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Epstein-Barr Virus Lytic Replication Is Controlled by Posttranscriptional Negative Regulation of BZLF1

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Regulation of the immediate-early gene BZLF1 is assumed to play a key role in triggering the lytic replication of Epstein-Barr virus (EBV). The expression of BZLF1 is regulated on multiple levels, including control of transcription by several positive and negative *cis*-acting elements as well as posttranslational modifications and protein-protein interactions. Localization of BZLF1 on one strand of the genome and the latent EBNA1 transcription unit on the complementary strand suggests a regulatory mechanism via hybridization of antisense RNA. With a plasmid encoding a defective BZLF1 RNA, which could not be translated, we were able to induce expression of endogenous BZLF1 gene product Zta and other proteins of the lytic cycle. Our data show for the first time that latent replication is stabilized by negative regulation of an immediate-early gene of the lytic cycle by a posttranscriptional mechanism. This might be a common theme of herpes simplex virus and EBV latency.

Receptor-mediated cell tropism, cell-type- and differentiation-dependent control of gene expression, and modulation of host immune responses are essential for Epstein-Barr virus (EBV) to evade the host immune response (for a review see reference 48). In differentiating epithelial cells, the lytic replication is frequently induced (3, 50), which ensures the production of progeny virus at some sites in the organism (43, 44, 49). In contrast, infection of lymphoid cells predominantly leads to a stage of latency where only a set of up to 11 latent viral genes which stabilize latent replication are expressed (for a review see reference 31). The activation of the replicative cycle of EBV starts with the expression of immediate-early *trans* activators Zta, Rta, and I′ta, encoded by the reading frames BZLF1, BRLF1, and BI′LF4 (4, 16, 23, 25, 39), respectively, which cooperate in activation of further early genes and finally result in expression of late viral proteins. In Burkitt's lymphoma (BL)-derived cell lines, Zta was shown to be the only viral *trans* activator able to interrupt latency and to induce the lytic replication (5, 7, 15). The key role of Zta in maintaining a balance between latent and lytic replication may be reflected in its control on different levels of expression. It was shown earlier that the BZLF1 promoter region contains several binding motifs for positive and negative regulatory factors as well as two Zta response elements which trigger positive autoregulation (10–12, 28, 38, 47). Besides transcriptional control, Zta is regulated by posttranslational modifications such as phosphorylation (8), cellular localization (3), and protein-protein interactions (13, 17, 18, 42, 51). Zta can be translated from two different transcripts starting in either the BZLF1 or BRLF1 promoter (22, 41), the latter giving rise to the expression of a bicistronic message coding for both Zta and Rta. Both transcripts are localized in a region covered also by the intron of the EBNA1 primary transcript. Since EBNA1 is expressed in every latently infected cell (32, 33), we investigated the possibility of a posttranscriptional regulatory mechanism for BZLF1 via antisense hybridization of EBNA1 and BZLF1 transcripts.

Incorrect processing of heterogeneous nuclear RNA (hnRNA) for BZLF1 encoded by transfected plasmids. To interfere with a proposed posttranscriptional regulatory mechanism that inhibits the endogenous BZLF1 RNA from translation, plasmid pCMVZmS, which allowed a constitutively high expression of a mutated hnRNA for BZLF1, was transiently transfected in different cell lines. Zta expression plasmid pCMVZ was constructed by inserting a BZLF1 fragment (positions 102118 to 103207, B95-8 sequence [2]) into the *Eco*RI and *Sph*I restriction enzyme sites of pcDNAamp (Invitrogen). A 1,417-bp BZLF1–cytomegalovirus promoter-enhancer fragment was subcloned into the *Nco*I-*Nco*I restriction enzyme site of plasmid pCMV (24) to generate pCMVZ. Plasmid pCMVZmS was constructed by modification of the initiation site for translation in plasmid pCMVZ by PCR, thus introducing a *Pst*I restriction enzyme site and two stop codons that abolish translation of a functional BZLF1 gene product. Expression plasmid pCMV CAT (24) was used as a mock control to ensure that the switch from latency to lytic EBV replication was not induced by high expression rates of any gene product.

