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The BRRF1 Early Gene of Epstein-Barr Virus Encodes a Transcription Factor That Enhances Induction of Lytic Infection by BRLF1

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The switch from the latent to the lytic form of Epstein-Barr virus (EBV) infection is mediated by expression of the viral immediate-early (IE) proteins, BZLF1 (Z) and BRLF1 (R). An EBV early protein, BRRF1 (Na), is encoded by the opposite strand of the BRLF1 intron, but the function of this nuclear protein in the viral life cycle is unknown. Here we demonstrate that Na enhances the R-mediated induction of lytic EBV infection in 293 cells latently infected with a recombinant EBV (R-KO) defective for the expression of both R and Na. Na also enhances R-induced lytic infections in a gastric carcinoma line (AGS) carrying the R-KO virus, although it has no effect in a Burkitt lymphoma line (BL-30) stably infected with the same mutant virus. We show that Na is a transcription factor that increases the ability of R to activate Z expression from the R-KO viral genome in 293 cells and that Na by itself activates the Z promoter (Zp) in EBV-negative cells. Na activation of Zp requires a CRE motif (ZII), and a consensus CRE motif is sufficient to transfer Na responsiveness to the heterologous E1b promoter. Furthermore, we show that Na enhances the transactivator function of a Gal4–c-Jun fusion protein but does not increase the transactivator function of other transcription factors (including ATF-1, ATF-2, and CREB) known to bind CRE motifs. Na expression in cells results in increased levels of a hyperphosphorylated form of c-Jun, suggesting a mechanism by which Na activates c-Jun. Our results indicate that Na is a transcription factor that activates the EBV Zp IE promoter through its effects on c-Jun and suggest that Na cooperates with BRLF1 to induce the lytic form of EBV infection in certain cell types.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus typically acquired early in life through salivary exchange. EBV is the causative agent of infectious mononucleosis and is associated with a variety of malignancies of B-cell and epithelial cell origin (48, 49). Like all herpesviruses, EBV can exist in either a latent or lytic state with respect to viral gene expression. An infection of B cells typically results in latency, in which only a subset of viral genes is expressed and progeny virus is not released. Latently infected B cells occasionally reactivate into the lytic cycle in response to stimuli such as B-cell activation (57) or differentiation (9). Differentiated epithelial cells are also permissive for lytic infection (28, 32, 55, 65). The induction of the viral lytic cycle in either B cells or epithelial cells results in the expression of the majority of viral genes and the release of progeny virus capable of infecting new cells.

Entry into the viral lytic cycle is initiated by expression of the immediate-early (IE) EBV proteins, BZLF1 (Z) and BRLF1 (R) (7, 8, 45, 50, 58, 68). Z, a bZIP protein with sequence homology to c-Jun and c-Fos, binds and transactivates promoters containing AP-1-like motifs (6, 13, 59, 61). R can also activate target promoters through direct binding (20, 21, 44); however, R also activates transcription indirectly through the induction of signaling cascades (1, 10). Stimuli that induce a lytic infection initially activate the transcription of both IE genes (57), and the expression of either IE protein in latently infected cells is sufficient to induce the lytic form of EBV infection (7, 8, 45, 58, 64, 68). Each IE protein activates the promoter of the other IE gene, and together the two IE proteins then activate the viral early genes and lytic viral replication (2, 14).

The ability of each IE protein to activate transcription of the other IE gene is essential for the disruption of viral latency by either protein (2, 14, 46, 68). Z transactivates the R promoter (Rp) by directly binding to Rp (2, 54). In contrast, R activates Z transcription by enhancing the transcriptional functions of cellular factors (ATF-2 and c-Jun) binding to a CREB response element (CRE) motif (ZII) in the Z promoter (Zp) (1). This effect is mediated through the induction of the stress-associated mitogen-activated protein (MAP) kinases (SAPKs) p38 and c-Jun N-terminal kinase (JNK) (1), which phosphorylate and activate the transcription factors ATF-2 and c-Jun, respectively (12, 23, 47, 62).

In addition to the Z and R genes, the IE locus of EBV contains another open reading frame, BRRF1 (also designated Na), which lies within the first intron of the R gene and is oriented in the opposite direction (38). Na mRNA appears with early kinetics following induction of the viral lytic cycle in several latently infected B-cell lines (38, 53). The promoter driving expression of Na (Nap) is located within the coding sequence for R, and reporter assays indicate that Nap is activated by Z (53). This activation may be mediated by the direct binding of Z to Nap, given that Z binds several sites in Nap between nucleotides −469 and +1 in electromobility shift as-

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says (53). The Na gene product is a 34-kDa protein that localizes to the nucleus in HeLa cells (53). However, no studies to date have identified a function for Na.

For this paper, we examined the role of Na in the viral life cycle. Utilizing several different cell lines that are latently infected with a recombinant EBV defective for expression of both R and Na, we demonstrate that the Na gene product cooperates with R to induce an efficient lytic EBV infection in certain cell lines. Furthermore, we show that Na by itself activates promoters containing CRE motifs (including the IE Z promoter) in EBV-negative cell lines and that this effect is mediated through an enhanced c-Jun transcriptional function. However, Na by itself cannot activate BZLF1 transcription in the context of the intact viral genome. Our results suggest the existence of a previously unexpected positive auto-regulatory circuit for R-mediated lytic EBV induction, in which R by itself initially activates BZLF1 transcription with a low efficiency, but subsequently activates BZLF1 transcription with a much higher efficiency (in conjunction with Na) after the Z gene product has induced Na transcription.

