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Identification and Mapping of Epstein-Barr Virus Early Antigens and Demonstration of a Viral Gene Activator That Functions in *trans*

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The *Bam*HI M DNA fragment of the Epstein-Barr virus (EBV) genome was inserted in two orientations into a simian virus 40-based expression vector, and the EBV-specific proteins produced in COS-7 monkey cells were examined. In one orientation, termed *Bam*HI-M rightward reading frame 1 (BMRF1), a set of phosphoproteins ranging in size from 47,000 to 54,000 daltons was synthesized. These proteins reacted with monoclonal and polyclonal antisera, defining them as components of the EBV early antigen diffuse set of proteins (EA-D). The *Bam*HI M DNA fragment in the opposite orientation, termed *Bam*HI-M leftward reading frame 1 (BMLF1), directed the synthesis of a nuclear antigen detected by antibodies in serum from a patient with nasopharyngeal carcinoma. The BMLF1 antigen was not detected by monoclonal or polyclonal antibodies directed against the EA-D complex. A series of deletion mutants were constructed in the *Bam*HI M DNA fragment, and the EA-D complex and BMLF1 antigen were mapped to discrete open reading frames in this DNA fragment. A test for several possible functions of these antigens showed that the BMLF1 antigen had the ability to activate or enhance, in *trans*, the level of expression of a gene under the control of the adenovirus early region 3 promoter or the simian virus 40 early promoter in the absence of its *cis*-acting enhancer. These experiments demonstrate a new gene function, encoded by EBV, that may be important in the positive regulation of viral or cellular genes.

Epstein-Barr Virus (EBV), like other herpesviruses, establishes latent infections in which the virus or viral genome coexist with the cell in a silent form until some external stimuli activate a productive infectious cycle (12, 17, 49). Induction of the EBV replicative cycle can be studied with cultures of lymphoblastoid cell lines, such as B95-8 or P3HR-1, which are then termed producer cell lines. These producer cells yield infectious virus upon exposure to one of several diverse chemical agents such as 12-*O*-tetradecanoylphorbol-13-acetate. Lymphoblastoid cells carrying the EBV genome express EBV-specific mRNAs (28, 43) representing about 10% of the coding capacity of the viral genome. In the latently infected cells, at least three distinct EBV-encoded antigens were identified and termed EBV nuclear antigens I and II (7, 10, 19, 22, 40) and membrane antigen (21, 25). In addition, a *cis*-acting site essential for the persistence or replication of the EBV DNA was mapped and identified (31, 39, 47). EBV nuclear antigen I acting at the *cis*-acting site (31, 48) is required for the long-term persistence of the EBV genome. Upon induction of such lymphoblastoid cell lines with chemical inducers, a new set of EBV-induced or -encoded proteins is synthesized and was detected by sera from patients with infectious mononucleosis, Burkitt lymphoma, or nasopharyngeal carcinoma. Among the antigens described as being produced shortly after 12-*O*-tetradecanoylphorbol-13-acetate induction of lymphoblastoid cells are the early antigens (EA components) (34, 49).

The available evidence suggests that the EA complex is probably composed of multiple protein entities (20, 38). Based upon the pattern of chemical fixation of cells, immunofluorescent staining, and cellular localization, the EA complex has been divided into diffuse (EA-D) and restricted (EA-R) components. EA-D protein is resistant to fixation with methanol and is localized in the nucleus and cytoplasm, whereas EA-R protein is sensitive to methanol fixation and is restricted to a cytoplasmic location (20) Soon after induction of latently infected cells, the EA complex is synthesized, even in the absence of EBV DNA replication (9, 33), suggesting an important or immediate role for these antigens in the switch from the latent state to a productive infection.

The first step in the clarification of this complex classification of antigens was the preparation by Pearson and his colleagues of monoclonal antibodies against EA-D and EA-R, which can now be used to define these entities (35). Using hybrid selection of mRNA and in vitro translation of proteins, Pearson et al. (35) mapped the EA-D proteins of 48,000 to 52,000 daltons (Da) to the *Bam*HI M restriction fragment of the EBV genome. Several other groups showed that the *Bam*HI M DNA fragment encodes an EBV nuclear antigen (4, 16, 41), and Cho et al. (5) prepared antiserum against a *Bam*HI-M-encoded protein synthesized in bacteria. This antiserum reacts with a set of 46,000- to 55,000-Da proteins in extracts from B95-8 cells or chemically induced Raji cells. In the present study, a simian virus 40 (SV40)-based expression vector was used to synthesize in monkey cells two different EBV-induced proteins encoded by different regions of the *Bam*HI M DNA fragment. The EA-D component was mapped to a single open reading frame (ORF) termed the *Bam*HI-M rightward reading frame 1 (BMRF1). This gene, localized by deletion mapping in the *Bam*HI M fragment, produces a heterogeneous collection of phosphoproteins. From the opposite orientation of the *Bam*HI M DNA fragment, in a single ORF termed the *Bam*HI-M leftward reading frame 1 (BMLF1), a distinct nuclear protein was mapped and identified. This antigen was shown to function as an activator or enhancer, in *trans*, of

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genes regulated by adenovirus or SV40 early promoters. The *trans* activation of viral promoters by this gene product could play a role in the induction of EBV from the latent to the productive state.

MATERIALS AND METHODS

Cell lines. COS-7 (13) and HeLa cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 100 U of penicillin, 100 μg of streptomycin per ml, and 10% heat-inactivated fetal calf serum (FCS).

