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A New Epstein-Barr Virus Transactivator, R, Induces Expression of a Cytoplasmic Early Antigen

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Several Epstein-Barr virus (EBV) early promoters respond to a new EBV transactivator encoded by BRLF1, designated R. Transactivation was measured in chloramphenicol acetyltransferase assays on Raji, BHK, and Vero cells that were cotransfected with the transactivator and target promoters linked to the *cat* gene. The divergent promoter of *Bam*HI-H was particularly responsive to R transactivation. This large promoter region consists of a leftward TATA box for the *Not*I repeat gene (BHLF1) and a probable rightward TATA box for the EA-R gene (BHRF1) separated by 940 base pairs of unusual sequence complexity. Sequences within this divergent promoter region appear to confer inducibility by EBV transactivators R and Z (BZLF1). The Z transactivator stimulated expression in both the leftward and rightward directions, and R stimulated expression primarily in the rightward direction, but the MS transactivator (BMLF1) had no activity in either direction. The adenovirus E3 promoter also responded to the R transactivator, but several other herpesvirus and human promoters were nonresponsive. When the divergent promoter was linked to the EA-R gene as it is in the EBV genome, the R and Z transactivators also induced the expression of EA-R in cotransfected cells. This cytoplasmic early antigen is encoded by BHRF1 and may be anchored in intracellular membranes by a carboxy-terminal transmembrane region.

Like other herpesviruses, Epstein-Barr virus (EBV) has both a lytic and a latent life cycle. In addition to B lymphocytes, EBV will infect the nasopharyngeal and cervical epithelium (55–57). Infection of epithelial cells results in cell death and production of progeny virus, which provides for transmission of the virus to a new host, where it can cause infectious mononucleosis. Infection of lymphocytes usually leads to a latent infection which, in conjunction with other factors such as immunosuppression, can lead to B-cell lymphomas (28). Together with environmental, genetic, or other factors which have not been clearly defined, EBV also contributes to nasopharyngeal carcinoma (26) and Burkitt's lymphoma (46, 63).

When human peripheral B lymphocytes are infected with EBV in vitro, several events occur: the cells are stimulated to produce antibody, a latent infection is established, and the cells are immortalized such that they will grow continuously in culture (36, 45, 48). Gene expression during the latent cycle is tightly regulated, and only a few of the approximately 100 genes are expressed (12). The events that occur in a B cell to prevent or shut down the lytic cycle have not been determined.

Because a permissive system for study does not exist, a commonly used technique is to induce immortalized B cells to enter the lytic cycle by treating them with various chemicals including 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (65). Thus, induction by TPA makes endogenous lytic cycle gene expression subject to experimentation. The mechanism of TPA activation is unknown. Furthermore, it is not known which EBV promoters are TPA responsive. TPA could serve to activate a transactivator or the target(s) of this transactivator. Several EBV genes have been reported to encode transactivators. The Z transactivator, encoded by BZLF1 (*Bam*HI-Z left reading frame no. 1), when transfected into latently infected cells, can induce early antigen production in Raji cells and production of infectious virus from X50-7 cells (8, 19). The MS transactivator, encoded by BMLF1, activates a number of different promoters from various sources (32). A latent-cycle gene product, EBV nuclear antigen no. 1 (EBNA1), encoded by an open reading frame in the *Bam*HI K fragment, has been shown to activate transcription (via *Bam*HI-C enhancer sequences) in addition to its role in maintaining the latent episome (47). There is also evidence to suggest that another latent-cycle product, EBNA2, induces expression of the human CD23 gene (61).

Takaki et al. (59) reported that an EBV subclone, 302-23, could induce the expression of an EBV cytoplasmic early antigen (EA-R) encoded by *Bam*HI-HF. They further suggested that this transacting activity of 302-23 was localized within a smaller fragment, *Hin*dIII I2. It has since become clear that all the transactivators described above (except EBNA2) are encoded within the EBV subclone 302-23. However, none of these transactivators is encoded within the *Hin*dIII I2 fragment. Takada et al. (58) activated the same cytoplasmic early antigen with the *Bam*HI-Z fragment (containing the Z transactivator gene). Because only the amino-terminal 25 residues of the Z gene (BZLF1) are encoded within the *Hin*dIII I2 fragment, it was possible that an additional transactivator was encoded by *Hin*dIII I2.

