

Identification of Proteins Encoded by Epstein-Barr Virus *trans*-Activator Genes

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Specific antisera were generated to characterize Epstein-Barr virus proteins reported to have *trans*-activating properties. Open reading frame BRLF1 was found to be expressed in two modifications in vivo, with molecular sizes ranging from 94 to 98 kilodaltons (kDa) depending on the cell line, whereas only one protein (Raji cells, 96 kDa) was detected by in vitro translation. Open reading frame BZLF1 encoded polypeptides of 38 and 35 kDa and additional smaller forms. A BZLF1-encoded 30-kDa protein could be detected under conditions in which expression was restricted to immediate early genes. Nuclear localization could be shown for the proteins derived from reading frames BZLF1 and BMLF1. BMLF1 expression gave a heterogeneous protein pattern, with molecular sizes between 45 and 70 kDa, including a predominant 60-kDa protein detected in different B-cell lines.

To understand the regulatory events of primary infection, maintenance of latency, or tumor development, it is essential to study the initial steps in Epstein-Barr virus (EBV) gene activation (2, 4, 35). In latently infected cells, viral gene expression is restricted to a few viral products: EBV nuclear antigens (3, 11, 16, 22, 34, 40), BNLF1 membrane protein ("latent" membrane protein) (26), EBV-encoded small RNAs (18), and terminal protein (23). The expression of these genes is dependent on the host cell (33), suggesting a close interaction of viral and cellular factors. Regulatory aspects of the lytic cycle of EBV based on localization of viral genes on the genome (7, 19, 37) and definition of immediate early (4, 35; K. Fuchs, M.S. thesis, Max von Pettenkofer-Institute, University of Munich, Munich, Federal Republic of Germany, 1986) or latency disrupting (8, 9) genes are now studied in detail by functional analysis (5, 24).

Transfection experiments revealed *trans*-acting properties for the poorly characterized gene products of the EBV open reading frames (ORFs) BZLF1 (5, 8, 9, 14, 39), BMLF1 (5, 21, 24, 27, 41), and BRLF1 (15). Expression of BZLF1 is sufficient to disrupt latency in B cells (5, 8). *trans*-Activation by the spliced BMLF1-BSLF2 product is nonspecific with respect to the target gene and host cell system, as shown by cotransfection assays using different promoters of herpes simplex virus or unrelated viruses (adenovirus, simian virus 40, and human immunodeficiency virus) (21, 24, 41). The BRLF1-encoded *trans*-activator is capable of inducing the expression of the early BHRF1 ORF (15).

These results indicate the important regulatory functions of BZLF1, BMLF1, and BRLF1, but little information has been given about the polypeptides mapping in these genes. Previously published results from our group described the in vitro translation pattern of hybrid-selected RNA specific for BZLF1 and BRLF1 (36, 37). In the present work we characterize in vivo proteins encoded by these ORFs and show their cellular localizations and molecular weights and the presence of modified forms in lymphoid cell lines.

Subfragments of the three ORFs were cloned in pUR vectors and expressed as fusion proteins with β -galactosidase. The ORF BMLF1 was subcloned from the *Bam*HI M fragment of EBV B95-8 DNA (38). A *Bam*HI-*Xho*I fragment

of 609 base pairs encoding 165 N-terminal amino acids was cloned in frame with β -galactosidase into pUR289 and digested with *Bam*HI-*Sal*I. The coding sequences for the expression of BRLF1 and BZLF1 were derived from the *Sal*I C fragment of EBV M-ABA DNA (31) and subcloned in pUC vectors as described previously (36). The *Hind*III-*Xba*I fragment encoding 400 C-terminal amino acids of BRLF1 was ligated with pUR288. For the expression of 110 C-terminal amino acids of BZLF1, a *Hind*III-*Xba*I fragment had to be subcloned in pINIII B1 to gain *Bam*HI-*Xba*I sites for insertion in pUR289.

Detection of the EBV-specific epitopes in Western blots (immunoblots) by a pool of human sera (patients with nasopharyngeal carcinoma) showed the presence of viral antigenic sites. The specificity of human antibodies to the EBV portion of the fusion proteins was ensured by using sera with low β -galactosidase reactivity. The fusion protein preparations were partially purified by the isolation of insoluble inclusion bodies (25) and were used to generate reading frame-specific rabbit antisera.