Plasmids. Plasmid pKCAT23 was obtained from J. Alwine (46). The pIE plasmid was derived from a *Sal*I fragment containing the immediate-early gene of pseudorabies virus which was obtained from J. Nevins (26). Plasmid pE1A was obtained from J. Logan (30), and pSVΔCAT (chloramphenicol acetyltransferase) was constructed from pSV2-CAT (14) by L. Duprey by deleting the 72-base-pair repeat SV40 enhancer from the *Acc*I site to the *Sph*I site. Plasmid pSVC (Fig. 1A) was derived from p404 (31) by deleting the neomycin resistance gene (from *Hin*dIII to *Pvu*II) and replacing it with *Bam*HI linkers. The 4.7-kilobase *Bam*HI M fragment of pDF516 (a gift from E. Kieff) (6) was cloned in either orientation into the *Bam*HI site of the plasmid pSVC (Fig. 1A). Baer et al. (1) identified three ORFs in the *Bam*HI-M region, two in the rightward orientation, known as BMRF1 and BMRF2, and one in a leftward orientation, known as BMLF1 (Fig. 1B). The orientation of the *Bam*HI M fragment relative to the SV40 early promoter governs which ORF(s) is expressed. In the plasmid pSVM'C, BMRF1 or BMRF2 or both are expressed (Fig. 1C), whereas in the plasmid pSVMC, BMLF1 is expressed. Plasmids B10, BB20, and BB8 are subclones of pSVM'C (Fig. 1C). The B10 clone contains a frameshift mutation in BMRF2, generated by filling in the overlapping single strands of DNA with Klenow polymerase at the unique *Bgl*II site and ligating the blunt ends together. The BB20 clone was obtained from pSVM'C by deleting the *Bgl*II-*Bam*HI fragment which contains BMRF2 and BMLF1, and the BB8 clone was obtained by deleting the *Bam*HI-*Bgl*II fragment containing BMRF1. The BH16 (Fig. 1C) clone was derived from the pSVMC plasmid by deleting the *Bam*HI-*Hin*dIII fragment containing BMRF1 and BMRF2.

DNA transfection procedures. Cells to be transfected were seeded at a density of 0.8×10^6 to 1×10^6 cells per 100-mm dish 1 day before transfection. COS-7 cells were fed with fresh medium (DMEM plus 10% FCS) 4 h before addition of DNA (2 to 5 μg) in DMEM containing 200 μg of DEAE-dextran per ml and 2 μM chloroquine diphosphate for 12 h. Cells were then washed twice and incubated in DMEM plus 10% FCS for 2 to 3 days posttransfection. HeLa cells were transfected by the calcium phosphate method (15) with modifications. For transient CAT assays, HeLa cells were fed 4 to 5 h before transfection. A 10-μg portion of plasmid DNA (5 μg of test plasmid and 5 μg of CAT plasmid), 15 μg of high-molecular-weight carrier DNA, and 31.5 μl of 2 M $CaCl_2$ were added to sterile water to a final volume of 0.25 ml. This mixture was added dropwise to 0.25 ml of 2× HBS (270 mM NaCl, 40 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], 1.4 mM Na_2HPO_4, 10 mM KCl, 10 mM glucose [pH 7.05]) while air was blown through a Pasteur pipette into the solution. The precipitate was allowed to form at room temperature for 30 min. The medium was aspirated from the cells, and the DNA precipitate (0.5 ml) was added to the cell monolayer for 30 min at room temperature with occasional rocking. Then 4.5 ml of medium was added, and incubation continued for another 4 h followed by a boosting with 20% glycerol in DMEM (vol/vol) for 1.5 min. Transfected cells were washed twice and incubated in DMEM plus 10% FCS for 60 h.

Immunoprecipitation. Cells were harvested 48 h after DNA transfections. For immunoprecipitations, transfected cells were first starved for 1 to 2 h in methionine- or phosphate-free DMEM supplemented with 0.5% heat-inactivated FCS and then labeled with 250 μCi of [^{35}S]methionine or $^{32}P_i$ per ml, respectively, for 4 h. Labeled cells were lysed as described elsewhere (11) in 0.05 M HEPES (pH 7.5)–2% sodium dodecyl sulfate (SDS)–9 mM EDTA (pH 7.5). Lysed cells were sonicated five times with a microtip on ice for 20 s, boiled for 5 min, and then centrifuged for 5 min to remove cell debris. Five volumes of lysis buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 150 mM NaCl, 2.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride) was added to the clear supernatant. [^{35}S]methionine or $^{32}P_i$ incorporation into high-molecular-weight proteins was determined by trichloroacetic acid precipitation of this fraction. The same number of counts per minute was used for immunoprecipitation with either monoclonal antibody directed against EA-D or EA-R (Biotech Research Laboratories, Inc.). The antibody incubation was performed at 4°C for up to 12 h, and the antibody-antigen complexes were precipitated by 50 μl of 10% protein A-sepharose 4B. For precipitation with EA-D monoclonal antibody, protein A-sepharose 4B was pretreated with 1 μl of rabbit anti-mouse immunoglobulin G1 (IgG1) for 1 to 2 h at 4°C. The immunoprecipitates were washed and separated by 12.5% SDS-polyacrylamide gel electrophoresis a described by Hearing et al. (18).

Immunofluorescence. Transfected cells were trypsinized 1 day after transfection and seeded on printed glass slides. After 1 day, cells were fixed at −20°C for 5 min in acetone and stained by indirect immunofluorescence (20). Fixed cells were incubated with anti-EA-D monoclonal antibody or human serum and stained with fluorescein isothiocyanate-conjugated anti-mouse IgG or anti-human IgG, IgM, and IgA, respectively. Serum no. 353 (from W. Henle) from a patient recovering from infectious mononucleosis had high-titered antibodies (greater than a 1/80 dilution) against viral encapsidated antigen and EA-D but not against EBV nuclear antigen. A serum sample from a patient with nasopharyngeal carcinoma (from J. C. Nicolas) was EA-D antibody positive and also contained antibodies to the BMLF1 nuclear antigen. Not every nasopharyngeal carcinoma serum tested was BMLF1 antibody positive.