EBV-positive lymphocyte cell lines (LCLs) Em, Wo, Ra, and Gu(S), BL cell lines Akata (45), P3HR1/16 (46), and Raji (30), and the EBV-negative BL cell line BJAB (19) were transfected with the plasmids described above (Fig. 1). Transfection was performed by electroporation essentially as described before (38). The transcription efficiency of transfected plasmids was controlled by reverse transcription PCR analysis. Primers located within the first and second exons of BZLF1 could detect both hnRNA and spliced mRNA. The primers used were 3′ primer ON2463 (5′-CGG CAT TTT CTG GAA GCC ACC CGA-3′; positions 102463 to 102487) and 5′ primer ON3194 (5′-ATT GCA CCT TGC CGG CCA CCT TTG-3′; positions 103170 to 103194). RNA was isolated by using the RNAzol B method (34) and treated with DNase to ensure that only RNA was amplified. Reverse transcription PCR conditions were chosen according to the *Tth* polymerase system of Boehringer Mannheim: denaturation for 30 s at 95°C, primer annealing for 45 s at 60°C, and extension for 90 s at 72°C. After amplification, the PCR products were digested with *Pst*I to differentiate be-

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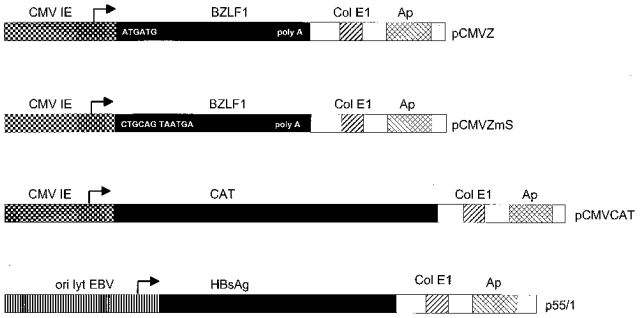

FIG. 1. Schematic drawing of the plasmid constructs, pCMVZ (positive control), pCMVZmS (mutated initiation site of translation), pCMVCAT (negative control), and p55/1 (Zta-inducible reporter plasmid), that were used in transfection assays. CMV IE, cytomegalovirus immediate-early promoter-enhancer; Ap, ampicillin resistance; HBsAg, hepatitis B virus surface antigen.

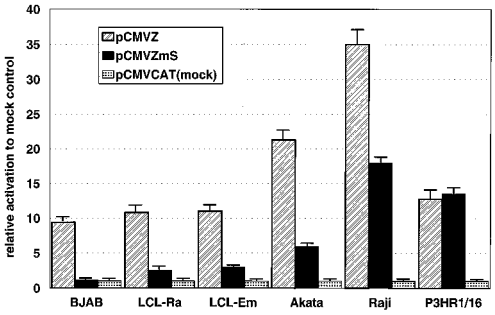

FIG. 3. Expression of endogenous Zta was stimulated by defective-plasmid-encoded BZLF1 transcripts. In transfection assays, reporter plasmid p55/1 was cotransfected in the cell lines indicated at the bottom. Transfection with pCMVZ was performed as a positive control; pCMVCAT transfection was used as a mock control. Each value indicates the mean activity of the hepatitis B virus surface antigen reporter plasmid, in three independent experiments, relative to the mock control, which was assigned an activity of 1. In EBV-positive cells, expression of the mutated hnRNA for Zta resulted in a cell line-dependent induction of Zta expression, which was highest in the BL lines P3HR1 and Raji.