MATERIALS AND METHODS

Plasmids. The Na expression vector pRC-FLAG-BRRF1 and the Z expression vector pSG5-Z were described previously (51, 53). pSG5-R (a gift from Diane Hayward) contains the genomic R sequence beginning with the second exon (including the ATG start codon for R) but lacking the first intron (containing the Na gene) (24). Appropriate control vectors, pRC (Invitrogen) and pSG5 (Stratagene), were used in all experiments. The pRK5-BALF4 expression vector encodes the EBV gp110 protein and was previously shown to enhance the infection efficiency of EBV virions derived from the B95-8 strain of EBV (42). Zp-CAT, Zp-CAT ΔZIA/ZIB, Zp-CAT ΔZII, and −65Zp-CAT (all gifts from E. Flemington) were described previously (17). Briefly, Zp-CAT contains the region of the BZLF1 promoter from −221 to +12 (relative to the transcription start site) linked upstream of the chloramphenicol acetyltransferase (CAT) gene. Zp-CAT ΔZIA/ZIB and Zp-CAT ΔZII represent mutants of Zp in which the ZIA and ZIB sites and the ZII site, respectively, have been mutated via site-directed mutagenesis. The plasmid −65Zp-CAT contains nucleotides −65 to +12 of Zp upstream of CAT. EApBS-CAT (also called BMRF1-CAT) contains the BMRF1 promoter sequence from −331 to +1 upstream of CAT and was described previously (44). CRE-CAT contains three consensus CRE sites upstream of CAT; CRE(m)-CAT is identical to CRE-CAT except for specific mutations in the CRE site. The SG424 vector encodes the Gal4 DNA binding domain alone, whereas Gal4-CREB, Gal4-ATF1, Gal4-ATF2 (all gifts from M. Green), and Gal4–c-Jun (a gift from A. Baldwin) contain the respective proteins linked in-frame to the Gal4 DNA binding domain. Gal4-EIB-CAT (a gift from M. Green) consists of five copies of the Gal4 DNA binding site linked upstream of the minimal EIB TATA box and CAT gene. The CR2/puro expression vector was a gift from L. Hutt-Fletcher; CR2 is the primary cellular receptor for EBV (15). CMV–c-Jun expresses full-length c-Jun under the control of the cytomegalovirus (CMV) promoter. Plasmids were purified by use of Qiagen Maxiprep kits (Qiagen) according to the manufacturer's instructions.

EBV wild-type, Z-KO, and R-KO viruses and cell lines. 293 cells infected with the R-KO virus (293 R-KO), Z-KO virus (293 Z-KO), and wild-type virus (293 WT) have been described previously (11, 14). In the R-KO virus, nucleotides 103,638 to 105,083 (B95.8 coordinates [accession no. V01555]) within the BRLF1 gene were removed via the insertion of a tetracycline resistance cassette into the virus (14). In the Z-KO virus, nucleotides 102,389 to 103,388 (B95.8 coordinates) within the BZLF1 gene were removed via the insertion of a kanamycin resistance cassette. The R-KO, Z-KO, and WT viruses also encode enhanced green fluorescent protein (GFP) and a hygromycin B resistance gene (both cloned into the B95.8 deletion of EBV, where the second copy of oriLyt normally resides). 293 R-KO, 293 Z-KO, and 293 WT cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (FBS), penicillin-streptomycin, and hygromycin B (100 μg/ml; Roche). HeLa cells are a cervical adenocarcinoma line and were maintained in Dulbecco's modified Eagle medium with 10% FBS and penicillin-streptomycin. AGS R-KO cells (see below) were maintained in F-12 medium containing 10% FBS, penicillin-streptomycin, and 100 μg of hygromycin B/ml. BL30 R-KO cells and LCL R-KO cells (see below) were maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin-streptomycin, and 400 μg of hygromycin B/ml. Raji cells, an EBV-positive Burkitt lymphoma cell line, were maintained in RPMI 1640 with 10% FBS and penicillin-streptomycin.

DNA transfection. Transfections of 293 R-KO and 293 WT cells were done by use of Lipofectamine 2000 (Invitrogen). Cells (6×10^5) were seeded the day prior to transfection in 2 ml of medium in a six-well plate. Transfections were performed according to the manufacturer's instructions, except that the reagent/DNA ratio was scaled down to 2 μl/1 μg. Transfections of HeLa cells for reporter assays were performed by use of Fugene 6 (Roche) according to the manufacturer's instructions. AGS R-KO cells were electroporated with 5 μg of total DNA in a Zapper electroporation unit as described previously (60). BL30 R-KO and LCL R-KO cells were transfected in buffer V (Amaxa Biosystems) with an Amaxa Nucleofector (Amaxa Biosystems). Cells were cultured for 24 h in antibiotic-free medium, followed by two washes in 1× phosphate-buffered saline (PBS). Cells (10^7) were resuspended in 100 μl of buffer V (per condition) and 5 μg of total DNA was added. Settings G-16 and T-20 were used for BL30 R-KO and LCL R-KO cells, respectively. After being transfected, cells were incubated overnight in 1.5 ml of antibiotic-free medium at 37°C in a 12-well plate; 2.5 ml of complete medium was then added.

Generation of R-KO cell lines. For the production of R-KO viral stocks, 5×10^6 293 R-KO cells were plated in 10 ml of RPMI 1640–10% FBS–penicillin-streptomycin in a 100-mm-diameter dish the day prior to transfection. The cells were transfected with 1.5 μg each of pSG5-R, pSG5-Z, pRC-FLAG-BRRF1, and pRK5-BALF4 expression plasmids by use of Lipofectamine 2000 (12 μl of reagent per condition) according to the manufacturer's instructions. At 72 h posttransfection, supernatants were filtered through 0.45-μm-pore-size filters and viral stocks were stored at 4°C. AGS cells (a gift from L. Hutt-Fletcher) are an EBV-negative gastric adenocarcinoma cell line. In order to create an AGS line infected with the R-KO virus, we first transfected AGS cells with 1 μg of an expression vector encoding CD21-puromycin resistance by using Fugene 6 (Roche) according to the manufacturer's instructions. At 48 h posttransfection, cells were selected with 0.5 μg of puromycin HCl (Roche)/ml; puromycin-resistant colonies were pooled. For infections with R-KO virus, puromycin-resistant cells were seeded at 50% confluence in a 60-mm-diameter dish and were incubated with R-KO viral stocks. Four days after infection, cells were selected with hygromycin B (100 μg/ml); one hygromycin B-resistant colony representing an AGS cell infected with the R-KO virus was isolated. BL30 cells are an EBV-negative Burkitt lymphoma line. For the generation of BL30 R-KO cells, approximately 5×10^6 BL30 cells were pelleted and resuspended in 5 ml of R-KO viral stock for 3 h at 37°C. After the incubation, the cells were diluted into 50 ml of medium. Selection with 400 μg of hygromycin B/ml was begun at 5 days postinfection. For the generation of LCL R-KO cells, 3×10^6 peripheral blood mononuclear cells (provided by the laboratory of Susan Fiscus, University of North Carolina at Chapel Hill) were resuspended in 3 ml of R-KO virus and incubated overnight at 37°C. Seven milliliters of RPMI 1640–20% FBS–penicillin-streptomycin was then added, and cyclosporine A (Sigma) was added to achieve a final concentration of 500 ng/ml. Fifty percent of the medium was changed every 7 days until transformation into LCLs was apparent (at ~3 to 4 weeks postinfection). LCLs were then maintained in RPMI 1640–10% FBS–penicillin-streptomycin.