CAT assays. CAT assays were performed at 60 h posttransfection by the method of Gorman et al. (14) with the following modifications. The monolayers of the cells were scraped in 1.5 ml of 0.25 M Tris hydrochloride (pH 8.0) and centrifuged for 2 min, and the cell pellets were suspended in 75 μl of 0.25 M Tris. The cells were frozen at −80°C for a minimum of 5 min and thawed at 37°C for 5 min, and the freeze-thaw cycle was repeated two more times. The cells were centrifuged for 5 min to remove cell debris. The total amount of cell protein in the supernatant was quantitated by a Bio-Rad protein assay (Bio-Rad Laboratories). For CAT assays, the same amount of protein in a 50-μl volume was added to 70 μl of 1 M Tris hydrochloride (pH 8.0)–0.1 μCi of [^{14}C]chloramphenicol and warmed up to 37°C, and 30 μl of 4 mM acetyl coenzyme A was added. The reaction was stopped by extracting the chloramphenicol in 1 ml of ethyl acetate. The organic layer was dried, suspended in 30 μl of ethyl acetate, spotted on a silica gel chromagram sheet

(Eastman Kodak Co.), and run in chloroform-methanol (95:5). For quantitative comparisons of CAT activity, all three acetylated chloramphenicol forms were scraped into Biofluor (New England Nuclear Corp., Boston, Mass.) and counted separately from the unacetylated form to obtain the percentage of acetylation.

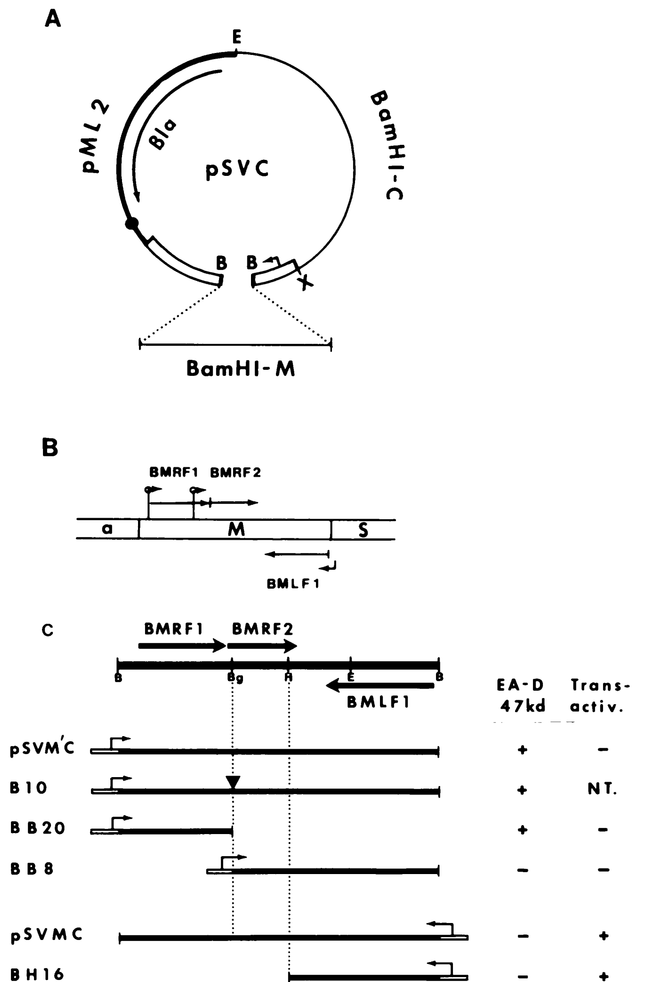

FIG. 1. Structure of the EBV *Bam*HI-M expression vectors. (A) Structure of the parent vector pSVC. ▬, pML2-derived sequences encoding the β-lactamase gene (βla); ●, the ColE1 replication origin; ——, EBV sequences (*Bam*HI-C is a 5.1-kb *Eco*RI-*Pvu*II fragment derived from pDK16, and *Bam*HI-M is a 4.7-kb *Bam*HI fragment derived from pDF516); ⊏⊐, SV40 early regulatory regions controlling the expression of the inserted *Bam*HI M fragment, with the arrow indicating the direction of SV40-promoted transcription. E, *Eco*RI; B, *Bam*HI; X, *Xho*I. (B) Schematic representation of the three putative ORFs within the *Bam*HI-M region (adapted from Baer et al. [1]). The three ORFs, BMRF1, BMRF2, and BMLF1, are represented by horizontal arrows. ➡, Promoters shown to function in EBV B95-8 cells; ↱, promoter predicted from DNA sequences (1). (C) Left panel: structure of *Bam*HI-M-expressing plasmids. The three ORFs, BMRF1, BMRF2, and BMLF1, are represented by horizontal arrows. ⊏⊐, SV40 regulatory region containing the early promoter and enhancer, with the arrow indicating the direction of transcription; ▼, a frameshift mutation in the B10 plasmid obtained by filling in the overlapping ends at the *Bgl*II site and ligating the ends together. B, *Bam*HI; G, *Bgl*II; E, *Eco*RI; H, *Hin*dIII. Right panel: summary of properties of these *Bam*HI-M-containing plasmids with respect to coding capacity for the 47-kDa EA-D or the *trans*-activating function. NT, Not tested.

