle. One obvious early role is to induce BZLF1 and BRLF1 expression. However, the inducing stimuli may also have important effects on events downstream of BRLF1 and BLZF1 expression. These downstream effects are likely to be more important for HDACi than for short-duration stimuli. A 16-h exposure to NaB is clearly superior to an 8-h exposure in stimulating viral DNA replica-

FIG. 11. Response time for detection of the number of lytically activated cells and lytic cycle proteins in Akata cells continuously treated with anti-IgG. (A) Cells were untreated or treated continuously with anti-IgG. The fraction of cells induced to express the EA-D (BMRF1) lytic antigen was determined by FACS with monoclonal antibody R3.1. (B and C) Akata cells were treated with anti-IgG (+); untreated cell extracts (–) were harvested at the indicated times after treatment. The immunoblots shown in both panels, prepared from aliquots of the same cell extracts, were probed with antibodies specific for EA-D and ZEBRA (B) or BALF5 and Rta (C). β-Actin controlled for protein loading.

tion (Fig. 3), whereas a 1-h exposure to TPA gives a nearly-maximal lytic viral DNA replication response (Fig. 8).
Mysteries about the mechanism of lytic cycle induction by AzaCdR. One of the most unexpected results from this investigation was that AzaCdR behaved as a short-duration stimulus (Fig. 5A). Moreover, AzaCdR could activate BRLF1 and BZLF1 mRNAs by 6 h, a time well before the onset of lytic

viral DNA replication (Fig. 5B and 6). Furthermore, when the methylation states of Zp and Rp were examined by bisulfite sequencing 8 h after treatment of HH514-16 cells with AzaCdR, a time when there was abundant lytic cycle induction, as detected by the levels of BRLF1 and BZLF1 mRNAs, we could detect no change in the methylation state of Zp or Rp (29). Interpretation of the latter result is complicated by the problem that it might be difficult to detect a demethylation event in a subset of viral genomes occurring in a subset of lytically responsive cells. For example, if 5% of cells were lytically induced at 8 h and only 20% of viral genomes were demethylated in the lytically responsive cells, we would need to detect a demethylation event in 1% of the viral genomes. This would not be possible by bisulfite sequencing of viral DNA in the total cell population. However, the short duration of exposure to the agent and, more impressively, the rapid lytic cycle response is not what would be expected if AzaCdR were acting primarily as a DNA methyltransferase inhibitor and activating the EBV lytic cycle by an epigenetic mechanism, as is often assumed for this compound. Demethylation as the result of inhibition of DNA methyltransferase would require that viral DNA in the lytically activated cells be replicated within a period of 4 to 8 h. It is more likely that 5AzaCdR is activating EBV lytic cycle gene expression by a novel mechanism that does not require viral DNA replication. Others have recently postulated in experiments on induction of fetal hemoglobin that AzaCdR acts by a novel mechanism that does not involve DNA hypomethylation (27, 39).

What these experiments tell us about early upstream events in activation of the EBV lytic cycle. The earliest events that activate the EBV lytic cycle occur from 1.5 to 6 h after exposure to an inducing agent, a time when new protein synthesis is required (44). They may also continue asynchronously after this time. For three of the stimuli that we studied, namely, TPA, anti-IgG, and AzaCdR, these early events are likely to include activation of one or more signal transduction pathways. For TPA this pathway includes PKC (15). Anti-IgG induces a wide array of signaling events, including activation of intracellular Src family tyrosine kinases; mitogen-activated kinases such as JNK, ERK, and p38 kinase, Ca²⁺/calmodulin dependent kinase; and one or more isoforms of PKC (6, 8, 25, 38). We may assume that since AzaCdR is a short-acting stimulus, it too activates a signal transduction cascade, the identity of which remains to be elucidated. Once the inducing agents activate signal transduction, they can be removed from the culture medium (Fig. 2, 3, 7C, and 8A and B). Presumably,

TABLE 2. Summary of stimulus durations and response times required to detect expression of BRLF1 and BZLF1 mRNAs

Cell line	Stimulus	Minimal stimulus duration	Response time (h)
HH514-16	NaB	2–4 h	6
	TSA	2–4 h	6
	AzaCdR	<15 min	4–6
B95-8	TPA	<15 min	6–8
Raji	TPA	<0.5 h	8
Akata	TPA + NaB	0.5–6 h	8–12
	Anti-IgG	<5 min	1.5 ^a –3

^a 1.5 h by qRT-PCR (data not shown).

initial signaling events are sufficient to set in motion the entire cascade leading to lytic viral DNA replication and eventually production of virions.