In this report we describe a new transactivator, designated R. This lytic-cycle transactivator is encoded by BRLF1, which is contained intact within the *Hin*dIII I2 fragment. R has specificity for a complicated divergent promoter in *Bam*HI-H, leading to stimulated synthesis of the cytoplasmic early antigen, EA-R, encoded by BHRF1.

MATERIALS AND METHODS

Plasmid construction. EBV DNA fragments from B95-8 or M-ABA were cloned into pGH52 (a simian virus 40 [SV40] expression vector), pCAT (a vector for the chloramphenicol acetyltransferase [CAT] gene [*cat*]), pGH56 (a derivative of pUC18 with *Hin*dIII-*Bgl*II-*Hin*dIII sites in place of the

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*Hin*dIII site), or pBR322. pGH52 is pSV2neo with *Hin*dIII-*Bgl*II/*Hin*dIII sites in place of the *Hin*dIII site. The *cat* vector pCATB′ was obtained from O. Andrisani, Purdue University, Lafayette, Ind. pCATC′ is the *Bam*HI-*Sal*I fragment of pCATB′ (containing the *cat* gene and SV40 polyadenylation signals) cloned into pUC13. Cosmid clones 780-35 and 302-23 of M-ABA (44) were obtained from G. Bornkamm, University of Freiburg, Freiburg, Federal Republic of Germany. pSL77 contains the *Bam*HI H fragment of B95-8 (22). pPL17 has the *Bam*HI Z fragment of B95-8 inserted at the *Bgl*II-*Bam*HI sites of pGH52. pPL23A has a 230-base-pair (bp) *Sau*3AI fragment of EBV B95-8 *Bam*HI-a (promoter region of the ribonucleotide reductase small subunit) ligated into the *Bam*HI sites of pCATB′. pTS6 contains the MS transactivator as described previously (10). pDH136 contains the *Sph*I-*Nhe*I fragment of B95-8 *Bam*HI-S inserted into pCATB′. A-10CAT was obtained from G. Khoury, National Institutes of Health, Bethesda, Md. E3-CAT contains the adenovirus type 5 E3 regulatory sequences fused to the *cat* gene as previously described (62). SAA-CAT contains the hormone-responsive promoter region of serum amyloid A protein fused to the *cat* gene and was obtained from C. Lowell and J. Morrow, Johns Hopkins Medical School, Baltimore, Md. TK-CAT (pPOH3) and 175KCAT (pPOH2) were constructed as described previously (37, 38). pMH1A contains the *Bcl*I fragment (promoter region for BMRF1) which crosses the *Bam*HI-aM boundary cloned into the *Bam*HI site of pCATB′. pMH16 was constructed by cloning the *Bgl*II W fragment (containing the EA-R gene) of EBV M-ABA into the *Bgl*II-*Bam*HI sites of pGH52. pMH17 was made by inserting the *Bam*HI-*Nae*I fragment of *Bam*HI Z into pCATB′. pMH24 contains the *Bam*HI R fragment of EBV M-ABA inserted at the *Bam*HI site of pBR322. pMH25 was generated by cloning the *Hin*dIII I2 fragment of EBV M-ABA into the *Hin*dIII site of pGH56. pMH10 contains the *Sph*I-*Stu*I fragment (*Not*I gene) of B95-8 *Bam*HI-H inserted at *Sph*I-*Hin*cII sites of pGH56. pMH39 was constructed by picking up the *Bam*HI-*Bgl*II fragment of pMH10 (containing the B95-8 subfragment) and inserting it into the *Bgl*II-*Bam*HI sites of pGH52. pMH44 has the *Msp*MI-*Cla*I fragment (regulatory sequences for BRLF1) of pMH24 inserted at the *Bam*HI and *Cla*I sites of pCATB′. pMH11 was constructed by ligating the *Stu*I-*Bgl*II fragment (divergent promoter, leftward orientation) of B95-8 *Bam*HI-H into the *Bgl*II-*Hin*cII sites of pGH56. pMH47 contains the *Bam*HI-H sequences of pMH11 which were excised at the *Bam*HI and *Bgl*II sites and ligated into the *Bam*HI site of pCATC′. pMH49 contains the same insert as pMH47, except in the opposite orientation, such that the rightward divergent promoter is driving *cat*. pMH48 (SV40 promoter driving BRLF1) was constructed by ligating the *Hin*dIII I2 fragment of EBV M-ABA into the *Hin*dIII sites of pGH52. pMH50, a deletion mutant of pMH48, lacks the *Sal*I-*Xho*I fragment which contains most of BRRF1 and spans the *Bgl*II site. pMH58, also a deletion mutant of pMH48, lacks the *Mst*II fragment that spans the two *Bam*HI sites between Z and R. pMH53 contains the *Bgl*II W fragment of EBV M-ABA inserted at the *Bgl*II site of pMH11 (divergent promoter driving the EA-R gene). pMH81 is a partial *Xma*I deletion mutant of pMH53 lacking 1,133 bp of the divergent promoter but retains the TATA box.