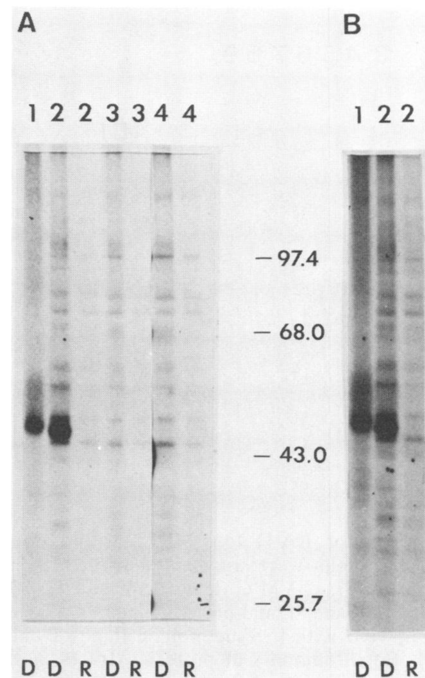

FIG. 2. Transient expression of EA-D in COS-7 cells transfected with the *Bam*HI-M plasmids. (A) COS-7 cells were transfected with the pSVM'C plasmid (lanes 1 and 2), the pSVMC plasmid (lane 3), or the parent vector pSVC (no EBV insert) (lane 4). Transfected cells labeled in vivo with $^{32}P_i$ (lane 1) or [^{35}S]methionine (lanes 2 to 4) were then immunoprecipitated with monoclonal antibodies directed against EA-D (D) or EA-R (R). (B) A longer exposure of the same autoradiogram of lanes 1 and 2.

RESULTS

Expression of EBV EAs from the *Bam*HI M DNA fragment. A variety of previous studies demonstrated that the *Bam*HI M DNA fragment of the EBV genome encodes viral early proteins which might be important in the switch from the latent state to productive infection (3, 4, 5, 35, 41). The *Bam*HI M DNA fragment is composed of three ORFs (1) that could encode reasonably sized proteins. Two of these ORFs can be transcribed in the rightward orientation and are called BMRF1 and BMRF2, and one ORF, BMLF1, is transcribed in the opposite or leftward orientation (Fig. 1B). To examine the proteins encoded by this region and their possible functions, the *Bam*HI M DNA fragment was cloned in either orientation (pSVM'C or pSVMC) into an expression vector plasmid (pSVC) by using the SV40 enhancer-promoter signals to regulate the EBV proteins (Fig. 1). These plasmids, termed pSVMC (for BMLF1) and pSVM'C (for BMRF1 and 2) (Fig. 1C) were transfected into COS-7 monkey cells, which express the SV40 large T antigen (13). Each plasmid contained the origin of SV40 DNA replication and was expected to replicate to a high copy number, producing a large number of template DNA molecules for maximal expression of the *Bam*HI-M-induced proteins. The proteins were labeled with either [^{35}S]methionine or $^{32}P_i$ from 44 to 48 h after DNA transfections. Soluble protein extracts were prepared and immunoprecipitated with monoclonal antibodies directed against EA-D or EA-R components of the EA complex. The immunoprecipitates were analyzed with SDS-polyacrylamide gel electrophoresis, and an autoradiogram of this gel is presented in Fig. 2. The pSVM'C plasmid, which

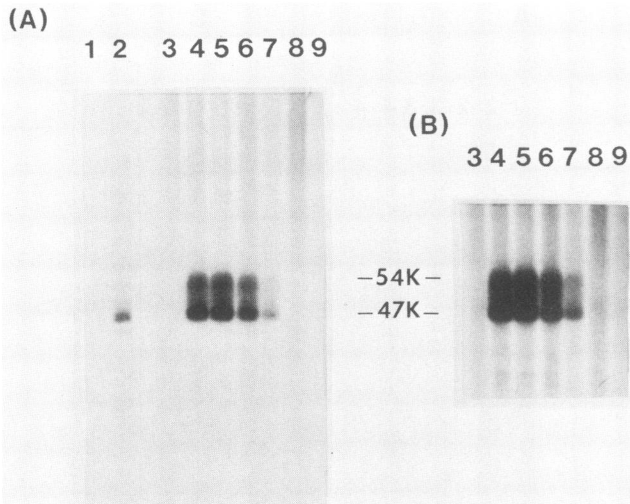

FIG. 3. Mapping of EA-D to BMRF1 within the *Bam*HI-M region. (A) COS-7 cells were transfected with the parent vector pSVC (lanes 1 and 3), pSVM'C (lanes 2, 4, and 5), B10 (lane 6), BB20 (lane 7), BB8 (lane 8), and pSVMC (lane 9). Transfected cells were labeled in vivo with [^{35}S]methionine (lanes 1 and 2) or $^{32}P_i$ (lanes 3 to 9). Equal amounts of protein extracts, normalized by counts per minute, were used for immunoprecipitation with anti-EA-D monoclonal antibody in lanes 3, 4, and 6 to 9. Lane 5 is a duplicate of lane 4, except that more protein was used in the immunoprecipitation. (B) A longer exposure of the same autoradiogram of lanes 3 to 9.

expresses proteins from BMRF1 and 2, produced in COS-7 cells a protein of about 47,000 Da that was labeled with $^{32}P_i$ (Fig. 2A, lane 1D) or [^{35}S]methionine (lane 2D) and immunoprecipitated with anti-EA-D antibody (D lanes) but not with anti-EA-R antibody (R lanes) (lane 2R). This protein was not synthesized by the parent vector pSVC (Fig. 1) which does not contain the *Bam*HI M restriction fragment (lanes 4D and 4R). The pSVMC plasmid which promotes the expression of the BMLF1 gene also failed to produce a protein detected by EA-D antibody (lane 3D) or EA-R antibody (lane 3R). Thus, BMRF1 or 2 or both stimulated the production of a 47,000-Da protein in COS-7 cells. The phosphate-labeled protein produced by this vector was slightly larger in size on SDS-polyacrylamide gels than the methionine-labeled protein, and this could be due to the heterogeneous modification of the protein (compare Fig. 2A, lanes 1D and 2D). A longer exposure of this same gel (Fig. 2B, lanes 1D, 2D, and 2R) showed a molecular-weight heterogeneity, especially with the phosphate-labeled proteins immunoprecipitated by the EA-D monoclonal antibody.

