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Late Gene Expression from the Epstein-Barr Virus BcLF1 and BFRF3 Promoters Does Not Require DNA Replication in *cis*

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Late gene expression follows and is dependent upon lytic replication of the viral genome. Although experimental evidence is lacking, lytic viral DNA replication is believed to remove modifications or binding factors from the genome which serve to repress late gene expression during latency or the early lytic cycle. We have developed a reporter assay to begin characterizing the mechanisms that regulate late gene expression in Epstein-Barr virus (EBV). In this model system, the activities of late promoter-reporter fusions are measured following transient transfection into tissue culture cells expressing EBV during different stages of the lytic cycle. This system faithfully recapitulates late expression patterns from the endogenous virus, implicating specific *cis*-active sequences in the control of late gene expression. In addition, these promoters respond only indirectly to the viral immediate-early transactivator, ZEBRA. This indirect response is mediated by other viral or virally induced activities downstream of ZEBRA in the lytic cascade. In this system, late gene expression is sensitive to inhibitors of the viral DNA polymerase such as phosphonoacetic acid, although the reporters lack a eukaryotic origin of replication and are not replicated under the assay conditions. Thus, replication of the transcriptional template is not a prerequisite for expression with late kinetics, a finding inconsistent with the current models which posit a *cis*-active relationship between lytic EBV DNA replication and late gene expression. Rather, analysis of this system has revealed a *trans* relationship between late gene expression and viral DNA replication and highlights the indirect and complex link between these two events.

The lytic cascade of Epstein-Barr virus (EBV) infection is divided into three phases of regulated gene expression, immediate early, early, and late (16). Synthesis of a single virally encoded transactivator, ZEBRA (3, 4), serves as a checkpoint for initiation of the replicative cycle. This immediate-early protein directly or indirectly activates expression of early viral genes, many of which regulate lytic replication of the viral genome (16). Amplification of viral DNA, initiating at the lytic origin of replication (5, 13), defines the boundary between early and late gene expression (16). Structural proteins synthesized during the late phase are assembled into virions which package the viral DNA and are ultimately released as infectious particles (16).

While events leading to the initiation of lytic replication of the viral genome have been extensively characterized at the molecular and biochemical levels, information regarding the regulation of late events in EBV infection is scarce. Late gene expression is blocked at the protein and steady-state mRNA levels by inhibitors of the viral DNA polymerase, BALF5 (19), such as phosphonoacetic acid (PAA) (24, 27), phosphonoformic acid (6), and acyclovir (14), suggesting a dependence upon the activity of BALF5 and presumably lytic replication of the viral DNA. In addition, the Raji cell line (22), which contains a deletion in the virally encoded single-strand binding protein BALF2 (1), is defective for lytic viral DNA replication and late gene expression (7). This link to viral DNA replication, however, appears to be independent of template methylation, as inhibitors of DNA methyltransferase do not induce synthesis of late products in Raji cells (28) or HH514-16 cells (11). These observations suggest that late gene expression is subject to uncharacterized regulatory mechanisms which likely constitute an important checkpoint within the viral lytic cascade.

In an effort to characterize these mechanisms, we have designed a transient-transfection-based reporter assay for late gene expression in an EBV-infected human B-cell line, HH514-16. This assay faithfully reproduced late regulatory mechanisms in EBV-infected B cells. The late promoters were active only in cell lines containing lytically replicating EBV and were not directly activated by ZEBRA alone in uninfected cell lines. Based on the information obtained in these experiments, the relationship of late gene expression and viral DNA replication appeared to be more complex than the removal of repressive modification or binding proteins from the transcriptional template. This assay has permitted us to analyze the *cis* regulation of late gene expression in detail (unpublished data).

MATERIALS AND METHODS

Cells. Lymphocyte cell lines were grown in RPMI 1640 medium supplemented with 8% fetal bovine serum. BJAB is an EBV-negative human B-cell line derived from a Burkitt lymphoma (20). P3J-HR-1 clone 16 (HH514-16) is a clonal human B-cell line derived from an EBV-positive Burkitt lymphoma (23). Raji is a human B-cell line derived from a Burkitt lymphoma harboring a defective EBV, which is unable to replicate viral DNA or express late viral genes (22).

Chemical induction and inhibition. Lymphocyte cell lines were subcultured 6 days prior to harvesting. Cells were treated on day 3 with 10 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) per ml and 3 mM sodium butyrate (NB) to induce the lytic cycle. In order to block viral DNA replication and, consequently, late gene expression, cells were treated with 0.4 mM PAA concomitantly with the inducing chemicals.

Antisera. SJ is an EBV-positive human serum from a healthy donor with reactivity to EBNA-1 and BFRF3 (26). R3.1 is a monoclonal antibody to BMRF-1 (21). C is a polyclonal rabbit antiserum raised to a trpE-ZEBRA (amino acids 1 through 167) fusion protein (29).

Reporter and expression plasmids. BcLF1 CAT was constructed by cloning a fragment from the *Bgl*I site (filled in with T4 DNA polymerase) to the *Xho*I site of 1,195 bp (nucleotides 138700 to 137505 in B95-8 sequence [1]) into a fragment from the *Hin*dIII site (filled in with T4 DNA polymerase) to the *Sal*I site of pCAT Basic (Promega). BFRF3 CAT was constructed by first cloning the *Hin*dIII-to-*Sal*I fragment (nucleotides 60585 to 61439 [1]) of BFRF3CZΔ (a gift

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of Jill Countryman) into a *Hin*dIII/*Xho*I digest of E4CAT to yield BFRF3/E4CAT. A fragment from the *Nde*I site (filled in with T4 polymerase) to the *Pst*I site from BFRF3/E4CAT was then cloned into pCAT Basic, which was digested with *Xba*I, filled in with T4 DNA polymerase, and digested with *Pst*I to yield BFRF3 CAT. BMRF1 CAT was generated by cloning a 333-bp fragment from the *Bam*HI site (filled in with T4 DNA polymerase) to the *Rsa*I site (nucleotides 79537 to 79871 [1]) into the *Sal*I site of pCAT Basic, which had been filled in with T4 DNA polymerase. Wp1168 luciferase, a 1,345-bp *Bam*HI-to-*Avr*II fragment of *Bam*HI W (nucleotides 13214 to 14559 [1]) that was cloned into pGL2 Basic (Promega) which had been digested with *Bgl*II and *Nhe*I, was a generous gift of Alan Rickinson. pGL2 Promoter (Promega) contains the simian virus 40 (SV40) minimal promoter. Basic + HMP, containing the human cytomegalovirus immediate-early minimal promoter cloned into the *Hin*dIII site of pGL2 Basic, was a kind gift of David Schatz. The vector, BXG1, and the ZEBRA expression plasmid, BXG1-gen ZEBRA, have been previously described (10). pBS + *Bam*HI H contains the *Bam*HI H fragment of EBV (nucleotides 48848 to 54853 [1]) in the *Bam*HI site of pBluescript KS(−) (Stratagene).