Cells and transfections. Vero, BHK, and NIH 3T3 cells were maintained in Dulbecco modified Eagle minimal essential medium with 10% fetal calf serum and were transfected by the calcium phosphate precipitation method (17, 37). At 24 h before transfection, cells were seeded into cluster dishes (each with six 35-mm wells) at 5×10^5 cells per well or into two-well slides at 2×10^5 cells per well. The medium was changed and antibiotics were added 3 to 4 h before transfection. The transfection cocktail contained 1 μg of each plasmid DNA, 125 μl of 2× HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 12 mM glucose, 42 mM HEPES [pH 7.04]), and H_2O to a final volume of 238 μl. While the transfection cocktail was bubbled, 12 μl of 2.5 mM $CaCl_2$ was added over a 30-s period. Samples were incubated at room temperature for 20 min to allow precipitate formation and then added dropwise to the culture medium. The cells were glycerol shocked 4 h posttransfection (39) by being treated for 30 s with 15% glycerol in medium without serum. The cells were washed with phosphate-buffered saline to remove the glycerol and incubated with complete medium plus antibiotics for 40 h.

B95-8 and Raji cells were maintained in RPMI 1640 with 10% fetal calf serum and were transfected by the DEAE-dextran procedure (34), modified by Takada et al. (58). Fresh medium was added 24 h before transfection. The transfection mix contained 2 μg of each plasmid DNA, 300 μl of calcium TBS (containing, per liter, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of $CaCl_2$, 8.0 g of NaCl, 0.38 g of KCl, 0.1 g of $Na_2HPO_4 \cdot 12H_2O$, and 3.0 g of Tris hydrochloride [pH 7.5]), and 300 μl of calcium TBS with 1 mg of DEAE-dextran per ml. Cells were washed with calcium TBS and suspended at 5×10^7 per ml of calcium TBS, and 100 μl was added to the DNA mix. Samples were incubated at room temperature for 30 min and mixed every 5 min. Transfected cells were washed with TBS (without Ca) and incubated for 40 h with 1.5 ml of medium plus antibiotics in a 24-well cluster dish.

CAT assays. Cell lysate was prepared and CAT assays were performed essentially as described by Gorman et al. (16) and O'Hare and Hayward (37). Transfected cells were washed with phosphate-buffered saline, suspended (suspension cultures) or scraped (monolayers) in 1 ml of TEN (0.04 M Tris hydrochloride [pH 7.4], 1 mM EDTA, 0.15 M NaCl), and kept on ice. Cells were pelleted and suspended in 50 μl (Raji and BJAB) or 100 μl (Vero and BHK) of 0.25 M Tris hydrochloride (pH 7.9). Lysates were prepared by sonication, and debris was removed by microcentrifuging (Eppendorf) at 14,000 rpm for 5 min in the cold. Each cell lysate (50 μl, unless otherwise stated) was mixed with 100 μl of CAT mix (70 μl of 1 M Tris hydrochloride [pH 7.4], 27 μl of H_2O, 2 μl of 40 mM acetyl coenzyme A, and 1 μl of [^{14}C] chloramphenicol [0.1 μCi, 60 mCi/mmol]) and incubated at 37°C for 60 min in Eppendorf polypropylene micro-test tubes. The chloramphenicol and its acetylated derivatives were extracted by adding 1 ml of ethyl acetate and vortexing for 30 s. The organic phase was separated by centrifugation and transferred to a new tube before being dried down in a vacuum. The dried samples were solubilized in 16 μl of ethyl acetate and spotted onto a silica gel 60 thin-layer plate. The acetylated forms were separated by chromatography in a chloroform-methanol solvent (95:5 [vol/vol]). Plates were exposed to XAR film, and the radioactivity was quantitated by liquid scintillation counting in 2,5-diphenyloxazole (PPO)–1,4-bis(5-phenyloxazolyl)benzene (POPOP)–toluene. The percent acetylation was determined as the percentage of total radioactivity found in the chloramphenicol-1-acetate, chloramphenicol-3-acetate, and chloramphenicol-1,3-diacetate forms. The fold increase in CAT activity was defined as the ratio of the percent acetylation of the sample to the percent acetylation of the control (pBR322).