Northern blotting. Total RNAs were prepared by use of RNAeasy kits (Qiagen), including DNase treatment, according to the manufacturer's instructions. Ten micrograms of total RNA was subjected to denaturing agarose gel electrophoresis, and fractionated RNAs were transferred to a membrane by use of the Turboblotter system (Schleicher and Schuell) according to the manufacturer's instructions. After being transferred, RNAs were cross-linked to the membrane via UV irradiation (1,200 J). The following double-stranded DNA fragments were used as probes for Z, Na, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively: a PstI/HindIII fragment from pSG5-Z, a BglII/XhoI fragment of pRC-FLAG-BRRF1, and a mouse GAPDH DECAtemplate (Ambion). Double-stranded DNA fragments were gel purified with a Qiagen gel extraction kit (Qiagen) and radiolabeled with a Prime-A-Gene kit (Promega), and unincorporated radioactivity was removed by the use of Sephadex G-50 columns (Amersham); all steps were performed according to the manufacturer's instructions. Prehybridization and hybridization (2×10^6 cpm of probe) were performed in Quikhyb solution (Stratagene) according to the manufacturer's instructions. Membranes were exposed to film overnight at −80°C.

Immunoblotting. Immunoblotting was performed as described previously (1). Briefly, cells were lysed in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors; equivalent amounts of protein were then separated in sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis gels. For the examination of c-Jun phosphorylation status, cells were kept at 4°C at all times

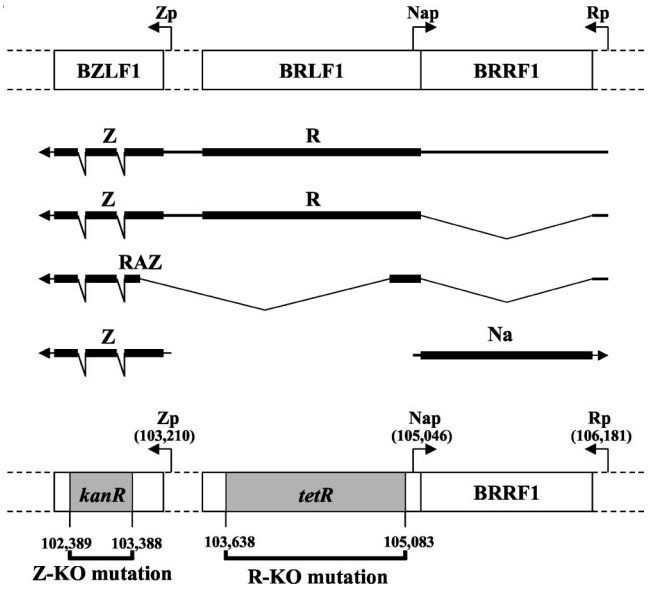

FIG. 1. Diagram of the IE region of the EBV genome. The IE locus of EBV is depicted, with the positions of the BZLF1, BRLF1, BRRF1 (Na), and RAZ genes indicated. Promoters for BZLF1 (Zp), BRLF1 (Rp), and BRRF1 (Nap) are designated by arrows. Transcripts originating from each of the various promoters are depicted, and the proteins encoded (Z, R, RAZ, or Na) by each transcript are indicated above their respective mRNAs. The insertional mutations carried by the Z-KO virus and the R-KO virus are indicated by the gray boxes in the bottom diagram; numbers represent coordinates of the EBV strain B95.8 genome (accession no. V01555).

during the harvesting procedure. After being transferred and blocked, membranes were incubated overnight at 4°C with the appropriate primary antibodies diluted in 5% milk in 1× PBS and 0.1% Tween 20 (PBS-T). Primary antibody dilutions were as follows: 1:100 anti-BRLF1 (Argene), 1:100 anti-BZLF1 (Argene), 1:100 anti-BMRF1 (Capricorn), 1 μg of M2 anti-FLAG (Sigma)/ml, and 1:5,000 anti-β-actin (clone AC-15; Sigma). Anti-c-Jun (Cell Signaling) and anti-phosphoSer73-c-Jun (Cell Signaling) antibodies were diluted 1:1,000 in 5% bovine serum albumin in 1× PBS-T. The appropriate horseradish peroxidase-conjugated secondary antibodies (Promega) were used at a dilution of 1:10,000 in 5% milk–1× PBS-T for 1 h at room temperature. After being washed, bound antibodies were visualized by use of ECL reagent (Amersham) according to the manufacturer's instructions.

CAT assays. CAT assays were performed as described previously (19) with cell extracts harvested at 48 h posttransfection. Chloramphenicol acetylation was determined by thin-layer chromatography followed by PhosphorImager (Molecular Dynamics) quantification.

Viral titration assay. Supernatants from 293 R-KO cells were harvested at 48 h posttransfection and filtered through a 0.45-μm-pore-size filter. Raji cells (2×10^5) were then incubated in 0.5 ml of virus for 3 h at 37°C in a 12-well plate. One and one-half milliliters of medium was then added to each well and the cells were incubated for an additional 48 h at 37°C. Phorbol-12-myristate-3-acetate (PMA; Sigma) and sodium butyrate (Sigma) were then added to achieve a final concentration of 50 ng of PMA/ml and 3 mM sodium butyrate. The number of GFP-expressing Raji cells was quantitated 24 h later by fluorescence microscopy.

RESULTS

293 R-KO cells cannot express Na. The phenotype of a recombinant EBV in which the majority of the R gene has been disrupted through insertional mutagenesis (293 R-KO) (Fig. 1) was previously described for 293 cells as well as primary B cells immortalized by this virus (14). The R-KO virus is similar to the wild-type virus in terms of its ability to immortalize primary B cells, but 293 cells carrying the R-KO virus could not be induced into the lytic form of EBV infection unless a vector expressing the BRLF1 gene product was supplied in *trans* (14). These results were interpreted as showing that the lytic defect of the R-KO virus is completely rescued by the BRLF1 IE protein in *trans* (14).

Notably, however, the EBV sequences deleted from the R-KO virus (nucleotides 103,638 to 105,083 in the B95.8 strain coordinates) not only include the BRLF1 gene, but simultaneously include essentially all of the Na promoter, except for the sequences located within 36 bp of the Na transcriptional start site (Fig. 1). Thus, we hypothesized that the R-KO virus cannot efficiently transcribe the Na message. Furthermore, since the BRLF1 expression vector which was previously used to rescue the lytic defect of the R-KO virus in 293 cells also contained the EBV sequences encoding the Na gene product as well as the Na promoter (14), this vector could potentially express both BRLF1 and Na.