Transfections. Lymphocyte cells were resuspended in RPMI 1640 supplemented with 8% fetal calf serum at 1.5×10^7 cells/0.5 ml in an electroporation cuvette with a 0.4-cm gap. Ten micrograms of each CAT reporter plasmid and 1 μg of each luciferase reporter plasmid were added to the cuvettes. In cases where ZEBRA expression was used to induce the lytic cycle, 5 μg of either BXG1 or BXG1-gen ZEBRA was also added to the cuvettes. The cells were exposed to 0.25 kV and 960 μF with a Bio-Rad Gene Pulser. Growth medium containing 10 ng of TPA per ml, 3 mM NB, and/or 0.4 mM PAA, where appropriate, was added to achieve a 10-ml total volume.

Analysis of mRNAs. Seventy-two hours after transfection and treatment with inhibitors and/or inducers of the lytic cycle, cytoplasmic RNA samples were prepared as previously described (18). RNA representing 2.5×10^6 cells per lane was electrophoresed in a 1% agarose–6% formaldehyde gel in 20 mM MOPS (morpholinepropanesulfonic acid; pH 7). The gel was transferred to nytran and hybridized with probes radiolabelled with ^{32}P by the random-priming method. The probe derived from BFRF3 was a 754-bp *Bam*HI/*Pst*I fragment (nucleotides 61501 to 62255 [1]), the probe derived from BcLF1 was a 600-bp *Sac*I/*Nsi*I fragment (nucleotides 137680 to 138066 [1]), and the probe derived from BMRF1 was an *Eag*I 531-bp fragment (nucleotides 80141 to 80672 [1]). The blots were also probed with a 1.8-kb portion of β-actin cDNA.

Reporter assays. Seventy-two hours after transfection and induction into the lytic cycle, cells were harvested by centrifugation and washed once in phosphate-buffered saline. Cells were resuspended in 1× reporter lysis buffer (Promega) at 8.1×10^4 cells/μl. The suspension was incubated at 25°C for 15 min and subsequently centrifuged at 12,000 × *g* at 25°C for 15 s. The supernatant was removed to a new tube, and the pellet was resuspended in sodium dodecyl sulfate (SDS) sample buffer at 10^6 cells/5 μl. Fifty-nine microliters of the supernatant was incubated at 60°C for 10 min to inactivate endogenous deacetylases. A chloramphenicol acetyltransferase (CAT) assay (12) was then performed on this extract in 500 mM Tris-HCl (pH 7.8)–0.5 mM acetyl coenzyme A–1 μCi of [^{14}C]chloramphenicol at 37°C for 3 h. The [^{14}C]chloramphenicol was then extracted with 0.5 ml of ethyl acetate, spotted on thin-layer chromatography plates (Baker Chemical Co.), and chromatographed ascending in chloroform-methanol (95:5) until the front reached the top of the plate. The thin-layer chromatography plates were exposed to Kodak XAR-5 film overnight, and the spots were quantitated by scintillation counting. Twenty microliters of the extract, which was not heated to 60°C, was used to measure luciferase activity by incubation with 100 μl of luciferase assay substrate (Promega). Light units were counted in duplicate for 10 s in a Lumat luminometer. Relative CAT activity was standardized to the corresponding luciferase activity.

Protein electrophoresis and immunoblotting. Extracts were heated to 100°C for 5 min and separated on discontinuous SDS–10% polyacrylamide gels. Separated proteins were electrotransferred to nitrocellulose membranes at 4°C. The filters were blocked in 5% nonfat dry milk at 4°C overnight. Membranes were incubated with serum diluted in 5% nonfat dry milk for 1 h at 25°C, washed in 1× TS (10 mM Tris [pH 7.5], 200 mM NaCl, 5% Tween 20), incubated with ^{125}I-protein A for 1 h at 25°C, and washed in 1× TS. Membranes were exposed to Kodak XAR-5 film in the presence of an intensifying screen overnight at −70°C.

Isolation of total DNA and Southern blotting. Total cellular DNA was isolated from 1.5×10^7 cells following transfection. Cells were pelleted by centrifugation, washed in 1× PBS, and resuspended in 4 ml of buffer (0.25 M Tris [pH 8.5], 0.125 M EDTA [pH 8.0]). Pronase was added to a concentration of 1.25 mg/ml, SDS was added to 1.25%, and the suspension was incubated at 60°C for 2 h. The reaction mixture was incubated on ice for 5 min, and 0.2 volume of 5 M potassium acetate was added. Following a 20-min incubation on ice, the sample was spun at 12,000 rpm at 4°C for 15 min. The supernatant was digested with RNase A at a concentration of 20 μg/ml at 37°C for 15 min. Two volumes of 95% ethanol was added, and the DNA was precipitated at −20°C overnight. The DNA was collected by centrifugation (12,000 rpm, 4°C, 15 min) and resuspended in H_2O. Ten micrograms of DNA was digested with either *Dpn*II or *Sau*3AI (New England BioLabs) at 37°C overnight. Digests were electrophoresed at 70 V overnight in a 1.5% agarose–1× Tris-borate-EDTA gel. The DNA was transferred to nitrocellulose (Schleicher & Schuell, Inc.) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) overnight and prehybridized in 50% formamide–1 M NaCl–50 mM Tris (pH 8.3)–1 mM EDTA (pH 8.0)–10× Denhardt's solution–0.1% SDS–0.25 mg of salmon sperm DNA per ml for 3 h at 42°C. The blots were hybridized with random-primed ^{32}P-labelled probes corresponding to the 563-bp *Nco*I-to-*Xba*I fragment of pCAT Basic (Promega), the *Bam*HI W fragment of EBV (nucleotides 13215 to 16287 in the B95-8 sequence), or the 484-bp *Pvu*I-to-*Ssp*I fragment of pBluescript KS(+) (Stratagene) at 42°C for 16 h. The blots were postwashed in 3× SSC–0.1% SDS–0.2% $NaPP_i$–1× Denhardt's solution for 5 min at 65°C, in 3× SSC–0.1% SDS–0.2%–$NaPP_i$ for 1 h at 65°C, and in 0.3× SSC for 20 min at 65°C. Blots were exposed to Kodak XAR-5 film for 4 h.

RESULTS

Expression of reporter genes from late viral promoters requires an active viral DNA polymerase. To characterize the regulatory mechanisms governing late gene expression in EBV, three viral promoters active during the EBV lytic cycle, namely, the major capsid antigen, BcLF1, and the small viral capsid antigen, BFRF3 (both of which are late promoters), and the early BMRF1 promoter for the viral DNA polymerase processivity factor, were cloned upstream of the coding sequence for CAT on plasmids lacking eukaryotic origins of replication (Fig. 1). The activities of these promoters were determined during the early phase or the late phase of the lytic cycle in a single EBV-positive cell line by adding or omitting, respectively, the viral DNA polymerase inhibitor PAA. However, a transfection control with strong activity in both early- and late-expressing cells was necessary in order to compare the activities of a given promoter during these two stages. We used a luciferase reporter under the control of the EBV latent promoter, Wp, for this purpose. In HH514-16 cells induced with a combination of TPA and NB the luciferase activity from Wp was identical early, in the presence of PAA, and late, in the absence of PAA, in the lytic cycle (Fig. 2). By cotransfecting this luciferase reporter with the lytic CAT reporters, we could control for transfection efficiency and, thus, compare the early and late activities of any lytic promoter.