The localization of viral antigens was shown by immunofluorescence tests with acetone-fixed, chemically induced Raji, P3HR-1, and B95-8 cells (40 nM phorbol-12-myristate-13-acetate [TPA]-3 mM butyrate; induction for 2 days) or superinfected Raji and freshly infected BJA-B cells (8 hours postinfection; virus prepared from P3HR-1 cells). In these cells specific signals could be obtained with each of the three antisera (rabbit sera were diluted 1:50 in BJA-B cell extracts for preadsorption; fluorescein-isothiocyanate-conjugated anti-rabbit antibodies from Dakopatts were used in a dilution of 1:100), whereas untreated latent Raji cells or EBV-negative BJA-B cells gave no positive reaction (Fig. 1A). When BRLF1-specific serum was used, antigens were detected in the cytoplasm and nuclei of induced P3HR-1 cells (Fig. 1B). In B95-8 cell preparations, BZLF1- as well as BMLF1-specific proteins could be demonstrated to be restricted to nuclear compartments (Fig. 1C and F). These results confirm earlier reports on the expression of nuclear antigens from the ORFs BMLF1 and BZLF1 (6, 9, 14, 41). In infected BJA-B cells (P3HR-1 virus), BMLF1- or BZLF1-specific fluorescence was detected in the nuclei and cytoplasm starting at between 6 and 12 h postinfection (Fig. 1D).

For further analysis of the proteins, we used Western

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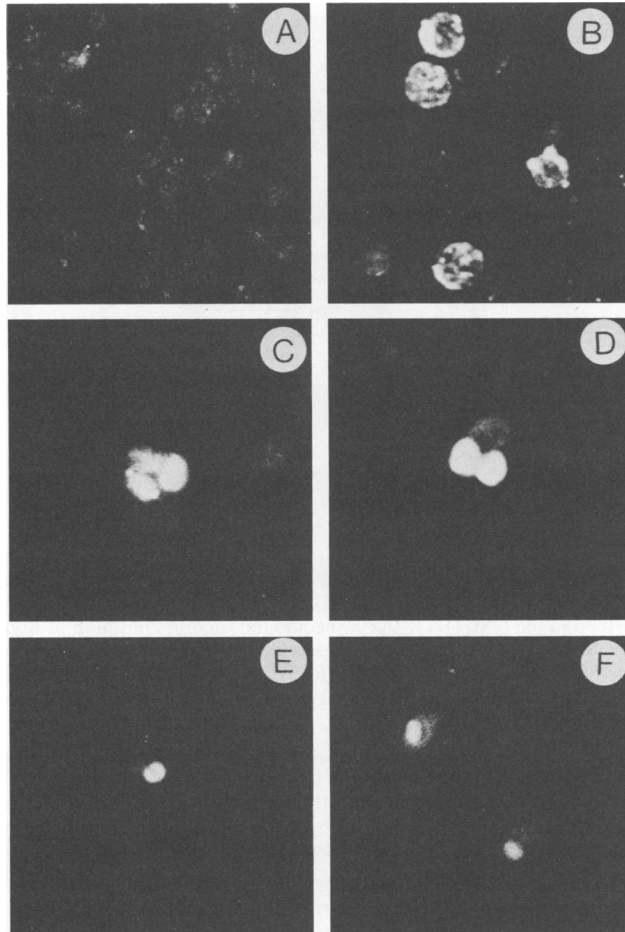


FIG. 1. Indirect immunofluorescence of acetone-fixed cells with ORF-specific rabbit sera and fluorescein-isothiocyanate-conjugated anti-rabbit antibodies. The cells were either infected with P3HR-1 virus (D) or induced with TPA-butyrate (A through C, E, and F). (A) Preimmune serum, Raji cells; (B) BRLF1-specific serum, P3HR-1 cells; (C) BZLF1-specific serum, B95-8 cells; (D) BZLF1-specific serum, infected BJA-B cells; (E) BZLF1-specific serum, P3HR-1 cells; (F) BMLF1-specific serum B95-8 cells.