Mapping EA-D to the BMRF1. The pSVM'C plasmid that promotes synthesis of the 47,000-Da EA-D entity could be derived from either BMRF1 or 2 (Fig. 1C). To localize this protein and determine whether any of the heterogeneity was due to the use of both ORFs (spliced mRNAs, etc.), subclones of the *Bam*HI M DNA restriction fragment were prepared and inserted into the SV40-based expression vector. Plasmid B10 contained a frame shift mutation in BMRF2 but left the BMRF1 region intact (Fig. 1C). BB20 contained a deletion of BMRF2 made by dropping the *Bgl*II to *Bam*HI site and retaining only BMRF1. BB8 (Fig. 1C) had the SV40 enhancer-promoter regulating the majority of BMRF2 but lacking the start codon (ATG) of BMRF2. The plasmids pSVM'C, B10, BB20, and BB8 were transfected into COS-7 cells, and 44 h later the cell cultures were labeled with $^{32}P_i$ or [^{35}S]methionine for 4 h. The EA-D monoclonal antibody was used to immunoprecipitate this protein, and the autoradiogram of the gel analyzing these immunoprecipitates is shown in Fig. 3. The parent vector pSVC, containing no inserts of EBV DNA, that was transfected into COS-7 cells labeled with either [^{35}S]methionine (Fig. 3A, lane 1) or $^{32}P_i$ (lane 3) produced no detectable protein when immunoprecipitated with the EA-D monoclonal antibody. The pSVM'C vector, containing the entire *Bam*HI M DNA fragment, synthesized a 47,000-Da protein labeled with [^{35}S]methionine (lane 2) and a heterogeneous collection of proteins labeled with $^{32}P_i$ (lanes 4 and 5). The vectors containing the B10 or BB20 inserts of EBV DNA synthesized similar spectrums of phosphate-labeled EA-D proteins (lanes 6 and 7, respectively), as was observed with wild-type *Bam*HI-M DNA (lanes 4 and 5). The BB20 construction synthesized less EA-D-specific protein (lane 7), but a longer exposure of the same gel (Fig. 3B, lanes 3 to 9) showed that BB20 produced the same heterogeneous collection of EA-D molecules but at a lower level (Fig. 3B, lane 7). Deletion of BMRF1 from pSVM'C in the BB8 vector (Fig. 1C) resulted in the failure to synthesize EA-D (Fig. 3A and B, lane 8). Transfection with pSVMC with the *Bam*HI-M inserted in the opposite orientation (Fig. 1C) also failed to produce any detectable EA-D protein (Fig. 3A and B, lane 9) in an assay using the anti-EA-D antibody.

These data map the coding region for the EA-D polypeptides detected by the EA-D monoclonal antibody to BMRF1 in *Bam*HI-M (Fig. 1C). The heterogeneity of the EA-D protein complex observed with phosphate labeling must therefore be accounted for by sequences in BMRF1. The BB20 deletion mutant, which contains only 113 base pairs of BMRF2, produces all the heterogeneous forms of EA-D observed in gel electrophoresis (Fig. 3B). The BB20 vector (Fig. 1C) did produce much lower levels of EA-D protein labeled by $^{32}P_i$, even though equal amounts of protein extracts (normalized by counts per minute) were immunoprecipitated by anti-EA-D antibody (Fig. 3, lanes 3 to 9). Transcription through the BMRF2 region may be important for mRNA stability, and this could lower the level of EA-D produced by the BB20 plasmid.

Nuclear localization of BMRF1 EA-D and a second nuclear antigen encoded by BMLF1 by using indirect immunofluorescence. To demonstrate the localization of the EA-D antigen, COS-7 cells were transfected with the parent vector with no EBV DNA (pSVC) or a variety of vectors previously shown to be positive or negative for EA-D expression (Fig. 1, 2, and 3). Antisera used to detect these antigens by immunofluorescence were the EA-D monoclonal antibodies, a human polyclonal antiserum (no. 353) that was shown to have a high titer against EA-D from a patient recovering from infectious mononucleosis, or serum from a patient with nasopharyngeal carcinoma also known to have antibodies against EAs. Cells transfected with the parent vector with no EBV DNA (pSVC) and stained with anti-EA-D monoclonal antibody were negative (Fig. 4A, a). Cells transfected with the vector containing the entire *Bam*HI M fragment in the BMRF1 orientation (pSVM'C) or the vector containing only the BMRF1 region (BB20) and stained with monoclonal anti-EA-D antibody contained nuclear fluorescence (Fig. 4A, b and c, respectively). Cells transfected with the parent vector pSVC with no EBV DNA and then stained with polyclonal human serum from an infectious mononucleosis patient were negative for immunofluorescence (Fig. 4B, a). Similar cells transfected with pSVM'C or BB20, shown previously to