The normalized activities of three lytic cycle promoters were determined in the early and late phases of the lytic cycle following transient transfection into HH514-16 cells (Fig. 3A). During the early lytic cycle in the presence of PAA, the activities of the late promoters, BcLF1 and BFRF3, were identical to that of the vector control, pCAT Basic. However, during the late phase of the lytic cycle, in the absence of PAA, both promoters strongly directed the expression of CAT. The induction of late expression in each case was roughly 20-fold over the early activity (Fig. 3A). In comparison, the early BMRF1 promoter efficiently induced CAT expression in the presence of PAA, a stimulation of 20-fold over the vector control, pCAT Basic (Fig. 3A). This early activity was unaltered during the late phase (Fig. 3A).

Expression of reporter genes mirrors expression from the corresponding endogenous viral gene. CAT activity was compared with expression of the corresponding gene products from the endogenous virus by collecting total RNA for Northern analysis, total cellular lysate for immunoblotting, and protein extracts for reporter assays from each transfection into HH514-16 cells (Fig. 3A). Stable messages for the BcLF1 and BFRF3 gene products were detected only in the absence of PAA (Fig. 3B, lanes 2, 4, and 6). In contrast, BMRF1 message was detectable in both the presence and absence of PAA (Fig. 3B, lanes 1 through 6). These results were also apparent at the protein level (Fig. 3C). In all cases, reporter activities exactly mirrored expression of the corresponding endogenous viral gene in the same cells. In addition, this assay did not affect expression from the endogenous viral genome; the same viral gene products were expressed with the same kinetics regardless of whether the plasmid transfected contained an early or a late

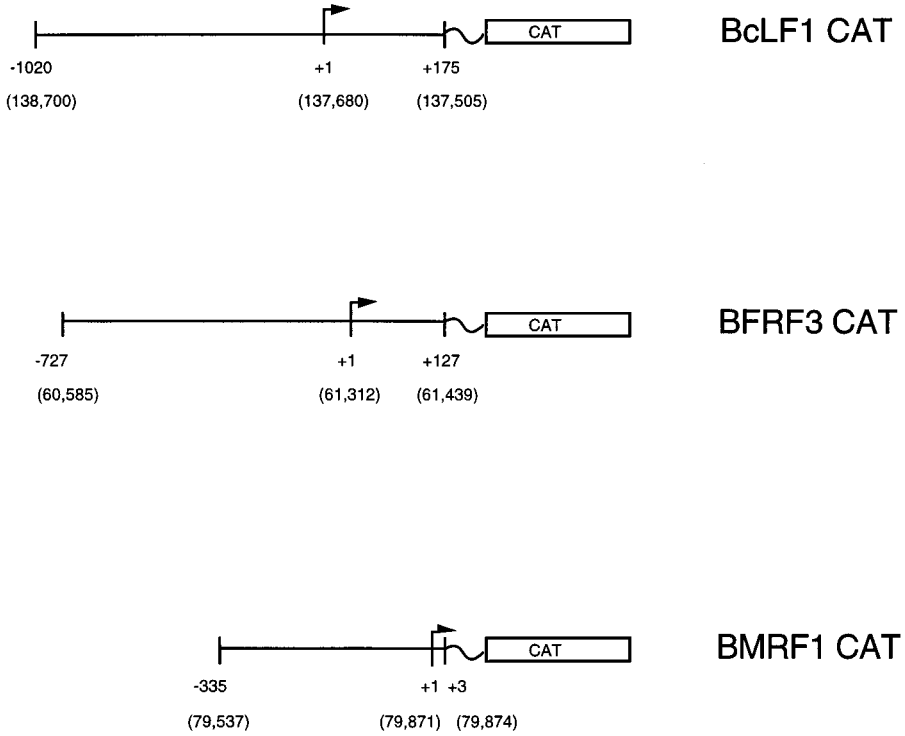

FIG. 1. Lytic promoter constructs. Shown are reporter constructs for three lytic cycle promoters, BcLF1 (late), BFRF3 (late), and BMRF1 (early). Numbering relative to the transcriptional start sites is shown beneath each construct, and position in the B95-8 sequence is shown in parentheses (1).

promoter (Fig. 3B or C; compare lanes 1 and 2 with 3 and 4 or 5 and 6). This reporter assay, therefore, faithfully mirrored lytic cycle expression patterns from the viral genome and was an accurate model for regulation of late gene expression.

Lytic cycle promoters are unresponsive to induction stimuli in the EBV-negative cell line BJAB. Next, we determined whether the lytic cycle promoter activities observed in HH514-16 cells (Fig. 3A) were virus dependent and not a response to the pleiotropic cellular activation caused by TPA and NB. The activities of these constructs were analyzed in the EBV-negative cell line BJAB. As shown in Fig. 4, the activities of the lytic cycle promoters, BcLF1, BFRF3, and BMRF1, were comparable to that of the vector control in BJAB cells. None of the lytic cycle promoters responded directly to treatment with TPA and NB in BJAB cells (Fig. 4). In contrast, the HCMV minimal promoter, Basic + HMP (Fig. 4, right side), was strongly induced (20-fold) by treatment with TPA and NB. We conclude, therefore, that the lytic cycle promoter activities observed in HH514-16 cells were an indirect response to TPA and NB likely mediated by a viral or virally induced *trans* function.

Lytic cycle promoters drive stage-specific expression of reporter genes in response to ZEBRA in HH514-16 cells. We analyzed the activities of the late gene reporters in HH514-16 cells induced into lytic replication by ZEBRA expressed from a heterologous promoter in order to confirm that their expression was specifically dependent upon the lytic cycle of EBV. The activity of Wp is repressed by the high levels of ZEBRA expressed from the heterologous promoter (15, 26a) and, therefore, was not suitable as an internal control. Instead, a luciferase reporter driven by the SV40 minimal promoter, pGL2 Promoter, was used as a transfection efficiency and standardization control in these experiments. The activity of pGL2 Promoter was unaltered by ZEBRA expression in the presence or absence of PAA in HH514-16 cells (Fig. 5A). The activities of the two late promoters, BcLF1 and BFRF3, were strongly induced (24- and 45-fold, respectively) by ZEBRA expression only in the absence of PAA, during the late phase of the lytic cycle (Fig. 5B). In contrast, the early BMRF1 promoter responded nearly identically to ZEBRA expression in both the presence and absence of PAA (Fig. 5B), representing a 30- to 45-fold induction relative to the vector control. The levels of ZEBRA expression from the SV40 promoter were unaltered