To determine if the R-KO virus is indeed defective for Na expression, we performed a Northern blot analysis of total RNAs from 293 WT, 293 Z-KO, and 293 R-KO cells transfected with an empty vector, a vector expressing Z (which induces the expression of Na [53]), and a vector expressing R that does not contain the intronic sequence in which the Na gene lies. As shown in Fig. 2, as expected, the expression of either Z or R in the 293 WT line induced transcription of the 1.3-kb Na message. R induction of the BZLF1 message, as well as Z induction of the BRLF1 message, in the 293 WT line could be detected upon a longer exposure (data not shown). In the 293 Z-KO line, Z induced the transcription of Na, whereas R did not, indicating that R cannot induce Na expression without the help of Z. In 293 R-KO cells, neither Z nor R expression induced the transcription of Na, confirming that 293 cells infected with the R-KO virus cannot transcribe Na under the conditions in which it is normally expressed. Thus, 293 R-KO cells provide an environment to study the effects of Na during the viral life cycle.

Expression of Na enhances the capacity of R to efficiently induce lytic infection in 293 R-KO cells. To determine if Na affects the ability of R to induce a lytic EBV infection in 293 R-KO cells, we transfected these cells with a vector control, a

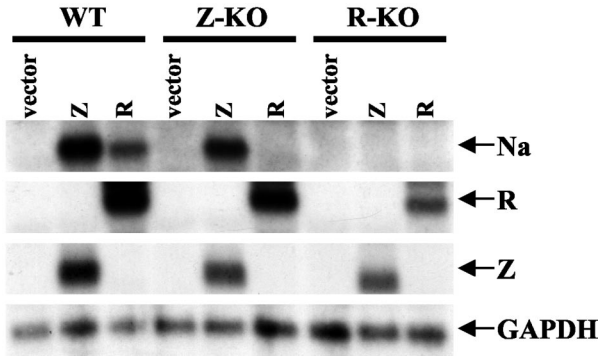

FIG. 2. 293 R-KO cells cannot transcribe BRRF1 mRNA. 293 WT, 293 Z-KO, or 293 R-KO cells were transfected with pSG5 (vector), pSG5-Z (Z), or pSG5-R (R). At 24 h posttransfection, total RNAs were harvested and Northern blotted as described in Materials and Methods. Membranes were hybridized with probes specific for BRRF1 (Na), R, Z, and GAPDH transcripts, as indicated by arrows.

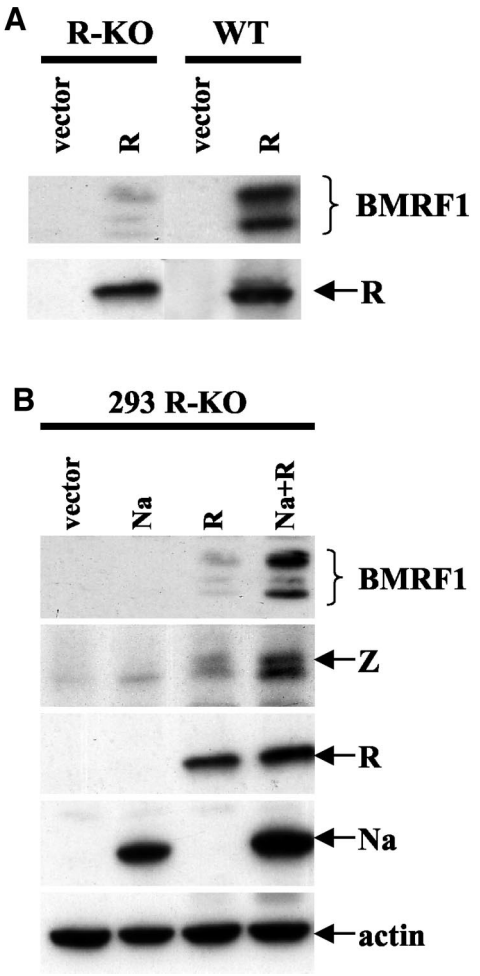

FIG. 3. Na enhances R-induced lytic infection in 293 R-KO cells. (A) 293 R-KO or 293 WT cells were transfected with pSG5 (vector) or pSG5-R (R). Cells were harvested at 48 h posttransfection, and 35 μg of protein was immunoblotted with antibodies specific for the early lytic protein BMRF1 (upper panel) or R (lower panel). (B) 293 R-KO cells were transfected with pSG5 (vector), pRC-FLAG-BRRF1 (Na), pSG5-R (R), or pRC-FLAG-BRRF1 and pSG5-R (Na+R). Cells were harvested at 48 h posttransfection, and 35 μg of protein was immunoblotted with antibodies specific for BMRF1, Z, R, FLAG (to detect FLAG-tagged Na), and β-actin, as indicated by arrows.

vector that expresses Na (FLAG-tagged), a vector that expresses R but not Na, or a combination of the R and Na vectors. The efficiency of early lytic gene induction was assessed by quantitating the expression of IE and early lytic proteins 48 h after transfection. As shown in Fig. 3A, the R vector alone induced only a low-level expression of the early BMRF1 protein in 293 R-KO cells, whereas 293 WT cells transfected with the R vector expressed considerably more BMRF1. Furthermore, although Na alone did not activate Z or BMRF1 expression in 293 R-KO cells, the combination of Na and R was much more effective than R alone in inducing both Z and BMRF1 expression (Fig. 3B).

As shown in Fig. 1, the R-KO deletion also removes a portion of the R-derived N terminus of RAZ (Fig. 1) as well as the first RAZ splice site, suggesting that the R-KO virus cannot express RAZ. RAZ is a chimeric protein consisting of the N terminus of R fused to the C terminus of Z that may function to repress Z activity (18, 52). To determine whether the absence of RAZ expression in 293 R-KO cells had an impact on the lytic infection induced by R, we transfected 293 R-KO cells with R alone, R and RAZ, R and Na, or R, Na, and RAZ in combination. The transfection of RAZ did not enhance the capacity of R alone or R in conjunction with Na to induce BMRF1 expression (data not shown). Thus, the inability of R to efficiently activate early lytic gene transcription in 293 R-KO cells likely reflects the loss of the Na, rather than RAZ, function in the mutant R-KO virus.