tween the mutated (693-bp) and unmutated (733-bp) recombinant BZLF1 transcripts (Fig. 2A). In all transfected cell lines, only the unspliced hnRNAs, not the spliced mRNAs, of the mutated as well as of the unmutated transcripts were detectable as early as 24 h after transfection, demonstrating correct transcription of the recombinant plasmids. pCMVCAT (mock)-transfected cell lines showed no BZLF1 signal. To confirm that the specific signal was due to RNA and not to contamination of DNA, in a control experiment the RNA preparations were additionally treated with RNase A and RNase T_1 prior to PCR (not shown). This treatment completely abolished the signal and demonstrated amplification of RNA. Using the same RNA preparation as above but primers specific for the spliced transcript (one primer covering a splice site) in PCR, only in P3HR1/16 (Fig. 2B) and Raji cells (data not shown) could the processed transcripts be detected for both recombinant constructs (3′ primer ON2511 [5′-CAG

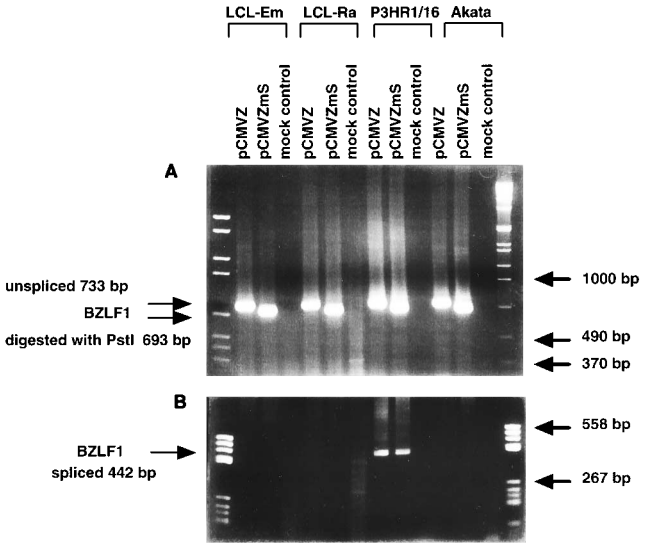

FIG. 2. Reverse transcription PCR with primer pairs either for the unspliced and spliced BZLF1 RNA (A) or specific for the spliced mRNA (B) 1 day after transfection of different cell lines. pCMVZ- and pCMVZmS-encoded RNAs were distinguished by digestion with restriction enzyme *Pst*I, yielding a 733-bp and a 693-bp signal (A). In all of the transfected cells except P3HR1/16 cells, only hnRNA, probably plasmid encoded, could be detected after reamplification, suggesting that the hnRNA could not be processed properly in those cell lines (B).

AAT CGC ATT CCT CCA GCG ATT-3′; positions 102511 to 102531 and 102655 to 102659] and 5′ primer ON3078 [5′-GAC CAA GCT ACC AGA GTC TAT CAG-3′; positions 103054 to 103078]). Hence, the (plasmid-encoded) BZLF1 hnRNA transcripts in the majority of these transfected cells could not be processed properly either because of overproduction of the hnRNAs or because of a so far unknown negative regulatory mechanism. To control rates of transfection and transcription of the plasmids in the different samples, the amount of unspliced transcripts was measured in PCR (not shown). Equal amounts of RNA from each transfected cell line were used, and aliquots of the reaction mixture were removed after various numbers of cycles. The amounts of transcripts were roughly similar, and no correlation was detected between the amounts of hnRNA and spliced mRNA. A possible correlation between the rate of hnRNA processing and activation of lytic replication of EBV is discussed below in the context of further results.