Downstream of the signal transduction cascade is a requirement for protein synthesis. We have previously shown that EBV lytic cycle induction by TPA and AzaCdR is inhibited by CHX (44). In recent experiments we have found that when CHX is present within the first 1.5 h after addition of anti-IgG, it also blocks lytic induction in Akata cells (data not shown). Therefore, the signal transduction cascades that are initiated by the short-duration stimuli such as TPA, AzaCdR, and anti-IgG not only act on preformed transcription factors but also lead to synthesis of new proteins that directly or indirectly activate Zp and Rp or remove repressors of these promoters.

The HDACi may or may not share with short-duration stimuli the property of activating signaling kinases. However, they do share a requirement for new protein synthesis. Based on experiments in which CHX was added at different times after addition of an HDACi, these new proteins required for EBV lytic activation are present within 4 to 6 h after addition of the HDACi. Moreover, HDACi facilitate the interaction of members of the Sp1 family of transcription factors with butyrate-responsive promoters such as the p21^{waf/cip} promoter and the promoter of the Kaposi's sarcoma-associated herpesvirus ORF50 gene (32, 45). HDACi activate and repress expression of a large number of cellular genes (22, 31). It is likely that these effects of HDACi on cellular gene expression profoundly alter the cellular environment to conditions that favor or inhibit EBV lytic gene expression. There is little evidence to support the idea that alteration of the histone acetylation state of Zp or Rp by HDACi is by itself a primary mode of EBV lytic cycle activation (9).

If short- and long-duration stimuli share a final common pathway, the common events may be revealed by understanding the patterns of de novo protein synthesis which characterize the subpopulation of cells which responds and the cellular subpopulation which is refractory to lytic cycle induction during the first few hours after exposure to an inducing stimulus.