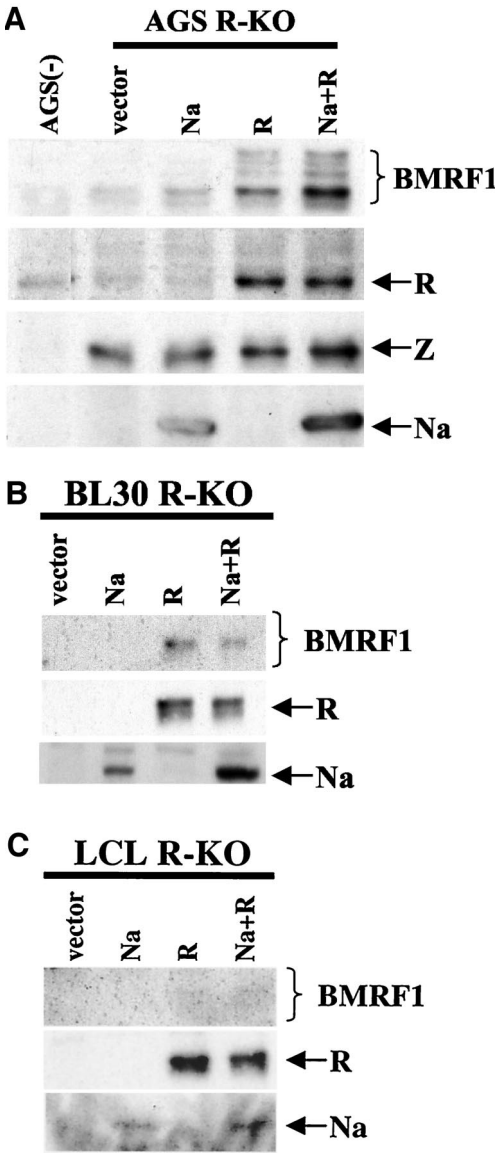

FIG. 4. Na enhances R-induced lytic infection in a cell-line-dependent manner. AGS R-KO (A), BL30 R-KO (B), or LCL R-KO (C) cells were transfected with pRC (vector), pRC-FLAG-BRRF1 (Na), pSG5-R (R), or pRC-FLAG-BRRF1 and pSG5-R (Na+R). Cells were harvested at 48 h posttransfection, and 35 μg of protein was immunoblotted with the indicated antibodies. Densitometric analysis was performed with NIH Image software. AGS(−), EBV-negative AGS cells.

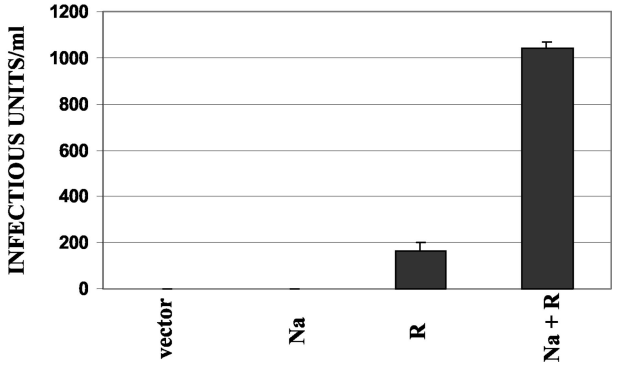

FIG. 5. Na increases the ability of R to activate EBV replication in 293 R-KO cells. Supernatants from the cells transfected for Fig. 3B were harvested, and the amounts of infectious virus present were quantitated as described in Materials and Methods. *x* axis labels represent the transfected plasmids and *y* axis labels represent the numbers of infectious units per milliliter of supernatant harvested. The data shown represent the results of two independent experiments.

Na enhances R-induced lytic infection in a cell-line-dependent manner. To determine if the ability of Na to enhance R-induced lytic infection is a universal phenomenon among various cell lines, we derived several other cell lines containing the R-KO virus, including a gastric carcinoma line (AGS R-KO), a lymphoblastoid cell line (LCL R-KO), and a Burkitt lymphoma line (BL30 R-KO), and examined whether the ability of transfected R to induce early viral gene expression was likewise enhanced by Na expression (Fig. 4). The results indicated that the synergy between Na and R in inducing BMRF1 expression is cell line dependent. AGS cells infected with wild-type EBV have a relatively high proportion of lytically infected cells (39). Interestingly, AGS cells infected with the R-KO virus demonstrated the expression of Z even in the absence of R (Fig. 4A), suggesting that cellular factors are sufficient to activate Z transcription in this cell line. Nevertheless, the ability of transfected R to induce BMRF1 transcription was enhanced 2.2-fold by the presence of Na in AGS R-KO cells (Fig. 4A). In contrast, in the BL30 R-KO line, although R alone induced some BMRF1 expression, the addition of Na did not enhance BMRF1 expression (Fig. 4B). Finally, in the LCL R-KO line, although transfected R was clearly visible, no significant BMRF1 expression was observed in the presence of R alone or with the combination of R and Na (Fig. 4C). Thus, the ability of R to induce a lytic EBV infection, as previously described (46, 68), is clearly cell line dependent. Furthermore, the synergistic effect of Na and R together also appears to be modified by either host-cell-dependent or virus-dependent conditions (such as epigenetic modifications of the viral genome) that are currently poorly defined.

Na increases the ability of R to activate EBV replication in 293 R-KO cells. The ability of Na to enhance R activation of lytic gene transcription in at least some cell types (particularly 293 R-KO cells) suggests that the combination of Na and R may likewise increase the amount of lytic viral replication. To determine if this increase in early gene transcription results in an increase in the amount of infectious virus, we harvested the supernatants of 293 R-KO cells transfected with either vector DNA, R alone, Na alone, or a combination of R and Na. The filtered supernatants were than used to infect Raji cells, which express the EBV receptor at a high level, and the amount of infectious virus in each 293 cell supernatant was quantitated by determining the number of GFP-positive Raji cells. As shown in Fig. 5, 293 R-KO cells transfected with the combination of R and Na produced fivefold more infectious EBV than cells transfected with R alone. Thus, the ability of Na to enhance R-mediated lytic gene expression in this cell type also correlates with increased lytic viral replication.

Na activates Zp in EBV-negative cells. The previous results suggested that Na is important for the efficient induction of lytic infection by R in some cell types. In addition, the ability of Na to enhance R activation of lytic viral genes in some cell types suggests that either Na enhances BRLF1 transcriptional function or Na itself is a transcriptional activator. To determine if Na itself is a transcriptional activator, we performed reporter gene assays with HeLa cells, using constructs in which either the BZLF1 (Zp) or BMRF1 (EAp) promoter was positioned upstream of the CAT gene. The Zp-CAT construct contains the BZLF1 promoter sequence from −221 to +12, which has been shown to be sufficient for the induction of Zp activity by a variety of stimuli (4, 17). Although the expression of Na had no effect on the activity of the construct driven by the BMRF1 (EAp) promoter, it increased the activity of the construct driven by Zp sevenfold (Fig. 6). In contrast, a Zp construct with a deletion of the Zp sequence 5′ of −65 was not activated by Na. These results indicate that Na specifically activates Zp and that sequences in Zp located between −65 and −221 are required for this effect.