Translation-defective RNA for BZLF1-induced expression of Zta. Since we have demonstrated that the transfected plasmids were correctly transcribed, we cotransfected reporter plasmid p55/1 with pCMVZmS, pCMVZ, and pCMVCAT to test if the transcribed RNAs could induce expression of endogenous Zta from latently replicating EBV. Plasmid p55/1 allowed expression of the surface antigen of hepatitis B virus from a Zta-inducible EBV viral regulatory region (ori *lyt*) (24). Since hepatitis B virus surface antigen was secreted into the culture medium, cell lysis was not required and transfection could be continuously monitored over 8 days, increasing the sensitivity even in poorly induced cell lines. As expected, in EBV-negative BL cell line BJAB, transfection of positive control expression plasmid pCMVZ showed a weak signal resulting from the plasmid-encoded Zta (Fig. 3). Transfection of mutated pCMVZmS showed no Zta signal, which demonstrated that the mutated RNA could not be translated (Fig. 3). However, expression of defective BZLF1 RNA resulted in a 2- to 3-fold enhancement of endogenous BZLF1 expression in EBV-positive LCLs and up to 12-fold enhancement in BL cell lines compared with the mock control (Fig. 3). Transfection of the positive control plasmid pCMVZ induced an approximately 35-fold induction of expression.

Expression of Zta was further monitored by Western immunoblot assays. The transfected cells were harvested by centrifugation (400 × *g*, 10 min at room temperature) and washed

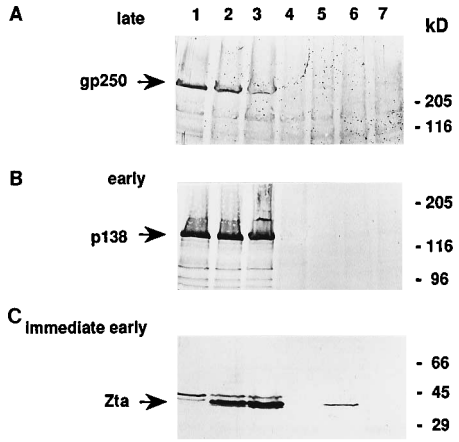

FIG. 4. Defective plasmid-encoded BZLF1 transcripts induced lytic cycle genes. Expression of immediate-early Zta (BZLF1), early p138 (BALF2), and late gp250/350 (BLLF1) viral proteins was monitored by Western blotting with specific monoclonal antibodies. Lane 1, TPA-treated P3HR1/16 cells (positive control); lanes 2 to 4, non-TPA-treated P3HR1/16 cells; lanes 5 to 7, EBV-negative BJAB cells. pCMVZ-transfected cells (lanes 2 and 5) show the same expression pattern as cells transfected with pCMVZmS (lanes 3 and 6). pCMVCAT (mock)-transfected cells (lane 3 and 7) were not induced.

twice with ice-cold phosphate-buffered saline (PBS). The cell pellet was resuspended by sonification in solubilization buffer (34). After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer to nitrocellulose membrane, immediate-early Zta (BZLF1) was detected by monoclonal antibodies. Figure 4 shows Western blot analysis of lymphoma-derived cell line P3HR1/16 and EBV-negative cell line BJAB transfected with plasmids CMVZ, pCMVZmS, and pCMVCAT. The molecular mass of Zta differs from 32 kDa up to 40 kDa depended on the viral strain and protein modification (29, 40) and shows one to three defined signals in Western blot assays. For a positive control, we used 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated P3HR1/16 cells (Fig. 4C, lane 1) that expressed predominantly the 40-kDa Zta, likely as a result due to phosphorylation caused by protein kinase C (8). Plasmids pCMVZ and pCMVZmS induced clearly visible expression of Zta. In contrast to the TPA control, however, the lower-molecular-mass variant of approximately 38 kDa was predominantly expressed (Fig. 4C, lanes 2 and 3). In EBV-negative BJAB cells transfected with pCMVZ, again only a weak signal from plasmid-encoded Zta was visible (Fig. 4C, lane 5). No signal could be detected when pCMVZmS was transfected in BJAB cells (Fig. 4C, lane 6) or in control mock-transfected P3HR1/and BJAB cells (Fig. 4C, lanes 4 and 7). pCMVZmS-transfected BL cell lines Raji and Akata showed a weaker signal in Western blot analysis than cell line P3HR1/16 (data not shown), which was in accordance with results from the reporter assay (Fig. 3). In LCLs Ra and Em, no Zta signal could be detected in Western blot analysis because of low abundance of Zta (data not shown).