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REFERENCES

- Adamson, A. L., and S. C. Kenney. 1998. Rescue of the Epstein-Barr virus BZLF1 mutant, Z (S186A), early gene activation defect by the BRLF1 gene product. *Virology* **251**:187–197.
- Allan, G. J., and D. T. Rowe. 1989. Size and stability of the Epstein-Barr virus major internal repeat (IR-1) in Burkitt's lymphoma and lymphoblastoid cell lines. *Virology* **173**:489–498.
- Bartkiewicz, M., H. Gold, and S. Altman. 1989. Identification and characterization of an RNA molecule that copurifies with RNase P activity from HeLa cells. *Genes Dev.* **3**:488–499.
- Ben-Sasson, S. A., and G. Klein. 1981. Activation of the Epstein-Barr virus genome by 5-aza-cytidine in latently infected human lymphoid lines. *Int. J. Cancer* **28**:131–135.
- Bhaduri-McIntosh, S., and G. Miller. 2006. Cells lytically infected with Epstein-Barr virus are detected and separable by immunoglobulins from EBV-seropositive individuals. *J. Virol. Methods* **137**:103–114.
- Campbell, K. S. 1999. Signal transduction from the B cell antigen-receptor. *Curr. Opin. Immunol.* **11**:256–264.
- Chang, L. K., and S. T. Liu. 2000. Activation of the BRLF1 promoter and lytic cycle of Epstein-Barr virus by histone acetylation. *Nucleic Acids Res.* **28**:3918–3925.
- Chatila, T., N. Ho, P. Liu, S. Liu, G. Mosialos, E. Kieff, and S. H. Speck. 1997. The Epstein-Barr virus-induced Ca²⁺/calmodulin-dependent kinase type IV/Gr promotes a Ca²⁺-dependent switch from latency to viral replication. *J. Virol.* **71**:6560–6567.
- Countryman, J. K., L. Gradoville, and G. Miller. 2008. Histone hyperacetylation occurs on promoters of lytic cycle regulatory genes in Epstein-Barr virus-infected cell lines which are refractory to disruption of latency by histone deacetylase inhibitors. *J. Virol.* **82**:4706–4719.
- Epstein, M. A., B. G. Achong, Y. M. Barr, B. Zajac, G. Henle, and W. Henle. 1966. Morphological and virological investigations on cultured Burkitt tumor lymphoblasts (strain Raji). *J. Natl. Cancer Inst.* **37**:547–559.
- Feng, W. H., and S. C. Kenney. 2006. Valproic acid enhances the efficacy of chemotherapy in EBV-positive tumors by increasing lytic viral gene expression. *Cancer Res.* **66**:8762–8769.
- Flemington, E., and S. H. Speck. 1990. Autoregulation of Epstein-Barr virus putative lytic switch gene BZLF1. *J. Virol.* **64**:1227–1232.
- Flint, S. J., L. W. Enquist, R. M. Krug, V. R. Racaniello, and A. M. Skalka. 2000. Principles of virology: molecular biology, pathogenesis, and control, p.236–284. ASM Press, Washington, DC.
- Francis, A. L., L. Gradoville, and G. Miller. 1997. Alteration of a single serine in the basic domain of the Epstein-Barr virus ZEBRA protein separates its functions of transcriptional activation and disruption of latency. *J. Virol.* **71**:3054–3061.
- Gao, X., K. Ikuta, M. Tajima, and T. Sairenji. 2001. 12-O-tetradecanoylphorbol-13-acetate induces Epstein-Barr virus reactivation via NF-kappaB and AP-1 as regulated by protein kinase C and mitogen-activated protein kinase. *Virology* **286**:91–99.
- Given, D., and E. Kieff. 1979. DNA of Epstein-Barr virus. VI. Mapping of the internal tandem reiteration. *J. Virol.* **31**:315–324.
- Gradoville, L., D. Kwa, A. El-Guindy, and G. Miller. 2002. Protein kinase C-independent activation of the Epstein-Barr virus lytic cycle. *J. Virol.* **76**:5612–5626.
- Gruffat, H., E. Manet, and A. Sergeant. 2002. MEF2-mediated recruitment of class II HDAC at the EBV immediate early gene BZLF1 links latency and chromatin remodeling. *EMBO Rep.* **3**:141–146.
- Heston, L., A. El-Guindy, J. Countryman, C. Dela Cruz, H. J. Delecluse, and G. Miller. 2006. Amino acids in the basic domain of Epstein-Barr virus ZEBRA protein play distinct role in DNA binding, activation of early lytic gene expression and promotion of viral DNA replication. *J. Virol.* **80**:9115–9133.
- Heston, L., M. Rabson, N. Brown, and G. Miller. 1982. New Epstein-Barr virus variants from cellular subclones of P3J-HR-1 Burkitt lymphoma. *Nature* **295**:160–163.
- Jenkins, P. J., U. K. Binne, and P. J. Farrell. 2000. Histone acetylation and reactivation of Epstein-Barr virus from latency. *J. Virol.* **74**:710–720.
- Joseph, J., G. Mudduluru, S. Antony, S. Vashistha, P. Ajitkumar, and K. Somasundaram. 2004. Expression profiling of sodium butyrate (NaB)-treated cells: identification of regulation of genes related to cytokine signaling and cancer metastasis by NaB. *Oncogene* **23**:6304–6315.
- Katz, D. A., R. P. Baumann, R. Sun, J. L. Kolman, N. Taylor, and G. Miller. 1992. Viral proteins associated with the Epstein-Barr virus transactivator, ZEBRA. *Proc. Natl. Acad. Sci. USA* **89**:378–382.
- Kolman, J. L., N. Taylor, L. Gradoville, J. Countryman, and G. Miller. 1996. Comparing transcriptional activation and autostimulation by ZEBRA and ZEBRA/c-Fos chimeras. *J. Virol.* **70**:1493–1504.
- Kurosaki, T. 1999. Genetic analysis of B cell antigen receptor signaling. *Annu. Rev. Immunol.* **17**:555–592.
- 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.
- Mabaera, R., M. R. Greene, C. A. Richardson, S. J. Conine, C. D. Kozul, and C. H. Lowrey. 2008. Neither DNA hypomethylation nor changes in the kinetics of erythroid differentiation explain 5-azacytidine's ability to induce human fetal hemoglobin. *Blood* **111**:411–420.
- Mentzer, S. J., S. P. Perrine, and D. V. Faller. 2001. Epstein-Barr virus post-transplant lymphoproliferative disease and virus-specific therapy: pharmacological re-activation of viral target genes with arginine butyrate. *Transpl. Infect. Dis.* **3**:177–185.
- Miller, G., A. El-Guindy, J. Countryman, J. Ye, and L. Gradoville. 2007. Lytic cycle switches of oncogenic human gammaherpesviruses. *Adv. Cancer Res.* **97**:81–109.
- Miller, G., and M. Lipman. 1973. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. *Proc. Natl. Acad. Sci. USA* **70**:190–194.
- Mitsiades, C. S., N. S. Mitsiades, C. J. McMullan, V. Poulaki, R. Shringarpure, T. Hideshima, M. Akiyama, D. Chauhan, N. Munshi, X. Gu, C. Bailey, M. Joseph, T. A. Libermann, V. M. Richon, P. A. Marks, and K. C. Anderson. 2004. Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications. *Proc. Natl. Acad. Sci. USA* **101**:540–545.
- Nakano, K., T. Mizuno, Y. Sowa, T. Orita, T. Yoshino, Y. Okuyama, T. Fujita, N. Ohtani-Fujita, Y. Matsukawa, T. Tokino, H. Yamagishi, T. Oka, H. Nomura, and T. Sakai. 1997. Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. *J. Biol. Chem.* **272**:22199–22206.
- Pearson, G. R., B. Vroman, B. Chase, T. Sculley, M. Hummel, and E. Kieff.