blotting techniques (methods modified from those described in reference 36). Samples (10^6 cells) were sonicated and separated on 10 to 15% sodium dodecyl sulfate-polyacrylamide gels. Western blots from gels with different cell extracts were incubated at room temperature overnight in a 1:50 dilution of rabbit antisera, and color reactions were achieved after incubation for 2 h with either peroxidase (POD)- or alkaline phosphatase (AP)-conjugated anti-rabbit antibodies (dilution, 1:1,000) with different substrates. Brown POD substrate was 0.5 mg of 3,3'-diaminobenzidine per ml, 0.01% hydrogen peroxide, and 50 mM Tris (pH 7.5). Red AP substrate was 1 mg of Fast Red (Bio-Rad Laboratories) per ml, 0.5 mg of 4-chloro-1-naphthol per ml, 50 mM Tris (pH 9.5), and 0.5 mM $MgCl_2$. Violet AP substrate was 0.3 mg of Nitro Blue Tetrazolium (Sigma Chemical Co.) and 0.15 mg of 5-bromo-4-chloro-3-indolyl phosphate (Sigma) per ml, both dissolved in dimethylformamide and diluted 1:100 in carbonate buffer (0.1 M $NaHCO_3$, 1 mM $MgCl_2$; pH 9.8).

Western blot analysis of BZLF1, BRLF1, and BMLF1 expression comparing EBV-positive (B95-8, P3HR-1, and

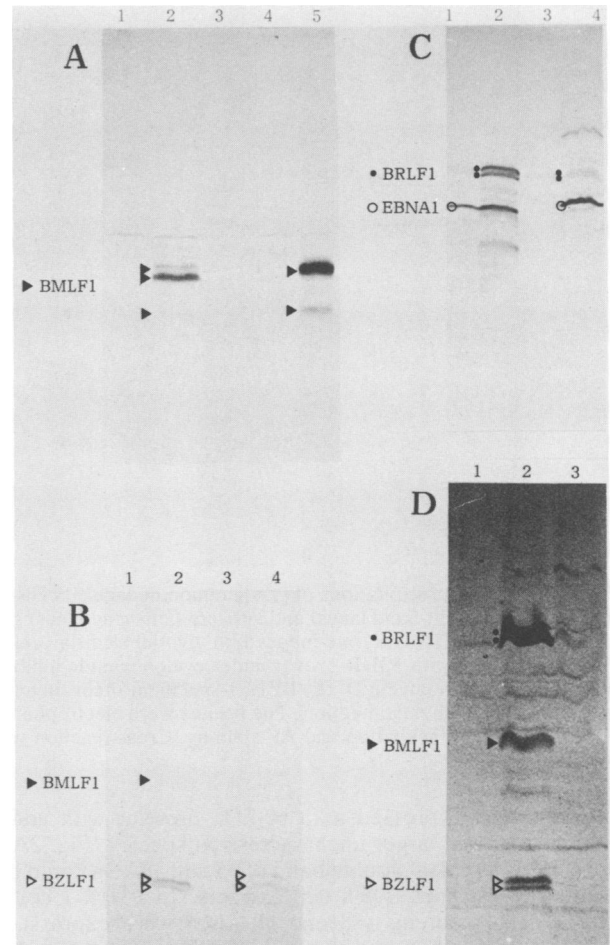


FIG. 2. Western blot analysis of different cell extracts after serial serum incubation and staining procedures as described in the text (POD and AP conjugates with specific color substrates). The molecular sizes of specific proteins are discussed in the text and summarized in Fig. 4. (A) BMLF1-specific serum (AP, violet). Lanes: 1, BJA-B; 2, P3HR-1 induced with TPA-butyrate; 3, B95-8; 4, Raji; 5, Raji induced with TPA-butyrate. (B) Incubation 1, BZLF1-specific serum (AP, violet); Incubation 2, BMLF1-specific serum (AP, red). Lanes: 1, Raji; 2, Raji induced with TPA-butyrate; 3, BJA-B; 4, P3HR-1 induced with TPA-butyrate. (C) Incubation 1, BRLF1-specific serum (AP, violet); incubation 2, EBNA1-specific serum (POD, brown). Lanes: 1, Raji; 2, Raji induced with TPA-butyrate; 3, BJA-B; 4, P3HR-1 induced with TPA-butyrate. (D) Incubation 1, BZLF1-specific serum (AP, red); incubation 2, BRLF1-specific serum (POD, brown); incubation 3, BMLF1-specific serum (AP, violet). Lanes: 1, Raji; 2, Raji induced with TPA-butyrate; 3, Raji.