Posttranscriptional control of BZLF1 gene expression repressed lytic replication of EBV. Expression of early protein p138 (BALF2) and late membrane protein gp250/350 (BLLF1) was monitored to measure the importance of this posttranscriptional mechanism for the control of lytic replication of EBV. In Fig. 4, Western blot analysis showed that in BL cell line P3HR1/16, expression of the defective RNA for BZLF1 induced the expression of p138 and gp250/350 (Fig. 4A and B, lane 3), indicating that the complete cascade of lytic gene expression was induced. Only poor signals were detected in

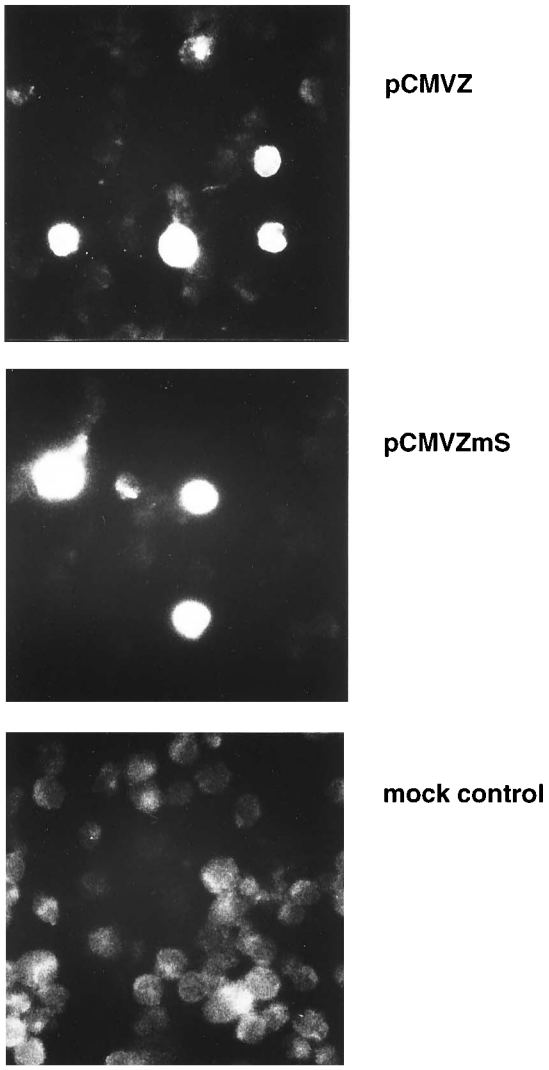

FIG. 5. Immunofluorescence analysis of transfected P3HR1/16 cells with monoclonal antibodies. In P3HR1 cells, expression of endogenous late gp250 membrane antigen is equally well induced by a functional (pCMVZ) and by a defective (pCMVZmS) plasmid-encoded transcript of BZLF1.

Akata cells, and no signals could be observed in Raji cells (data not shown) because of a deletion in the BALF2 region. Interestingly, in the P3HR1/16 cells either chemically induced by TPA treatment or transfected with pCMVZ and pCMVZmS, only gp250 was found.

To determine the rate of expression of gp250 and induction of the whole lytic cycle in transfected P3HR1/16 cells, pCMVZ-, pCMVZmS-, and pCMVCAT (mock)-transfected cells were analyzed by immunofluorescence. Transfected cells were fixed in ice-cold acetone-methanol for 5 min before monoclonal antibodies and secondary fluorescein isothiocyanate-linked rabbit anti-mouse antibodies (Dako) were applied each in a dilution of 1:50 for 1 h at 37°C. Incubation was stopped by rinsing cells twice with ice-cold PBS for 15 min. The slides were analyzed under the microscope at a magnification of 250. Figure 5 shows for the P3HR1 cell line that transfection with the mutated construct (pCMVZmS) resulted in almost the same number of cells positive for gp250 expression (approximately 5 to 7%) as in the control transfection (pCMVZ). These results

indicate that in most cells that could be transfected with the translation-defective construct, the lytic replication of EBV was started.