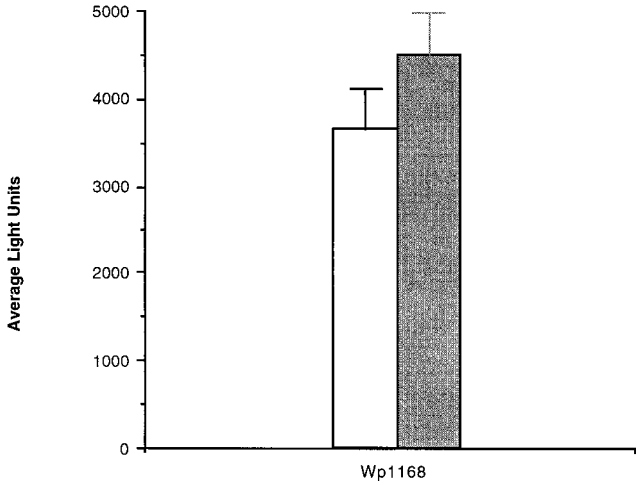

FIG. 2. Response of Wp1168 luciferase to chemical induction in HH514-16 cells. The white bar represents the activity of Wp1168 luciferase in HH514-16 cells treated with a combination of PAA, TPA, and NB, while the gray bar represents activity in HH514-16 cells treated with TPA and NB only. Values are averages from eight separate transfections, with error bars indicating standard errors of the means.

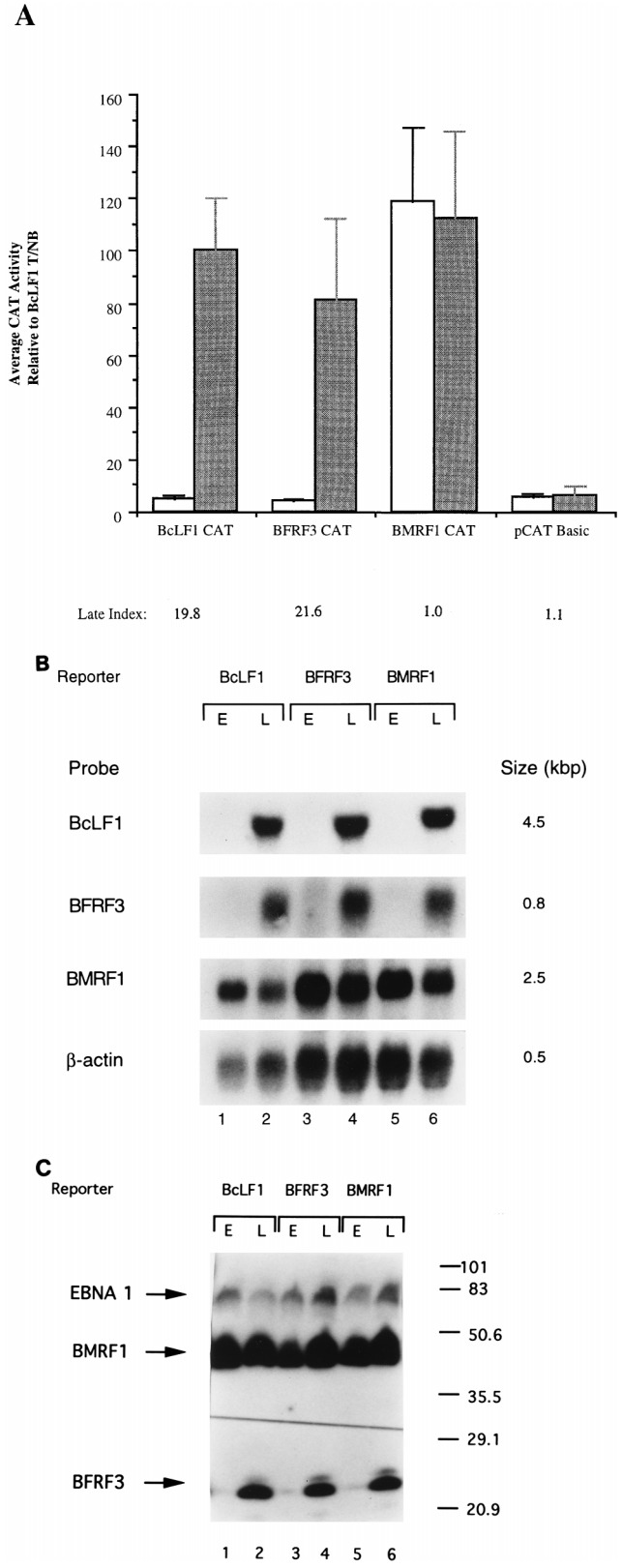

FIG. 3. Comparison of expression patterns from lytic reporter constructs and the endogenous HH514-16 virus. (A) Activities of BcLF1 CAT, BFRF3 CAT, BMRF1 CAT, and pCAT Basic in HH514-16 treated with PAA, TPA, and NB (white bars) or with TPA and NB (T/NB) alone (gray bars). Values are standardized to the luciferase activity from the cotransfected Wp1168 construct. Values represent five separate transfections, and error bars indicate standard errors of the means. For each construct, the late index was calculated by dividing the second value by the first. (B) Northern blots of total RNA prepared from the same cells transfected with the reporter plasmid indicated above each lane. E, early (cells treated with PAA, TPA, and NB); L, late (cells treated with TPA and NB alone). β-Actin was included as a control. (C) Immunoblots of protein extract isolated from HH514-16 cells transfected with the indicated reporter plasmid and treated as described for panel B. The immunoblotting sera were R3.1 (anti-BMRF1) and SJ (anti-EBNA1 and anti-BFRF3). Molecular sizes are in kilodaltons.

by the presence of PAA (Fig. 5C). Since the BcLF1 and BFRF3 promoters responded identically to induction of the lytic cycle by either chemical manipulation (Fig. 3A) or heterologous ZEBRA expression (Fig. 5B), their late behavior represented a direct response to the viral lytic cycle rather than a nonspecific stimulation by cellular factors affected by the inducing agents.

Late promoters are unresponsive to heterologous ZEBRA expression in the EBV-negative cell line BJAB. The ability of ZEBRA to stimulate the activities of the lytic cycle promoters in the absence of other viral products was determined in the EBV-negative cell line BJAB. The activity of the HCMV minimal promoter, as assayed by luciferase expression, was unaltered by the heterologous expression of ZEBRA in BJAB cells (Fig. 6A) and served as a control for these experiments. The late promoters, BcLF1 and BFRF3, did not respond to ZEBRA expression in BJAB cells and were completely inactive in contrast to the vector control, pCAT Basic (Fig. 6B). In contrast, the early BMRF1 promoter was stimulated 20-fold by the expression of ZEBRA in BJAB cells (Fig. 6B). Although the expression of ZEBRA was variable in this experiment (Fig. 6C), in other experiments large amounts of ZEBRA, comparable to those in lane 4, were never able to activate expression from the late promoters in BJAB cells (data not shown). Since ZEBRA expression was not sufficient to activate the late promoters in the absence of EBV, these promoters must have responded indirectly to ZEBRA expression in HH514-16 cells (Fig. 5B), through another viral or virally induced function downstream of ZEBRA in the lytic cascade.