Raji) and EBV-negative (BJA-B) B-cell lines showed that each ORF encoded proteins in at least two different modifications. Untreated Raji or B95-8 cells representing the latent state of infection did not express measurable amounts of *trans*-activator proteins. Chemical induction with TPA and butyrate led to the expression of specific polypeptides which varied in quantity relative to each other, as shown by serial incubation of ORF-specific sera on Western blots (Fig. 2). The virus strain-specific molecular size of EBNA1 (68 to 85 kilodaltons [kDa]) was used as marker for the examined cell lines (Fig. 2C). The molecular sizes of all detected polypeptides could be determined by comparison with a protein standard (205, 116, 97.4, 66, 45, and 29 kDa; Sigma).

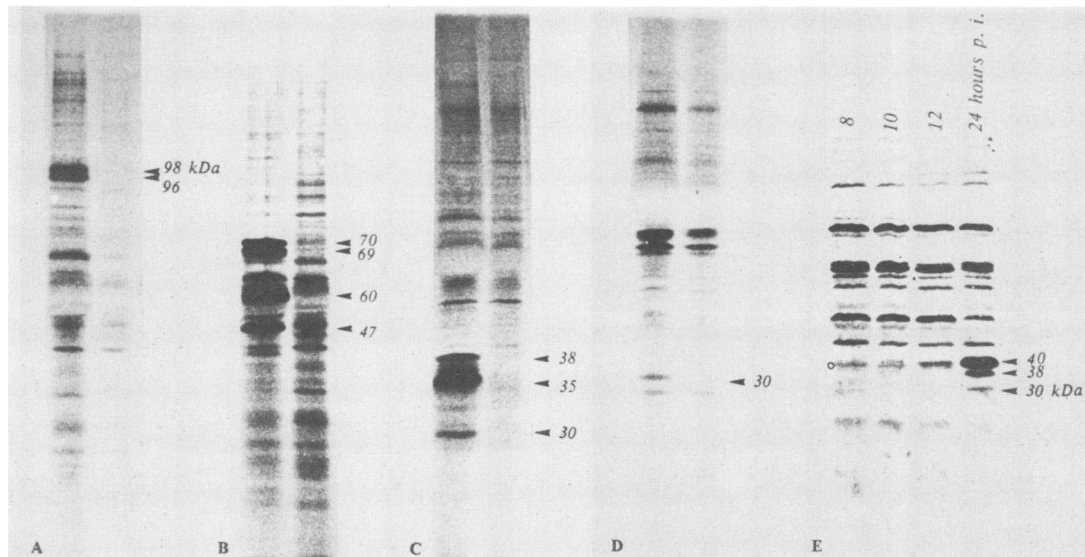


FIG. 3. Immunoprecipitations of [35 S]methionine-labeled cells (A through D) and Western blot showing time course of infection (E). The preimmune sera (right-hand lanes) and antisera (left-hand lanes) generated against BRLF1 (A), BMLF1 (B), and BZLF1 (C) were incubated with extracts from TPA-butyrate-induced, *in vivo*-labeled Raji cells as described in the text. (D) BZLF1-specific immunoprecipitation of Raji cells superinfected with P3HR-1 virus under cycloheximide inhibition followed by removal of the cycloheximide and labeling of proteins in the presence of actinomycin D. (E) BZLF1-specific proteins in a time course of infected BJA-B cells (P3HR-1 virus). A total of 10^6 cells was harvested at 8 to 24 h postinfection. The lysates were electrophoresed, blotted onto nitrocellulose, and analyzed for BZLF1-specific proteins (\blacktriangle) by antiserum incubation and AP staining. Cross-reaction with a cellular protein is indicated (\circ).

BMLF1 was expressed as a 60-kDa protein, with additional smaller and larger uncharacterized species (Fig. 2A). For BZLF1, a typical double band of 35 and 38 kDa could be detected in Raji and B95-8 cell extracts. In P3HR-1 cells, larger BZLF1 proteins (38 and 40 kDa) were expressed, possibly because of the expression of WZhet DNA (8, 20, 36) (Fig. 2B). BRLF1-specific polypeptides could be demonstrated as double bands with molecular sizes between 94 and 98 kDa depending on the cell line (Fig. 2C). In some preparations, only the smaller species was detectable by BRLF1-specific sera.