Na activates Zp through a CRE site. Zp has been extensively characterized, and a number of different positively acting regulatory elements have been mapped within the Zp sequences located between −65 and −221 relative to the transcription start site (Fig. 7A). Sequences previously shown to be important for Zp activation by a variety of different stimuli include a CRE motif (ZII) (17), which binds CREB, ATF1, ATF2, and c-Jun (1, 34, 37, 63); a series of ZI sites (5, 16, 17), which bind MEF2D, Sp1, and Sp3 (22, 35, 36); and ZIII sites, which bind

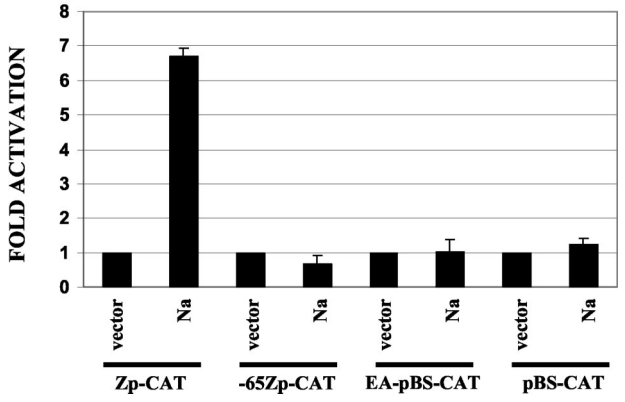

FIG. 6. Na activates the Z promoter (Zp) in EBV-negative cells. HeLa cells were transfected with one of the indicated reporter constructs (Zp-CAT, −65Zp-CAT, EA-pBS-CAT, or pBS-CAT) in conjunction with either pRC (vector) or pRC-FLAG-BRRF1 (Na). Cells were harvested at 48 h posttransfection and CAT assays were performed. The data represent the results of two independent experiments.

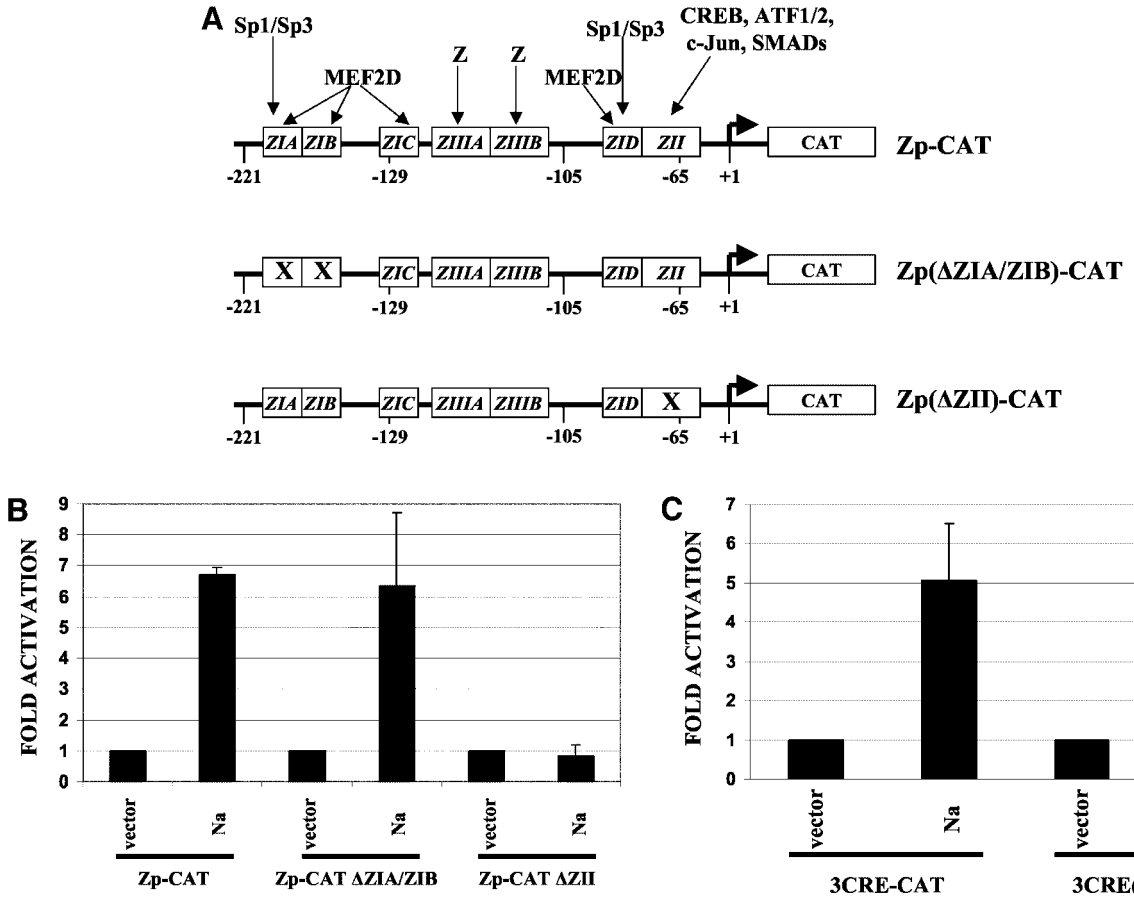

FIG. 7. Na activates Zp through a CRE site. (A) Diagram of the Zp reporter constructs used. The primary elements that positively regulate Zp are denoted in boxes, with the known transcription factors that bind them represented above. Site-directed mutations in either ZIA and ZIB or ZII are indicated with an "X." (B) HeLa cells were transfected with one of the indicated reporter constructs (Zp-CAT, Zp-CAT ΔZIA/ZIB, Zp-CAT ΔZII) in conjunction with either pSG5 (vector) or pRC-FLAG-BRRF1 (Na). Cells were harvested at 48 h posttransfection and CAT assays were performed. The data represent the results of two independent experiments. (C) HeLa cells were transfected with one of the indicated reporter constructs [3CRE-CAT or 3CRE(mut)-CAT] in conjunction with either pRC (vector) or pRC-FLAG-BRRF1 (Na). Cells were harvested at 48 h posttransfection and CAT assays were performed. The data shown represent the results of two independent experiments.