1983. Identification of polypeptide components of the Epstein-Barr virus early antigen complex with monoclonal antibodies. *J. Virol.* **47**:193–201.
34. **Raab-Traub, N., and K. Flynn.** 1986. The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation. *Cell* **47**:883–889.
35. **Ragoczy, T., L. Heston, and G. Miller.** 1998. The Epstein-Barr virus Rta protein activates lytic cycle genes and can disrupt latency in B lymphocytes. *J. Virol.* **72**:7978–7984.
36. **Sculley, T. B., M. Buck, B. Gabrielli, P. G. Parsons, and K. G. Krauer.** 2002. A histone deacetylase inhibitor, azelaic bishydroxamic acid, shows cytotoxicity on Epstein-Barr virus transformed B-cell lines: a potential therapy for posttransplant lymphoproliferative disease. *Transplantation* **73**:271–279.
37. **Serio, T. R., N. Cahill, M. E. Prout, and G. Miller.** 1998. A functionally distinct TATA box required for late progression through the Epstein-Barr virus life cycle. *J. Virol.* **72**:8338–8343.
38. **Speck, S. H., T. Chatila, and E. Flemington.** 1997. Reactivation of Epstein-Barr virus: regulation and function of the BZLF1 gene. *Trends Microbiol.* **5**:399–405.
39. **Stresemann, C., and F. Lyko.** 2008. Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *Int. J. Cancer* **123**:8–13.
40. **Takada, K.** 1984. Cross-linking of cell surface immunoglobulins induces Epstein-Barr virus in Burkitt lymphoma lines. *Int. J. Cancer* **33**:27–32.
41. **Takada, K., K. Horinouchi, Y. Ono, T. Aya, T. Osato, M. Takahashi, and S. Hayasaka.** 1991. An Epstein-Barr virus-producer line Akata: establishment of the cell line and analysis of viral DNA. *Virus Genes* **5**:147–156.
42. **Taylor, N., J. Countryman, C. Rooney, D. Katz, and G. Miller.** 1989. Expression of the BZLF1 latency-disrupting gene differs in standard and defective Epstein-Barr viruses. *J. Virol.* **63**:1721–1728.
43. **Westphal, E. M., W. Blackstock, W. Feng, B. Israel, and S. C. Kenney.** 2000. Activation of lytic Epstein-Barr virus (EBV) infection by radiation and sodium butyrate in vitro and in vivo: a potential method for treating EBV-positive malignancies. *Cancer Res.* **60**:5781–5788.
44. **Ye, J., L. Gradoville, D. Daigle, and G. Miller.** 2007. De novo protein synthesis is required for lytic cycle reactivation of Epstein-Barr virus, but not Kaposi's sarcoma-associated herpesvirus, in response to histone deacetylase inhibitors and protein kinase C agonists. *J. Virol.* **81**:9279–9291.
45. **Ye, J., D. Shedd, and G. Miller.** 2005. An Sp1 response element in the Kaposi's sarcoma-associated herpesvirus open reading frame 50 promoter mediates lytic cycle induction by butyrate. *J. Virol.* **79**:1397–1408.
46. **Yoshida, M., M. Kijima, M. Akita, and T. Beppu.** 1990. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *J. Biol. Chem.* **265**:17174–17179.
47. **zur Hausen, H., F. J. O'Neill, U. K. Freese, and E. Hecker.** 1978. Persisting oncogenic herpesvirus induced by the tumour promotor TPA. *Nature* **272**:373–375.