In extracts from TPA-butyrate-induced Raji cells, polypeptides encoded by the ORFs BRLF1, BMLF1, and BZLF1 could be demonstrated in parallel on one Western blot by serial incubation of the three specific sera and staining in different colors (Fig. 2D). In step 1, specific bands of 35 and 38 kDa were detected by incubation with anti-BZLF1 serum. When anti-BRLF1 serum was used, proteins of 96 and 98 kDa were detected. A BMLF1-specific 60-kDa protein could be shown in step 3.

The protein patterns of the single ORFs in induced Raji cells were further characterized by immunoprecipitation after *in vivo* labeling with [35 S]methionine (50 μ Ci/ml for 12 h, 2 days after induction) (Fig. 3). The lysate from 10^6 cells was incubated with 10 μ l of antiserum (preadsorbed with extract from 10^7 EBV-negative BJA-B cells). Immune complexes were bound on protein A-Sepharose beads, separated in polyacrylamide gels, and exposed to autoradiography films.

Two BRLF1-specific polypeptides of 96 and 98 kDa were immunoprecipitated in extracts from induced Raji cells (Fig. 3A). The molecular sizes were shown to be virus strain specific, ranging from 94 to 98 kDa *in vivo* and from 92 to 96 kDa *in vitro* (Fig. 4). *In vitro* translation of BRLF1-specific RNA from induced Raji cells (hybrid selection) and immunoprecipitation with anti-BRLF1 serum identified a 96-kDa

protein (36). Comparison of these results indicates that *in vivo* the smaller form represents the precursor of the additional larger protein in Raji, P3HR-1, and B95-8 cells.

Several proteins heterogeneous in molecular size were reported to be synthesized from the ORF BMLF1 (6, 24, 28). We precipitated polypeptides with molecular sizes of between 45 and 70 kDa in extracts from TPA-butyrate-induced Raji, P3HR-1, or B95-8 cells. The high sensitivity of the immunoprecipitation tests (exposure for 3 months in some cases) enabled us to identify faint BMLF1-specific bands which could be detected only occasionally in Western blots (Fig. 2 and 3). RNA data suggest alternative splicing, including the short ORF BSLF2 upstream of BMLF1 (6, 12) (Fig. 4). Further posttranslational modifications such as phosphorylation and truncated proteins possibly lead to the heterogeneity in expression. Proteolysis may create additional fast-migrating species. A characteristic protein of 60 kDa is the predominant BMLF1 product (Fig. 2).

By *in vitro* translation of BZLF1-specific RNA (Raji and B95-8), a polypeptide of 38 kDa could be immunoprecipitated (36). *In vivo*, two proteins of 38 and 35 kDa were identified (Fig. 2B and D and 3C). Smaller forms of about 30 kDa, which might represent truncated or degraded products of the 38-kDa precursor, were detectable at times. In EBV-negative BJA-B cells freshly infected with P3HR-1 virus in which the initially translated proteins could be examined with reading frame-specific antisera, the largest polypeptide (of 40 kDa; P3HR-1 virus specific) was found to be expressed first. (A total of 10^6 cells was infected with virus prepared from 50 ml of P3HR-1 culture. The virus had been pelleted by centrifugation for 2 h at $20,000 \times g$ from the culture supernatant after chemical induction for 10 days. Virus penetration was achieved by incubating the infection assay for 1 h at room temperature under gentle movement before the cells were transferred into fresh medium.) The 40-kDa protein was detectable 10 h postinfection in Western blots,