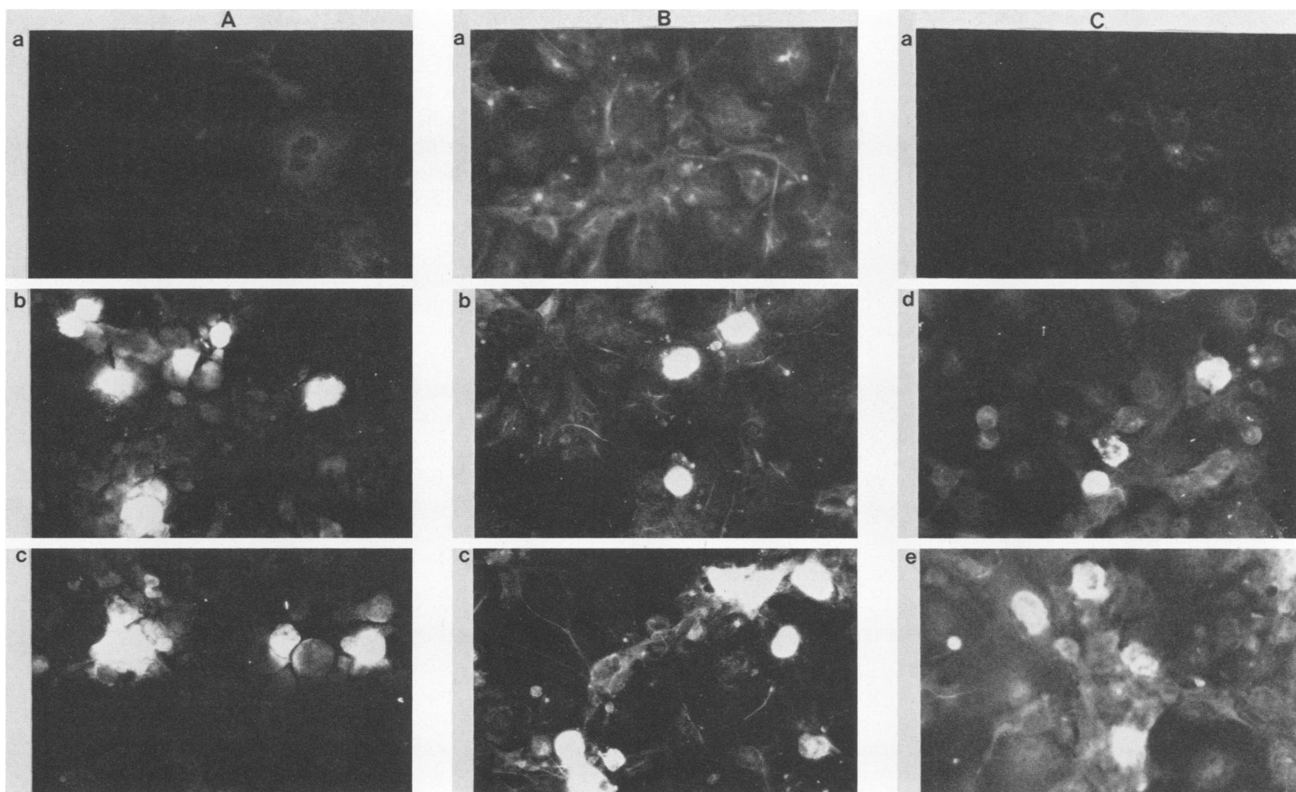

FIG. 4. Indirect immunofluorescent staining of COS-7 cells transfected with *Bam*HI-M-expressing plasmids. (A) Staining with monoclonal antibodies against EA-D. (B) Staining with human serum no. 353 obtained from a patient with infectious mononucleosis. EA-D titer for this serum is 80-fold dilution. (C) Staining with serum no. 49054 from a patient with nasopharyngeal carcinoma which has a high EA titer of 1,280-fold dilution. COS-7 cells were transfected with the parent vector pSVC (a), pSVM'C (b), BB20 (c), pSVMC (d), and BH16 (e).

produce EA-D, also stained positively in the nucleus with this human polyclonal serum (Fig. 4B, b and c, respectively). However, the same EA-D-specific polyclonal human serum did not detect any nuclear fluorescence in cells transfected with the pSVMC or BH16 vector in which a protein derived from BMLF1 (from the opposite strand of DNA) was expressed (Fig. 1C) (data not shown). Antibodies in serum from a nasopharyngeal cancer patient, however, did react with the nuclear protein produced by BMLF1 in vector pSVMC or BH16 (Fig. 1C), as seen in Fig. 4C, d and e, respectively. This same polyclonal serum from the nasopharygeal cancer patient did not detect any antigens in COS-7 cells transfected with the parent vector containing no EBV DNA (Fig. 4C, a).

These results demonstrate that the *Bam*HI-M DNA fragment of EBV expresses, in addition to EA-D, a second protein in an ORF with an orientation opposite to that of the EA-D protein. The ORF encoding this nuclear protein is BMLF1, as mapped by the BH16 expression vector (Fig. 1C). Cho et al. (4) showed that BMLF1 also encodes a nuclear protein, and these data agree with their observations.

Identification of a *trans*-activating function promoted by the BMLF1 gene product. The *Bam*HI M DNA fragment encodes the EA-D complex of proteins, which is thought to be involved in the switch from a latent to a productive infection. This switch is accompanied by the activation of many additional EBV genes and the synthesis of new EBV gene products. The adenoviruses and herpesviruses encode gene products E1A (2, 27, 32) and ICP-4 (8, 36, 45), respectively, which can transcriptionally activate early viral genes after infection. An experiment was devised to determine whether either of the *Bam*HI-M gene products, BMLF1 (Fig. 1C, BH16 vector) or BMRF1 (Fig. 1C, BB20 vector), could provide a gene enhancer or activator in *trans*. The test plasmid was a hybrid vector containing the adenovirus E3 region promoter linked to the CAT structural gene, termed pKCAT23 (46). HeLa cells were cotransfected with the pKCAT23 plasmid and, as a control, the parent vector pSVC with no EBV sequences, BB20 expressing the EA-D antigen, or BH16 expressing the BMLF1 gene product. As a positive control, pE1A expressing adenovirus E1A, or pIE, expressing the pseudorabies *trans*-activator intermediate-early product, was cotransfected into HeLa cells with the E3-CAT plasmid. At 60 h posttransfection, pooled cell extracts (from five culture dishes) were prepared and assayed for CAT enzyme activities. The autoradiogram of the products produced by acetylation of chloramphenicol is shown in Fig. 5. The lane marked CAT is the addition of commercial CAT enzyme to a substrate, used as a positive control in this experiment. In each sample, the percentage of substrates acetylated was quantitated by determining the radioactivity levels of all three acetylated products (Fig. 5) by using scintillation counting procedures. The percentage of acetylation for the products is also presented in Fig. 5.