Late gene expression requires lytic viral DNA replication in *trans* but not in *cis*. Characterization of this reporter system clearly demonstrated that late gene expression was controlled by specific *cis*-active regulatory sequences present in the BcLF1 and BFRF3 promoters and absent from the BMRF1 promoter. Moreover, late gene expression was dependent upon a viral or virally induced function downstream of ZEBRA in the lytic cascade. Since late gene expression from the viral genome is sensitive to inhibitors of the viral DNA polymerase, replication itself was a strong candidate for the required activity. Paradoxically, the activities of BcLF1 CAT and BFRF3 CAT were also sensitive to PAA (Fig. 3A and 5B), but these constructs lacked eukaryotic origins of replication.

Although the reporter constructs lack a eukaryotic origin of replication, we monitored the methylation state of the constructs following transient transfection as a marker for DNA replication to ascertain if a cryptic origin of replication was present. BcLF1 CAT, BFRF3 CAT, BMRF1 CAT, and pCAT Basic DNAs, detected by Southern blotting for CAT sequences, were sensitive to cleavage by *Sau*3AI but resistant to cleavage with *Dpn*II prior to transfection into HH514-16 cells (Fig. 7A, top, lanes 1 and 2, 7 and 8, 13 and 14, and 19 and 20), indicating methylation at *dam* sites (GATC). Following transfection into HH514-16 cells, all constructs retained this methylation pattern and the corresponding sensitivity to *Sau*3AI and resistance to *Dpn*II (Fig. 7A, top, lanes 3 through 6, 9

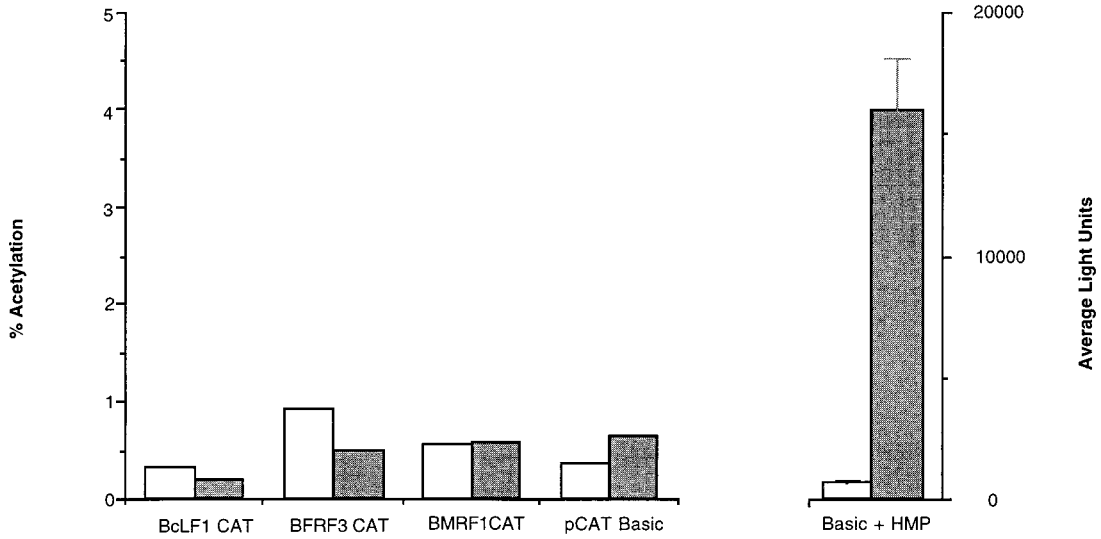

FIG. 4. Response of lytic reporter constructs to TPA-NB treatment in BJAB cells. The activities of constructs in BJAB cells that were untreated (white bars) or treated with TPA and NB (gray bars) are shown. The HCMV minimal promoter, Basic + HMP, was cotransfected with the CAT plasmids. Error bars represent standard errors of the means.

through 12, 15 through 18, and 21 through 24). Phosphorimaging and longer exposures to Kodak XAR film did not reveal sensitivity to *Dpn*II cleavage for any of the plasmids following transfection (data not shown). These results confirm that the reporters did not replicate under the assay conditions, in contrast to the endogenous viral genome, which amplifies *Bam*HI W sequences in the absence of PAA (Fig. 7A, bottom, lanes 5 and 6, 11 and 12, 17 and 18, and 23 and 24; compare lanes 3