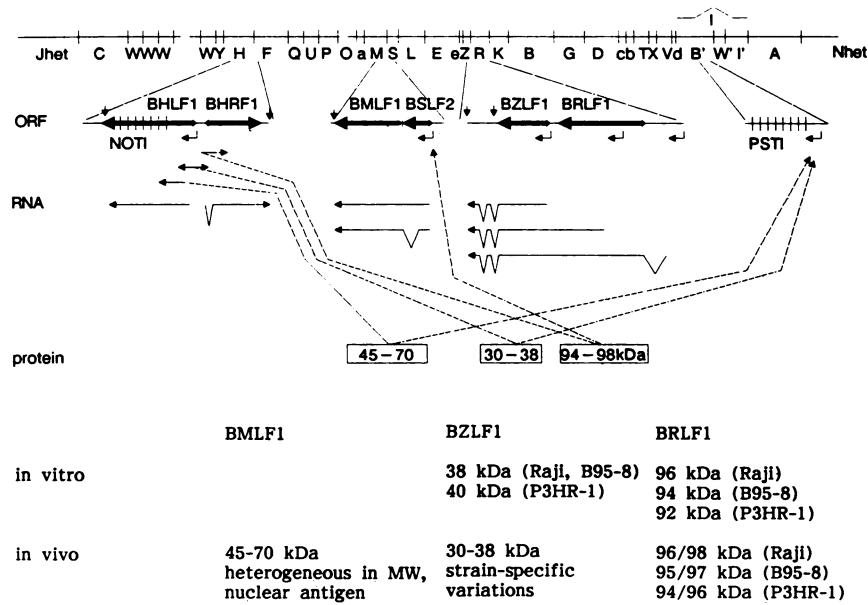


FIG. 4. Summary of physical and functional aspects of *trans*-acting genes of EBV, including *Bam*HI fragments, ORFs, transcripts and proteins involved in *trans*-activation, polyadenylation signals (↓), promoter sequences (↵), spliced RNAs (↘), and *trans*-activation (---→). The regulatory properties were derived from references 5, 8, 9, 13-15, 17, 21, 24, 27, 29, 30, 35, 39, and 41 and are described in detail in the text. Sequence and RNA data were derived from reference 12 and P. J. Farrell (personal communication). The molecular sizes of the proteins were calculated from the results of separate experiments not shown here. In vitro translation results have been published elsewhere (36, 37).

whereas at 24 h postinfection, three products of BZLF1 could be identified (Fig. 3E). In Western blot experiments analyzing different extracts from TPA-butyrate-induced cells, mainly the 38- and 35-kDa proteins (P3HR-1 cells; 40 and 38 kDa) were detected (Fig. 2B), with additional BZLF1-specific forms of about 30 kDa in lower amounts.

Low expression of this ORF during conditions favoring immediate early gene expression could also be shown. For this, Raji cells were superinfected with P3HR-1 virus for 6 h under cycloheximide translational block (50 µg/ml). The cycloheximide was then removed, and the cells were labeled with [³⁵S]methionine in the presence of actinomycin D (2 µg/ml) for inhibition of transcription for 3 h. A 30-kDa polypeptide was immunoprecipitated in these extracts by using BZLF1-specific serum (Fig. 3D). Since larger proteins were not detected in this test, the role of different BZLF1-encoded products remains to be clarified. Our results confirm recent reports (4; Fuchs, M.S. thesis) that showed transcription of BZLF1 in Raji cells superinfected with P3HR-1 virus in the presence of anisomycin and that suggested the immediate early status of this gene.

The expression patterns and known functions of the EBV *trans*-activator ORFs BMLF1, BZLF1, and BRLF1 are summarized in Fig. 4. RNAs and proteins are expressed in a complex manner. The BMLF1 *trans*-activator is expressed from exons of the ORFs BMLF1 and BSLF2 as a 60-kDa protein with additional modified forms. Sequence analysis identified BMLF1 homologs in related virus systems (a 52-kDa protein of herpesvirus saimiri, varicella-zoster virus gene 4, and herpes simplex virus ICP27) (10). BZLF1- and BRLF1-specific proteins are encoded by one polycistronic mRNA that is transcribed under anisomycin conditions (4, 35), which distinguish between genes of the early and immediate early regulatory classes. In this work we demonstrated the synthesis of a BZLF1-specific 30-kDa immediate early protein. The BRLF1-encoded proteins were identified

as a doublet which represents a precursor and a modified higher-molecular-weight form.

The identified *trans*-activator proteins showed heterogeneity in their molecular weights and strain-specific variations. Each reading frame was found to be expressed in at least two modifications. To clarify the significance of in vivo modifications, protein detection in functional assays is required next. We were able to localize BZLF1 and BMLF1 products in the nuclei of B cells, a position presupposed for DNA interaction. The activities of these EBV *trans*-acting proteins were demonstrated to be dependent on the host cell system (5, 15, 24). Cooperation with cellular, type-specific factors (1) and their limitation to certain states of cell differentiation could explain the cell-specific regulation of EBV *trans*-activators. The tropism of EBV for B lymphocytes on the one hand and epithelial cells on the other has to be taken into account in future studies of the expression and activity of *trans*-acting proteins.

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