Z and may mediate positive autoregulation (16), although this point is controversial (29, 31, 68). In addition, several SMAD binding elements, including one which overlaps the ZII site, have been implicated in the activation of Zp in response to transforming growth factor beta (33). To determine if a ZI or ZII site is required for Na transactivation of Zp, we examined the ability of Na to activate transcription from wild-type Zp-CAT versus a Zp-CAT plasmid containing specific mutations in two ZI sites (Zp-CAT ΔZIA/ZIB) or a Zp-CAT construct containing a mutant CRE (ZII) site (Zp-CAT ΔZII) (Fig. 7A). Mutation of the ZIA and ZIB sites had no effect on the Na induction of Zp, whereas mutation of the ZII CRE site completely eliminated Na's ability to activate Zp (Fig. 7B). These results suggest that Na activates Zp through the ZII CRE site.

To determine if a consensus CRE motif is sufficient to mediate Na transactivation, we compared the ability of Na to transactivate a reporter plasmid containing three consensus CRE sites upstream of a minimal adenovirus E1b promoter versus a construct containing three copies of a mutant CRE motif (Fig. 7C). These results indicated that the CRE motif is sufficient to mediate the Na transactivator effect.

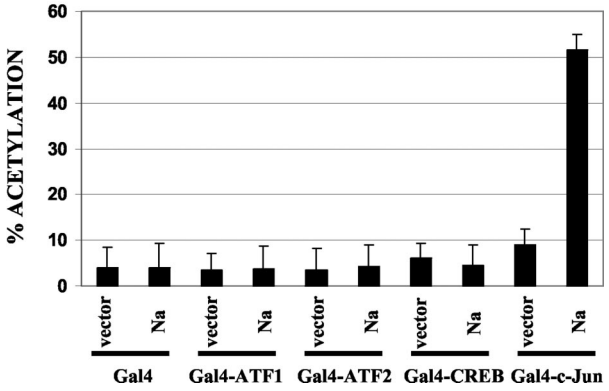

FIG. 8. Na activates c-Jun transactivator function. HeLa cells were transfected with Gal4-EIB-CAT in conjunction with a vector expressing one of the Gal4-fusion proteins (Gal4-ATF1, Gal4-ATF2, Gal4-CREB, or Gal4–c-Jun) or the control vector expressing Gal4 alone (Gal4). In addition, either pRC-FLAG-BRRF1 (Na) or pRC (vector) was also transfected into the cells. Cells were harvested and assayed for CAT activity at 48 h posttransfection. The data shown represent the results of two independent experiments.

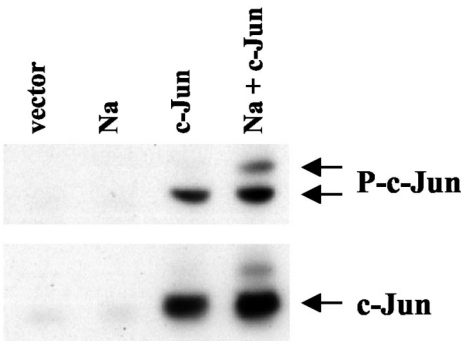

FIG. 9. Na increases levels of hyperphosphorylated c-Jun. HeLa cells were transfected with pRC (vector), pRC-FLAG-BRRF1 (Na), pCMV-c-Jun (c-Jun), or pRC-FLAG-BRRF1 plus pCMV-c-Jun (Na+c-Jun). Harvesting occurred at 48 h posttransfection, and 35 μg of protein was immunoblotted for c-Jun phosphorylated on Ser73 (P-c-Jun) (upper panel) or total c-Jun (lower panel).

Na activates c-Jun transactivator function. The ZII CRE site of Zp binds the transcription factors CREB, ATF-1, ATF-2, and c-Jun (1, 34, 37, 63), all of which have been shown to activate Zp in reporter assays (1, 34, 63). Since Na activates Zp through the ZII CRE site, we examined whether Na can activate CREB, ATF-1, ATF-2, or c-Jun fusion proteins linked to the Gal4 DNA binding domain. The abilities of ATF-1, ATF-2, CREB-1, and c-JUN Gal4 fusion proteins to activate a reporter plasmid containing five copies of the Gal4 DNA binding site upstream of the CAT gene, in the presence or absence of cotransfected Na, were examined in HeLa cells. As seen in Fig. 8, Na specifically activated the c-Jun transcriptional function, while it did not significantly affect the function of ATF-1, ATF-2, or CREB-1. Since the DNA binding activity in these experiments was mediated through the Gal4 DNA binding domain of the fusion proteins, these results suggest that Na activates promoters by enhancing the c-Jun transcriptional function.

Na increases levels of hyperphosphorylated c-Jun. Efficient c-Jun transcriptional functioning requires the phosphorylation of serines 63 and 73 in c-Jun by JNK (12, 25, 30). c-Jun phosphorylated at Ser63 and Ser73 is more stable (41) and has an enhanced ability to transactivate target promoters (43). We therefore determined if Na expression affects c-Jun phosphorylation. The c-Jun phosphorylation status was monitored by using an antibody specific for c-Jun phosphorylated at Ser73 versus an antibody that recognizes total c-Jun. Although HeLa cells transfected with c-Jun alone displayed a low level of basal c-Jun phosphorylation (Fig. 9, P-c-Jun, lower arrow), the presence of Na significantly increased the amount of heavily phosphorylated c-Jun (P-c-Jun, upper arrow). The specificity of the phospho-c-Jun antibody for phosphorylated c-Jun was verified by using blocking peptides specific for phospho-c-Jun (data not shown). The slightly increased level of total c-Jun in the presence of Na possibly reflects the increased stability of c-Jun in response to phosphorylation (41).

DISCUSSION

The switch from a latent to lytic infection during the EBV life cycle is a tightly regulated process in which the expression of the IE genes Z and R plays a pivotal role in initiating the transition. The expression of either IE protein results in expression of the other, and this ability of Z or R to turn on the promoter of the other corresponding IE gene is critical for the efficient induction of lytic infections (2, 14, 68). The IE region of EBV expresses a gene within the intronic sequence of R, designated Na or BRRF1, that is transcribed with early kinetics in response to Z (53). Na encodes a 34-kDa nuclear protein whose function in the viral life cycle was previously unknown. In this report, we demonstrated that Na functions as a viral transactivator that enhances the ability of R to disrupt latency in a 293 cell line carrying a recombinant EBV defective for expression of both R and Na. The expression of R in conjunction with Na results in increased levels of viral lytic genes compared to expression of R alone. In addition, the presence of Na also results in a fivefold increase in the amount of infectious virus produced after an R-induced lytic infection.