Immunofluorescent staining. Transfected cells were fixed

with acetone at −20°C for 10 min. The acetone was removed by washing with phosphate-buffered saline, and nonspecific sites were blocked with 0.1% bovine serum albumin. All antibodies were diluted in phosphate-buffered saline–0.1% bovine serum albumin. Nasopharyngeal carcinoma sera 145 (anti-EA-R titer, 240) and 406 (anti-EA-R titer, 160) were obtained from S. Spring, National Institutes of Health, Bethesda, Md. EA-R monoclonal antibody 5B11 was a generous gift from G. Pearson and was prepared against a fusion peptide containing sequences from BHRF1 (40). Each sample was incubated for 30 min at room temperature with 20 μl of NPC serum (1:25 dilution) or 5B11 (1:500 dilution), washed, and incubated again with fluorescein isothiocyanate-conjugated goat anti-human or goat anti-mouse second antibody. Slides were washed and mounted in 1 mg of *p*-phenylenediamine per ml to retard the fading of fluorescence.

Isolation and analysis of RNA. B95-8 cells were treated with TPA (40 ng/ml) for 48 h and washed twice with phosphate-buffered saline. RNA was isolated from whole cells with guanidine thiocyanate (9). Cells (0.2 to 0.4 ml) were treated with 3 ml of RNA buffer (4 M guanidine thiocyanate, 50 mM sodium acetate [pH 5.0], 10 mM EDTA, 136 mM sarcosyl, 7% β-mercaptoethanol) and vortexed for 60 s to shear the DNA. The sample was mixed with 1 g of CsCl, layered onto 1 ml of CsCl cushion solution (5.7 M CsCl, 50 mM sodium acetate [pH 5.0], 10 mM EDTA), and overlaid with 200 μl of 10% CsCl in 50 mM sodium acetate (pH 5.0). RNA pellets were suspended in 0.5 ml of TES (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 20% sodium dodecyl sulfate) and ethanol precipitated with 0.2 M LiCl. Poly(A)$^+$ RNA was separated on oligo(dT)-cellulose type 7 (Pharmacia, Inc.) by following the protocol described by Maniatis et al. (33). mRNAs were separated on formaldehyde gels (5 μg per lane), transferred to nitrocellulose, and hybridized to EBV DNA probes (33). Probes were sequenced by chemical degradation to confirm their origin. Labeled probes were generated by isolating DNA restriction fragments from agarose gels and end labeling them with T4 polynucleotide kinase or Klenow fragment (International Biotechnologies, Inc.) as recommended by the manufacturer. Radioactively labeled material was separated from unincorporated radioactivity on a NENsorb column (New England Nuclear Corp.). Labeled probe was eluted with 50% methanol and dried under vacuum. Heat-denatured probes were incubated with RNA blots for 2 to 4 days.

RESULTS

Mapping the cytoplasmic early antigen (EA-R) gene. Our preliminary experiments confirmed an earlier report by Takaki et al. (59) who showed that the cosmid clone 302-23 (Fig. 1A and C) of EBV could induce the expression of an early antigen (EA-R) encoded by cosmid clone 780-35 (Fig. 1A and B). These cosmids were cotransfected into BHK cells, and EA-R was detected by staining with human sera. Since both of these subclones contain many different genes, we prepared a series of constructs to better define the effects of specific genes and to eliminate the possible cooperative effects of gene combinations. The EBV sequences in these constructs are represented diagrammatically in Fig. 1.