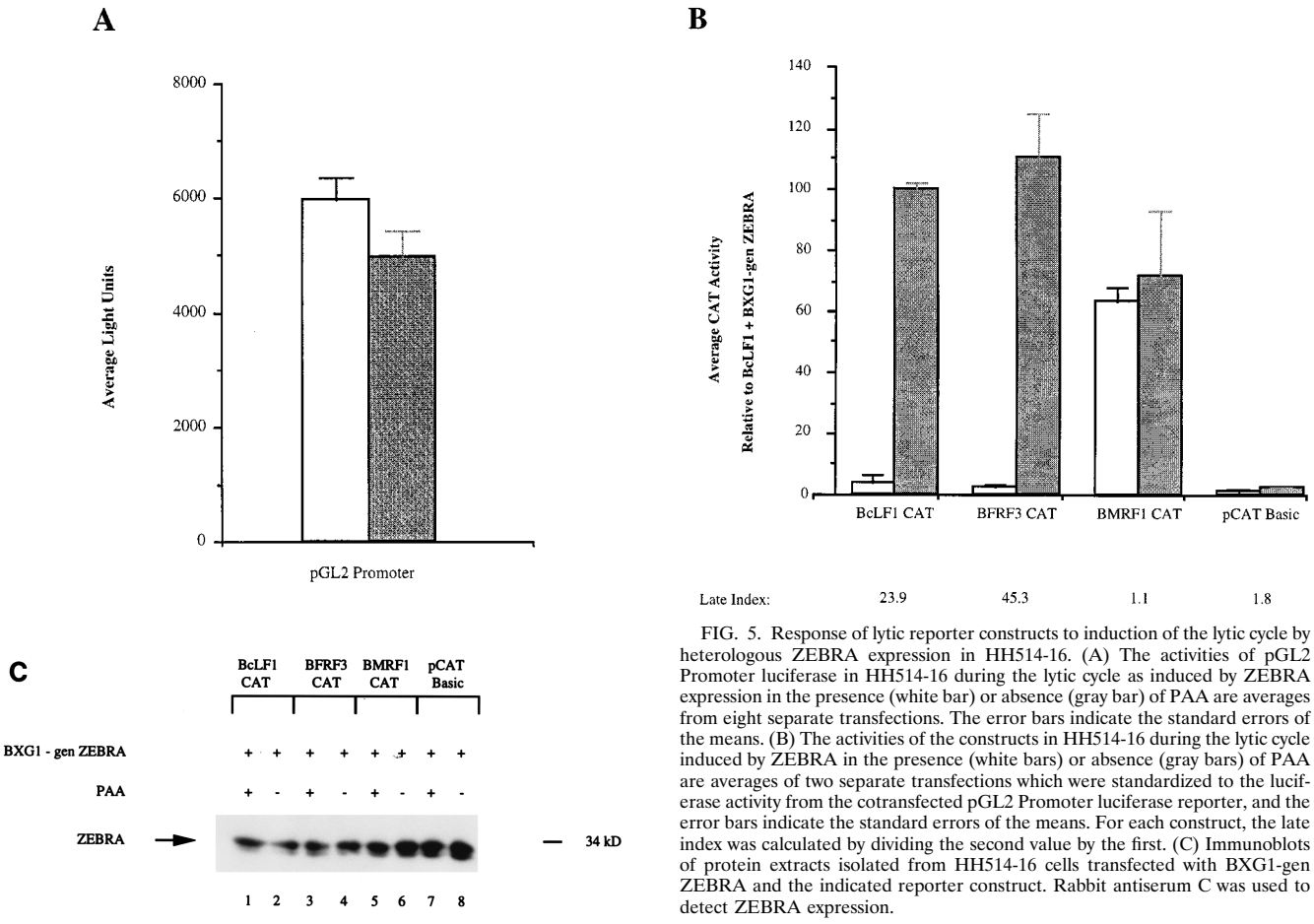

FIG. 5. Response of lytic reporter constructs to induction of the lytic cycle by heterologous ZEBRA expression in HH514-16. (A) The activities of pGL2 Promoter luciferase in HH514-16 during the lytic cycle as induced by ZEBRA expression in the presence (white bar) or absence (gray bar) of PAA are averages from eight separate transfections. The error bars indicate the standard errors of the means. (B) The activities of the constructs in HH514-16 during the lytic cycle induced by ZEBRA in the presence (white bars) or absence (gray bars) of PAA are averages of two separate transfections which were standardized to the luciferase activity from the cotransfected pGL2 Promoter luciferase reporter, and the error bars indicate the standard errors of the means. For each construct, the late index was calculated by dividing the second value by the first. (C) Immunoblots of protein extracts isolated from HH514-16 cells transfected with BXG1-gen ZEBRA and the indicated reporter construct. Rabbit antiserum C was used to detect ZEBRA expression.

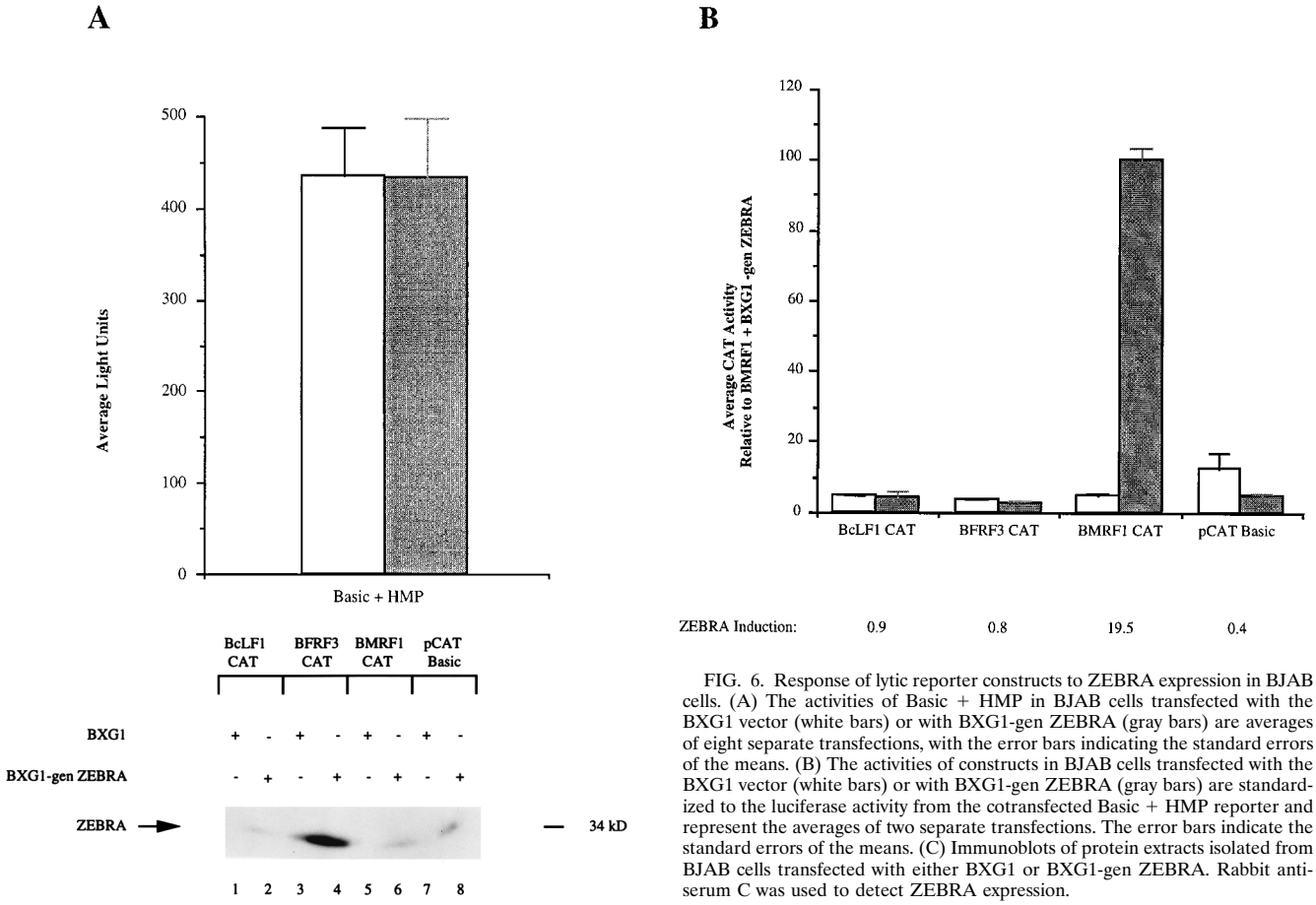

FIG. 6. Response of lytic reporter constructs to ZEBRA expression in BJAB cells. (A) The activities of Basic + HMP in BJAB cells transfected with the BXG1 vector (white bars) or with BXG1-gen ZEBRA (gray bars) are averages of eight separate transfections, with the error bars indicating the standard errors of the means. (B) The activities of constructs in BJAB cells transfected with the BXG1 vector (white bars) or with BXG1-gen ZEBRA (gray bars) are standardized to the luciferase activity from the cotransfected Basic + HMP reporter and represent the averages of two separate transfections. The error bars indicate the standard errors of the means. (C) Immunoblots of protein extracts isolated from BJAB cells transfected with either BXG1 or BXG1-gen ZEBRA. Rabbit antiserum C was used to detect ZEBRA expression.

and 4, 9 and 10, 15 and 16, and 21 and 22). This assay was sensitive enough to detect replication of a transfected plasmid containing the EBV lytic origin of replication, pBS + *Bam*HI H, in the absence of PAA (Fig. 7B, top, lane 5; compare lane 3) by probing for vector sequences within the bacterial f1 origin of replication following 4-h exposure to Kodak XAR film in the absence of an intensifying screen. As a control, BcLF1 CAT DNA produced in a *dam* mutant strain of *E. coli* had identical sensitivity to *Dpn*II and *Sau*3AI both before (Fig. 7A, lanes 25 and 26) and after (Fig. 7A, lanes 27 through 30) transfection into HH514-16 cells. This assay effectively uncoupled DNA replication and late gene expression, implicating a *trans* rather than a *cis* dependence.