The ability of Na to enhance R-induced lytic infection in 293 cells appears to be at least partially mediated through Na's ability to activate the Z promoter. Increased levels of Z were observed in the presence of R and Na in combination compared to R alone, suggesting that the presence of Na increased the transcription of Z from the endogenous genome. Although Na alone could not activate Z transcription in the context of the intact viral genome, Na alone was able to specifically transactivate a Zp reporter construct in EBV-negative HeLa cells. The ability of Na to activate Zp required the CRE (ZII) site, and the consensus CRE site was shown to be sufficient to confer Na responsiveness to a heterologous promoter. These data suggest that the CRE element is the primary target of Na-induced transactivation.

The Zp CRE site binds ATF-1, ATF-2, c-Jun, and CREB (1, 34, 37, 63). Of these, the phospho-ATF-2/phospho-c-Jun heterodimer may represent the predominant complex responsible for R activation of the Zp CRE site (1). We showed here that Na specifically activates the transcriptional function of c-Jun while not affecting the transcriptional function of ATF-1, ATF-2, or CREB-1. Thus, Na likely activates Zp by enhancing c-Jun transcriptional functioning. This activation of c-Jun by Na appears to be independent of changes in c-Jun DNA binding, as the activity of a Gal4–c-Jun fusion protein (in which DNA binding was mediated by Gal4) was also activated by Na. Since the phosphorylation of c-Jun residues Ser63 and Ser73 is required for efficient c-Jun transcriptional functioning, we examined the effect of Na on c-Jun phosphorylation. We found that Na increases the levels of a hyperphosphorylated form of c-Jun, suggesting that Na activates c-Jun by increasing its phosphorylation. Since the phosphorylation of c-Jun residues Ser63 and Ser73 is commonly mediated by JNK (12), these results indicate that Na may activate JNK. However, the exact mechanism by which Na modulates c-Jun phosphorylation has not yet been determined. Given that the ZII element is responsive to transforming growth factor beta and binds SMADs (33), it is possible that Na modulates SMADs in addition to c-Jun in order to transactivate Zp through ZII; this possibility remains to be examined.

An interesting result of these studies was the finding that while Na efficiently activates Zp in reporter gene assays, it does not by itself activate BZLF1 transcription from the intact viral genome in cells that are latently infected with EBV. Instead,

Na primarily enhances the ability of R to activate BZLF1 transcription from the endogenous viral genome in certain settings. We speculate that the Na transactivator function, which is mediated through the c-Jun transcription factor, is inhibited by the epigenetic modification of the EBV genome in latently infected cells, which includes DNA methylation of the BZLF1 promoter region as well as an inactive (deacetylated) state of the chromatin surrounding Zp (22). R-mediated activation of Zp is also thought to be mediated through cellular factors binding to the CRE motif, suggesting that the relative inability of R to efficiently activate Z transcription in certain cell types (such as lymphoblastoid lines) may likewise reflect the effect of inhibitory epigenetic modifications of the viral genome. Notably, MEF2D binding to the ZI motifs in Zp has been previously shown to modulate the histone acetylation of Zp in the context of the intact viral genome (22). Zp reporter constructs may not accurately mimic the state of Zp in the context of the intact viral genome, particularly in regard to the effects of chromatin structure and DNA methylation.

The relative contributions of the various cellular transcription factors (ATF-1, CREB-1, ATF-2, and c-Jun) which activate Zp transcription through the CRE motif to reporter gene assays are not currently understood with regard to their importance for inducing BZLF1 transcription in the context of the intact viral genome. It is possible that the state of Zp methylation and/or chromatin acetylation differentially influences the ability of these cellular factors to activate Zp transcription from the endogenous viral genome. In addition, cell-type-dependent factors may influence the relative importance of each transcription factor in activating BZLF1 transcription from the viral genome. In situations in which the combination of ATF-2 and c-Jun is sufficient for activating BZLF1 transcription from the endogenous viral genome, the synergistic effect of R and Na together may reflect the ability of R to activate p38 kinase (which phosphorylates and activates ATF-2) combined with the ability of Na to enhance the level of heavily phosphorylated c-Jun. In cell types in which R and Na together do not efficiently induce BZLF1 transcription (such as lymphoblastoid cells), it remains possible that activated CREB-1 and/or ATF-1 could efficiently induce BZLF1 transcription from the intact viral genome. Our finding that Na enhances R-induced lytic infections in certain epithelial lines, but not in two B-cell lines, suggests that c-Jun activation may be more important for BZLF1 transcription in some cell types than in others. Hence, Na may be more important for viral replication in some cells and/or tissues than in others.

Our results suggest (but do not prove) that Na may activate JNK. Alternatively, Na may inhibit the dephosphorylation of, or degradation of, phosphorylated c-Jun. JNK, along with p38, is a member of the SAPK family, and both of these kinases activate c-Jun and/or ATF-2 in response to cellular stress (12, 23, 47, 62). SAPK activation is a common theme among herpesviruses, with herpes simplex virus type 1, CMV, Kaposi's sarcoma-associated herpesvirus, and EBV all inducing p38 and/or JNK during infection (1, 3, 26, 40, 66, 67). Cross-linking of the B-cell receptor, an event that is thought to be a physiologically important stimulus for the in vivo reactivation of EBV (57), also results in JNK and p38 activation (56). Inhibition of the SAPKs results in a reduced efficiency of lytic infection for alpha-, beta-, and gammaherpesviruses (1, 27, 40), suggesting that viruses have evolved to utilize the cellular SAPK pathway to both trigger and facilitate lytic infection. Members of our lab have previously demonstrated that both Z and R activate the SAPKs and that R-mediated SAPK induction is required for the efficient activation of Zp by R (1). The ability of yet another lytic EBV protein, Na, to increase the levels of hyperphosphorylated c-Jun suggests that activated c-Jun is indeed important for efficient lytic EBV infection and that there may be multiple, redundant mechanisms encoded by the virus to ensure that this activation occurs.

In summary, we demonstrated here for the first time a function of the early Na gene product of EBV. Na enhances the ability of R to induce lytic infection by cooperating with R to activate the Z promoter, and this effect of Na appears to be more important in some cell types than in others. Whether there are additional Na-responsive viral promoters in the EBV genome (in addition to Zp) remains an open question. Our results suggest the existence of a previously unrecognized autostimulatory pathway for lytic EBV induction in which an initial activating stimulus (e.g., differentiation) which induces a low-level activation of either Zp or Rp would subsequently be amplified by the ability of Na to further induce Zp activation. Future insights into the mechanism of the Na function should allow a more thorough understanding of lytic cycle regulation, and given the cell type dependence of Na activity, could shed light on the cellular and viral characteristics that influence viral permissiveness.

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