The cytoplasmic EA-R was presumed to be encoded by one of the four open reading frames in 780-35 (Fig. 1B). The leftmost reading frame may not encode a protein, but short exons in this vicinity encode a latent-cycle nuclear protein, LP or EBNA5 (14, 49). The open reading frame which spans

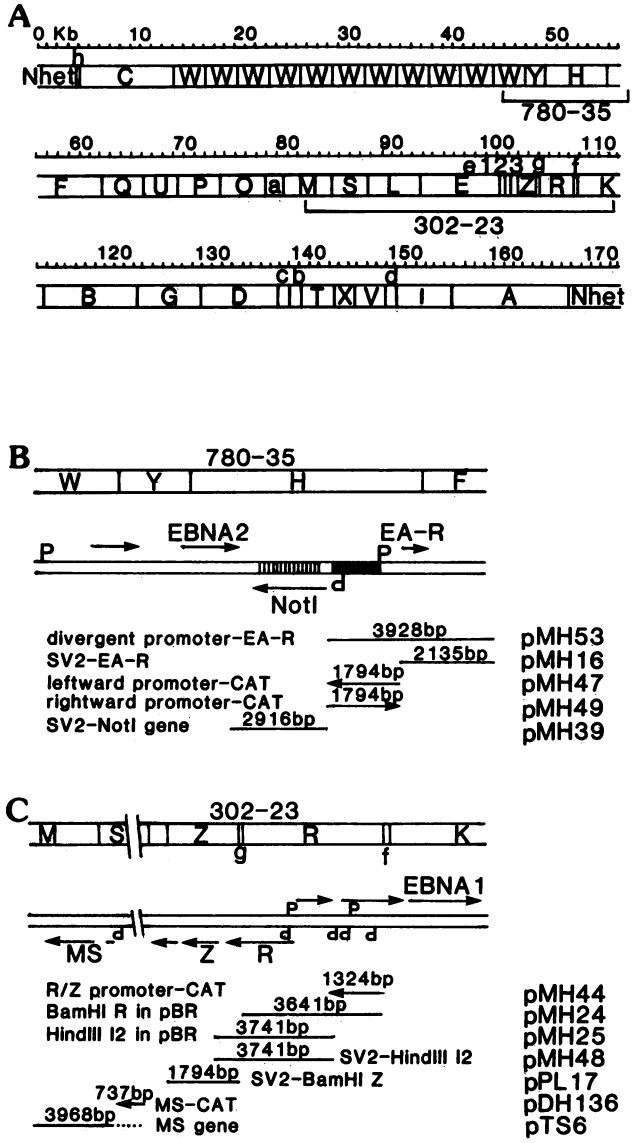

FIG. 1. Maps of EBV genomic DNA and EBV-containing plasmid constructions. (A) Map of EBV B95-8 genome. Letters indicate *Bam*HI fragments in order of size (A to Z, a to h). The three e fragments are designated e1 to e3. Numbers indicate length in kilobase pairs. Brackets mark the analogous sequences present in EBV M-ABA cosmid clones. M-ABA has an additional *Bam*HI site at approximately 50,000 bp on the B95-8 map. (B and C) Maps of cosmid clones 780-35 (panel B) and 302-23 (panel C) and subclones containing either M-ABA or B95-8 sequences. Cosmid clone 780-35 is a *Bgl*II (partial)-*Sal*I fragment of M-ABA. Cosmid clone 302-23 resulted from a partial *Hin*dIII digest of M-ABA (44). Arrows above or below the open bar represent open reading frames identified by DNA sequencing (3). Symbols: ▭, *Not*I repeat units; ▬, divergent promoter; P, TATA boxes. The length, a brief description, and the clone name of each subclone from these regions are indicated. The direction of transcription from EBV promoters cloned into CAT-vectors is indicated by arrows. A detailed description of the plasmid constructions can be found in Materials and Methods.

the *Bam*HI-YH boundary (BYRF1) encodes another latent-cycle nuclear antigen, EBNA2 (35, 50). The remaining two open reading frames (BHLF1 and BHRF1) were placed