Raji cells do not support late gene expression from reporter constructs. To examine the possibilities that late gene expression was dependent on a function of the DNA polymerase other than replication or that the activities of these reporter constructs were nonspecifically inhibited by PAA, their activities were analyzed in Raji cells, which were deficient in lytic viral DNA replication due to a deletion in BALF2, the virally encoded single-strand binding protein (7), but still expressed the viral DNA polymerase, BALF5. Although BcLF1 and BFRF3 CAT were induced approximately 2.5-fold by ZEBRA expression in Raji cells (Fig. 8A), BMRF1 CAT was more highly induced (roughly eightfold) by ZEBRA expression in Raji cells (Fig. 8A). Relative to the vector control, BcLF1 CAT was induced 3.7-fold, and BFRF3 CAT was repressed 1.2-fold, in contrast to BMRF1 CAT, which was stimulated nearly 12-fold (Fig. 8A). This data was comparable to early levels in HH514-16 cells where BcLF1 CAT was 3.1-fold and BFRF3 CAT was 1.8-fold more active than the vector controls (Fig. 5B). The levels of ZEBRA expressed were comparable for each transfection (Fig. 8B; compare lanes 2, 4, 6, and 8). This experiment circumvented the use of a viral DNA polymerase inhibitor and demonstrated that the late promoters did not respond to another activity of the viral DNA polymerase. Taken together with the above results, this observation indicates late gene expression from cloned BcLF1 and BFRF3 promoters required lytic replication of the viral genome in *trans* but not in *cis.*

DISCUSSION

We have developed a reporter assay which accurately recapitulated the late gene expression patterns of EBV in lytically infected cells. This system eliminated the complications of cell line-specific effects, allowing a direct comparison of early and late activities within a single cellular background. Expression from the endogenous virus, at both the RNA and protein levels, as well as replication of the viral genome served as internal controls for this system (Fig. 3A and B and 7). Attempts to reproduce the control mechanisms regulating late gene expression in a reporter assay have never been successful in EBV (15) or any other virus. Establishment of this system alone, therefore, has greatly increased our knowledge of the regulation of late gene expression.

This analysis of cloned viral promoters has generated insight into the *cis* regulation of late gene expression. Late promoters

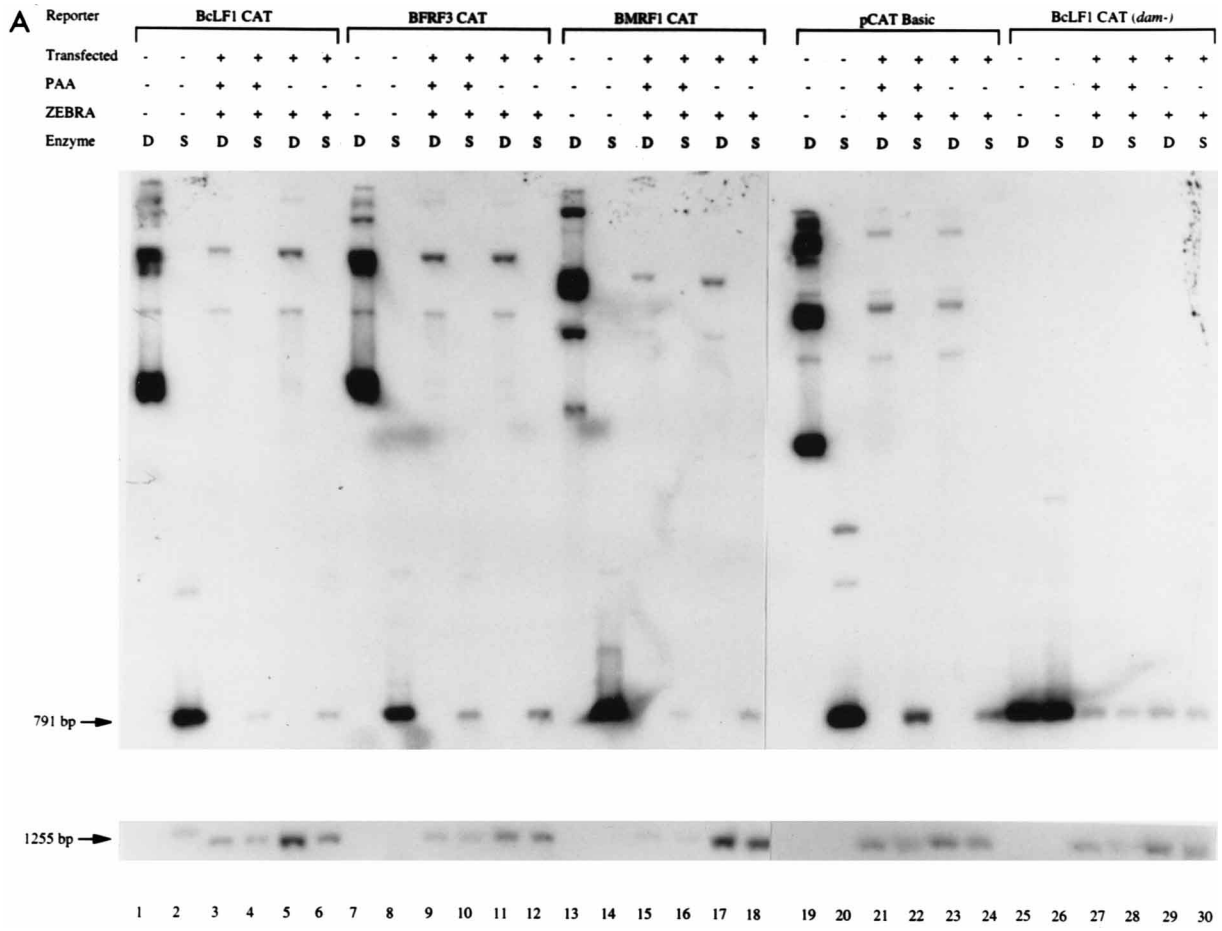

FIG. 7. Analysis of the methylation state of lytic reporter constructs following transfection into HH514-16. (A) Southern blots of plasmid DNA (lanes 1 and 2, 7 and 8, 13 and 14, 19 and 20, and 25 and 26) or total cellular DNA (lanes 3 to 6, 9 to 12, 15 to 18, 21 to 24, and 27 to 30) isolated from HH514-16 cells transfected with BXG1-gen ZEBRA (ZEBRA) and the indicated reporter constructs in the presence or absence of PAA were probed for CAT (top panel) or the *Bam*HI W fragment of EBV (bottom panel). DNAs were first digested with either *Dpn*II (D) or *Sau*3AI (S). (B) Southern blot of plasmid DNA (lanes 1 and 2) or total cellular DNA (lanes 3 to 6) isolated from HH514-16 cells transfected with BXG1-gen ZEBRA and pBS + *Bam*HI H in the presence or absence of PAA probed for the f1 origin of replication (top panel) or the *Bam*HI W fragment of EBV (bottom panel). DNAs were digested as described for panel A prior to blotting.