The EA-D protein produced by the BB20 vector had no effect upon the E3-CAT activity in HeLa cells. Cotransfection with the BB20 vector yielded the same background level of acetylation as with the pSVC vector, which contains no EBV DNA (Fig. 5). By contrast the BH16 vector, which

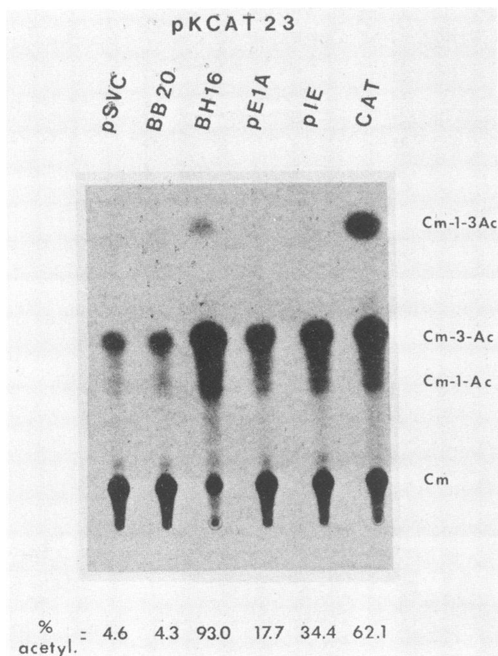

FIG. 5. Identification and mapping of an EBV *trans* activator to BMLF1 by using transient CAT assays. Plasmid pKCAT23 (E3-CAT) was cotransfected with each of the indicated plasmids into HeLa cells which were subsequently harvested at 60 h posttransfection. CAT is commercial CAT. The percent acetylation is calculated as the percentage of the ratio of counts of acetylated products (Cm-1-Ac, Cm-3-Ac, and Cm-1-3-Ac) over total counts (acetylated plus unacetylated chloramphenicol).

synthesized the BMLF1 protein (Fig. 4C, 2), produced an extract that acetylated 93% of the chloramphenicol and showing a marked stimulation (20-fold) in E3-CAT activity. The positive controls, the adenovirus E1A (pE1A), pseudorabies gene product (pIE), and commercial CAT enzyme itself, stimulated 4- to 14-fold above the background. As a further control, the E3-CAT DNA plasmid concentrations in these HeLa cell extracts after transfection were determined by slot blot hybridization of the low-molecular-weight DNA found in the Hirt extraction (23). The amount of plasmid DNA in each extract did not differ more than 1.0- to 1.5-fold, demonstrating that roughly equal levels of template DNA for E3-CAT were present in each extract (data not shown).

Thus, the BMLF1 gene product can act upon E3-CAT to activate, in *trans*, the level of the CAT gene product. To determine whether this *trans* activator would also stimulate other promoters, similar experiments described with E3-CAT cotransfections were repeated with the CAT gene regulated by the SV40 early promoter in a construction where the SV40 enhancer was deleted (pSVΔCAT). These results, studying the impact of EA-D and BMLF1 gene products upon E3-CAT and pSVΔCAT activities, are summarized and compared in Table 1. The parent vector pSVC, with no EBV sequences, did not stimulate CAT activity with either the E3 or SV40 early promoters regulating the CAT gene. Similarly, the EA-D gene product, synthesized by BB20 or pSVM'C, failed to stimulate the E3 or SV40 early promoters (Table 1). By contrast, the BMLF1 gene product produced by pSVMC and BH16 (Fig. 1C) stimulated both the E3-CAT and pSVΔCAT gene constructions between 11- and 24-fold in several replicate experiments. The pseudorabies *trans* activator made by the pIE plasmid also enhanced both E3-CAT and pSVΔCAT activities (7- to 23-fold), but the adenovirus E1A *trans* activator stimulated only the E3 promoter but not the SV40 early promoter (Table 1). This result is in agreement with those of Velcich and Ziff (44), who showed that the E1A gene product failed to act upon the SV40 early promoter in the absence of its enhancer.

These results (Fig. 5 and Table 1) demonstrate that the gene product from BMLF1 can function as an activator, in *trans*, upon two diverse promoters, the adenovirus E3 and SV40 early promoters. In addition, these results discriminate among the specificities of different *trans*-acting enhancer activities.

DISCUSSION

Expression of EA marks the switch from the latent state to the lytic cycle in EBV infection. Since the *Bam*HI M fragment of the EBV genome encodes the EA-D complex (5, 35), detailed characterization of this region of the genome could prove to be fruitful in understanding the events leading out of the latent state and into the initiation of the replicative cycle of EBV.