Two posttranscriptional regulatory mechanisms are possible. Our data suggest that in BL cell lines, the full lytic cycle of EBV is induced by a mutated recombinant BZLF1 RNA which overcomes a posttranscriptional control mechanism. In LCLs, Zta expression can be induced, but this was not sufficient to lead to the production of progeny virus. These observations are in accordance with earlier findings that LCLs seem to be less permissive for lytic replication than BL cells (14, 27), suggesting interaction of so far unknown cellular factors with viral expression patterns. Experiments described above clearly demonstrate that a posttranscriptional regulation plays a key role in controlling viral replication at least in some cell lines. This is in accordance with previous observations (21) that demonstrated a quantitative and qualitative difference in nuclear and cytoplasmic RNAs. Remarkably, only in those cell lines that showed a maximum of activation in cotransfection assays (P3HR1 and Raji) could spliced (plasmid-encoded) mRNA for Zta be detected. This finding suggests that processing of BZLF1 hnRNA is a limiting step in expression of BZLF1 and directly correlates with Zta expression, since transcription efficiency of the (plasmid-encoded) hnRNAs was the same in all cell lines tested. Differential splicing of the hnRNA of BZLF1 has been found earlier in BL-derived cell lines (20) and nasopharyngeal carcinoma cells (6).

Two different mechanisms may be responsible for retaining the unprocessed BZLF1 hnRNA within the nucleus. First, there may be *trans*-acting factors of cellular or viral origin. Corresponding activities are described for the well-characterized adenovirus system (1, 26). Second, the endogenous hnRNA for BZLF1 could be complexed by transcripts with complementary sequences that belong to the EBNA1 transcription unit. In the experimental approach outlined above, the expression of high numbers of recombinant mutated BZLF1 RNAs saturated the regulatory mechanism either by complexing the EBNA1 intron by antisense binding or, alternatively, by binding negatively acting regulatory proteins, thus releasing the endogenous BZLF1 hnRNA for processing.

A related posttranscriptional mechanism has been documented for herpes simplex virus (HSV) latency. During latency, only a single region of the HSV genome is transcribed in untranslated RNAs, termed the latency-associated transcripts (LAT) transcripts. These are in antisense orientation to the immediate-early gene ICP0 and can function to down regulate ICP0 production in transient assays (9). Furthermore, LAT-negative HSV mutant strains were shown to have a reduced ability for reactivation from infected ganglia in mice which was due to a lower number of latently infected neurons. Therefore, the often described effect on reactivation may be indirect (36, 37). In homology to the antisense BZLF1 EBNA1 intron hybridization, a recent study found the major 2-kb LAT also to be an intron. So far, we cannot exclude one or the other of the proposed mechanisms. The antisense theory seems to be suggestive because of close analogy to HSV. However, observations of Sandvej et al. (35) demonstrating simultaneous expression of EBNA1 and Zta in the differentiated layers of epithelial tissues in oral hairy leukoplakia might favor *trans*-acting cellular factors.

In conclusion, our results clearly demonstrated for the first time that the key protein in lytic replication, Zta, is controlled by a posttranscriptional mechanism, which can be overcome by a translation-defective BZLF1 hnRNA transcript. In distinct cell lines like P3HR1/16, this mechanism seems to be much more important in controlling the switch to lytic replication of EBV than in several LCLs or the BL-derived line Akata. Considering our results that show induction of Zta and lytic replication of EBV after abrogation of the described posttranscriptional negative mechanism, we suggest that in a population of latently infected lymphoid cells, there is a low level of BZLF1 transcripts which are subject to complete negative control by posttranscriptional mechanisms, depending on the type and differentiation of the infected cell. This second level of control in addition to the control on the transcriptional level ensures in the host the ability of EBV to persist without causing persistent generalized viremia.

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