are intrinsically different from early promoters (Fig. 3A and 5B), suggesting a regulation by specific *cis*-active sequences present in the late promoter fragments in these constructs (Fig. 1). This functional difference between cloned viral promoters suggests that a specific late regulatory mechanism exists and that late gene expression does not result from nonspecific stimulation of transcription brought about by a global change in the basal machinery.

This assay may have provided insight into finer regulation of late gene expression in EBV. For example, the BcLF1 late promoter directs low-level early expression of reporters in response to ZEBRA (Fig. 5B and 8A; 3.1-fold and 3.7-fold, respectively, compared to the vector control). This activation is dependent upon the presence of EBV and is not a direct response to ZEBRA expression (Fig. 6B). In contrast, the BFRF3 late promoter does not direct early expression of CAT (Fig. 5B and 8A, 1.8-fold and 0.8-fold, respectively, compared to the vector control). The differences in activities of these two

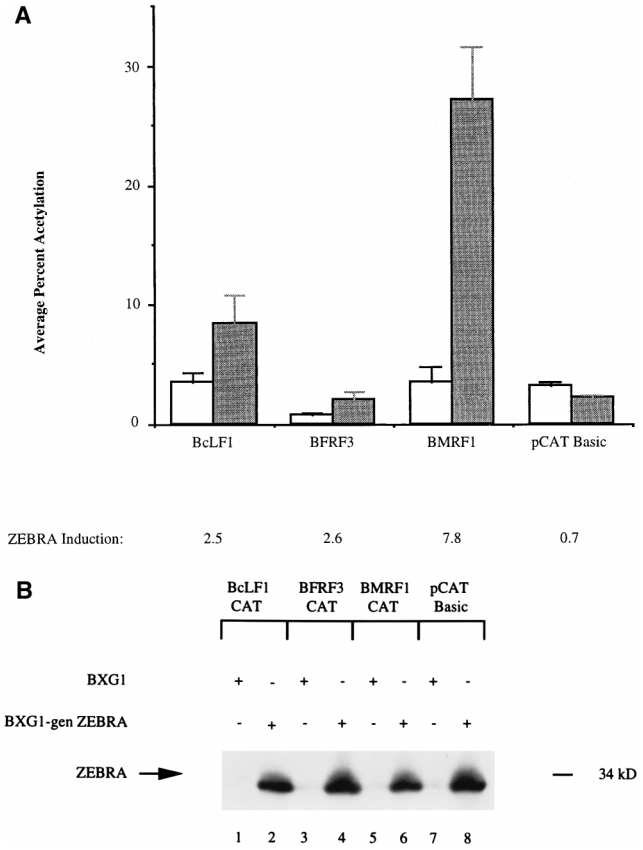

FIG. 8. Response of lytic reporter constructs to ZEBRA expression in Raji cells. (A) The activities of constructs in Raji cells that were cotransfected with BXG1 (white bars) or BXG1-gen ZEBRA (gray bars) were standardized to pGL2 Promoter luciferase activity, corrected for ZEBRA induction. (B) Immunoblots of protein extract isolated from Raji cells transfected with the indicated reporter plasmid and either BXG1 or BXG1-gen ZEBRA. Rabbit antiserum C was used to detect ZEBRA expression.

late promoters point to possible substages of late gene expression during the lytic cycle of EBV as have been previously described for herpes simplex virus (25).

This assay has also revealed preliminary information about the *trans* regulation of late gene expression in EBV. Despite the presence of ZEBRA response elements in some late promoters (17), ZEBRA likely stimulates the promoters' activities through an indirect mechanism which requires other viral activities (compare Fig. 5B and 6B). The required *trans* activity may actually be lytic DNA replication itself, as expression with late kinetics is strictly dependent upon this event in *trans* (Fig. 3A and 5B; compare Fig. 6B and 8A). The BcLF1 and BFRF3 promoters direct late expression in this system without alteration of the promoter itself by passage of a replication fork. By contrast, a lytic origin of replication, oriS, is required in *cis* for expression from late promoters in herpes simplex virus (13a). The widely accepted link between viral DNA replication and late gene expression in EBV is obviously more complex than previously thought.

A *trans* link between DNA replication and late gene expression in EBV is not a surprising result considering the infection cycle in B cells *in vitro*. During latency, the viral genome replicates once per cell cycle from oriP (30, 31) without expression of late products; therefore, DNA replication in *cis* is not sufficient to induce late gene expression. Rather, some unique aspect(s) of lytic DNA replication must stimulate the expression of late products. Comparison of latent and lytic replication of the EBV genome reveals a number of distinctive features of these two events. Latent and lytic replication proceed from different origins (13, 31) and are directed by different enzymatic machineries (8, 9, 30). Latent replication occurs in cycling cells in the presence of few viral products, while lytic replication may occur in arrested cells (2) in the presence of immediate-early and early lytic products. Finally, latent replication results in the production of episomal genomes, while lytic replication yields a 100- to 1,000-fold amplification of the viral genome in a linear form (13). These distinctions between latent and lytic replication likely form the basis of regulation for the expression of late products in *trans*. Manipulation of the assay described here may lead to more precise understanding of this *trans* relationship in the future.

The *trans* stimulation of late gene expression by lytic viral DNA replication may occur by either an activation or antirepression event. In the activation model, lytic DNA replication in *cis* could induce the expression of a positive regulator of late gene expression, analogous to an immediate-early factor. Alternately, lytic viral DNA replication in *cis* could induce the expression of an antirepressor. This factor could potentially alleviate the active repression of late promoters through modification or protein-protein interaction. Finally, amplification of the viral genome by lytic replication may serve to titrate a repressor from late promoters. Further studies are required to discriminate between these models.

The information obtained through the establishment of this system has broadened our understanding of late regulatory mechanisms. The fidelity of this assay in recapitulating late events in the EBV infection cycle provides a tractable experimental model to address the regulation of late gene expression in greater detail. Using this system, we have identified *cis*-active regulators of late gene expression (unpublished data).

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