Using antiserum raised against a *Bam*HI-M product synthesized in bacteria, Cho et al. (5) reported the mapping of the 48- to 50-kilodalton (kDa) EA-D protein to BMRF1. In this study, we used DNA transfection to map the coding region of EA-D directly to BMRF1. This EA-D component was specifically recognized by an anti-EA-D monoclonal antibody. The same monoclonal antibody was used by Lin et al. (29) in Western analysis to identify components of the EA-D complex in 12-*O*-tetradecanoylphorbol-13-acetate-induced B95-8 and Raji cells. A family of four polypeptides with molecular weights of 46,000, 49,000, 52,000, and 55,000 was identified as the major components of EA-D. The same monoclonal antibody was also used by Pearson et al. (35), and it recognized polypeptides of 44 and 47 kDa in in vitro translated products from B95-8 RNA hybrid selected with the *Bam*HI M fragment. Lin et al. (29) suggested that the difference in size between the in vitro translated products and the in vivo viral products might be due to posttranslational modifications. The results presented here demonstrate that a single gene in BMRF1 encodes a family of 47- to 54-kDa phosphoproteins which belongs to the EA-D complex. Transfection of the B10 subclone, which contained a frameshift mutation at the *Bgl*II site in BMRF2 (Fig. 1C), resulted in the expression of the same set of polypeptides as when the full-length *Bam*HI M fragment was used for transfection. In addition, transfection with the BB20

TABLE 1. *Trans* activation of pKCAT23 (E3-CAT) and pSVΔCAT (SV40 enhancerless) genes

Cotransfected test plasmid	Fold induction[a]	
	pKCAT23	pSVΔCAT
pSVC	1.0	1.0
BB20	0.9–2.9	0.5
pSVM'C	2.4–2.9	0.6–1.3
BB8	3.7–4.1	2.3–3.6
pSVMC	12.3–20.3	13.2–24.0
BH16	10.8–22.4	13.1–23.9
pE1A	3.8–9.9	1.1–2.1
pIE	7.5–7.6	13.0–22.9

[a] Fold induction is based on a comparison of percent acetylation (of all three forms) in each of the test plasmid cotransfections with that in the cotransfection with the parent vector pSVC. The ranges derive from multiple experiments.

subclone, which contained only BMRF1 coding sequence, resulted in the expression of the same set of polypeptides but at a lower level (Fig. 3, compare lanes 4 and 7). The reduced expression of EA-D in COS-7 cells transfected with the BB20 plasmid may be due to truncation of sequences downstream of the *Bgl*II site (Fig. 1C). The BB20 subclone may lack some portion of the 3′ noncoding region of the EA-D gene which may be essential for the stability of the mRNA. Consequently, the reduced level of mRNA is translated into a reduced level of protein. Qualitative and quantitative analyses of RNA expressed from these transfected plasmids in COS-7 cells will clarify this issue. One possible explanation for multiple proteins derived from only one ORF is that this set of phosphoproteins may be the products of differentially spliced RNA species expressed from BMRF1. However, a recent report by Sample et al. (37) indicates that there are three unspliced mRNAs spanning the BMRF1 region. A second possibility is that BMRF1 produces one protein recognized by the EA-D monoclonal antibody, but additional cellular proteins are associated with this viral gene product in a complex that is coimmunoprecipitated. The immunoprecipitations in this study used 2% SDS in the cell lysis buffer, and these conditions tend to minimize the possibility of high-molecular-weight complexes of proteins which would coimmunoprecipitate. The third possibility, as has been suggested by Lin et al. (29), is posttranslational modifications of the BMRF1 protein. The results reported here support this conclusion because a major 47-kDa polypeptide was observed when the transfected cells were labeled with [^{35}S]methionine. However, a more heterogeneous collection of proteins was immunoprecipitated with the same monoclonal antibody when protein extracts were derived from cells labeled with $^{32}P_i$. These results are consistent with the hypothesis that the higher-molecular weight species are poorly labeled with methionine (due to low protein concentrations) but highly modified with phosphate, which alters their mobility in SDS-polyacrylamide gels (Fig. 2 and 3).

The *Bam*HI M fragment encodes a second nuclear protein encoded by BMLF1, as reported here and previously (4). Deletion mapping within the *Bam*HI-M DNA localized it to an ORF (BMLF1) in expression vector BH16 (Fig. 1C). During the search for possible functions for this gene product, it was shown that this nuclear protein was able to enhance or activate the level of the CAT gene product under the control of the adenovirus E3 promoter (11- to 22-fold) or under the control of the SV40 early region promoter (with no enhancer) by 13- to 24-fold. This EBV-encoded *trans* activator shares some similarities and some differences with other viral *trans* activators, such as the adenovirus E1A gene product or the pseudorabies intermediate-early product (26). The adenovirus E1A gene product stimulated only the E3 promoter but not the SV40 early promoter (Table 1), in agreement with the results of Velcich and Ziff (44). The pseudorabies and EBV *trans* activators stimulated both the SV40 early promoter and the adenovirus E3 promoter. The differences in specificities of the *trans* activator functions may imply different mechanisms or the involvement of different cellular factors in this gene regulation process.

It is very likely that the *trans*-activating functions described here could play a role in the switch from a latent state to a productive infection. To investigate this, it will be important to examine the specificity of the BMLF1 *trans* activator on various promoters in the EBV genome. Until more specific information is available, it remains unclear whether the BMLF1 activator is involved in latency (in a positive or negative fashion) or in the switch to a productive infection. It is of some interest that the adenovirus E1A gene function may be involved with immortalization of cells in culture (24, 42) and EBV can also immortalize lymphocytes in culture. Whether the BMLF1 gene product is involved in this process remains to be tested.

Finally, it is useful to be concerned with the nomenclature of these viral proteins and gene functions. Cho et al. (4) have described the BMLF1 gene product as part of the EA-D complex. In this communication, the results have clearly shown that the EA-D-specific monoclonal antibody does not react with the BMLF1 gene product. In addition, a human polyclonal serum (from a patient recovering from infectious mononucleosis) defined as EA-D positive was shown here as BMLF1 antigen negative. It is, after all, simply a matter of definition whether to include the BMLF1 protein in the EA-D complex. However, with the evidence available to date, it seems better to define EA-D by a consistent criterion, i.e., the use of an EA-D-specific monoclonal antibody.

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