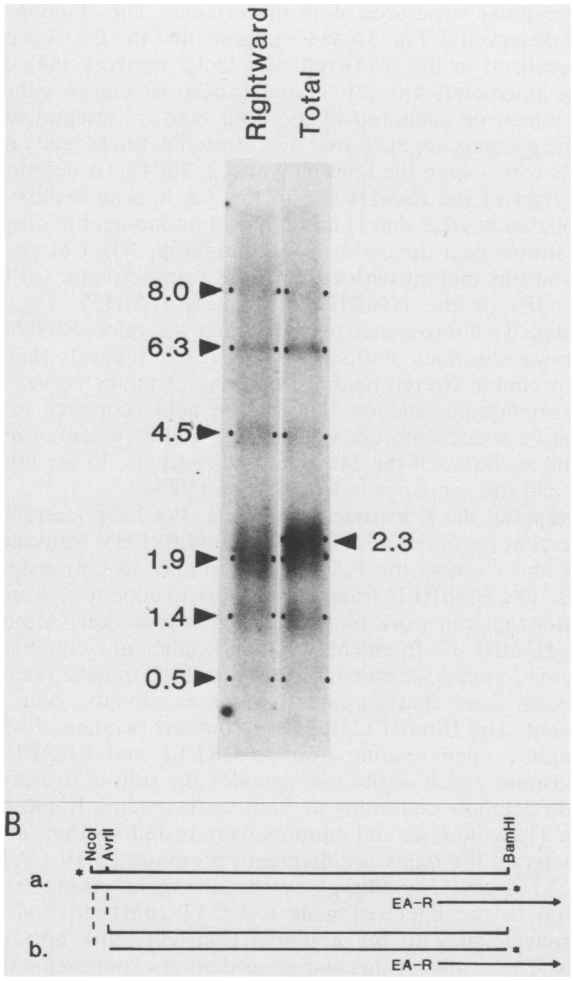

FIG. 2. Analysis of RNA transcribed from the divergent promoter. (A) Northern blot analysis of poly(A)$^+$ RNA from TPA-induced B95-8 cells. RNA sizes (in kilobases) were determined by comparison with DNA fragments of *Hin*dIII-digested lambda bacteriophage. (B) Map of probes used for Northern blot analysis. Probe "a" (bp 3708 to 6006 of *Bam*HI-H) was used to detect transcription in both the rightward and leftward directions. Probe "b" (bp 3719 to 6006 of *Bam*HI-H) was used to detect transcription in the rightward direction only.

under the regulation of the SV40 early promoter (pMH39 and pMH16, respectively). No product of the leftward *Not*I gene (BHLF1 [27]) was detected in transfected BHK cells. However, the rightward reading frame expressed a cytoplasmic antigen detected by serum samples from nasopharyngeal carcinoma patients. The immunofluorescence pattern was the same as that obtained with the entire 780-35 cosmid (data not shown). Therefore, an EA-R is encoded by BHRF1. These results confirm a recent report by Pearson et al. (40) in which the early antigen recognized by an EA-R monoclonal antibody was mapped to BHRF1.

Identification of a potential EA-R promoter. The EA-R gene was expressed only when cells were cotransfected with cosmid 302-23 or when EA-R was driven by the strong SV40 promoter. Presumably, the EBV DNA sequence that controls the expression of EA-R is responding to a gene product from the cosmid clone 302-23. To identify the promoter region for EA-R, we isolated poly(A)$^+$ RNA from TPA-induced B95-8 cells and analyzed it by Northern (RNA) blot hybridization (Fig. 2A). The probe contained the entire noncoding region between the *Not*I and EA-R genes plus most of the EA-R open reading frame (Fig. 2B). To detect transcription in the rightward direction only, we cleaved the double-strand probe with *Avr*II, which removed the label and 11 bp from the left end. The probe was sequenced to confirm its unidirectionality (data not shown). Several rightward mRNAs were detected including a 1.9-kilobase (kb) mRNA. This size correlates with the predicted size of approximately 1,750 bases plus poly(A) for an EA-R transcript initiating 30 bases down from a putative TATA box (TATTAA) and terminating near a predicted poly(A) adenylation signal in *Bam*HI-F. A smaller mRNA of 1.4 kb could represent a spliced version of the 1.9-kb mRNA (see the Discussion). A similar, presumably rightward RNA of 1.95 kb from BHRF1 was recently reported by Pfitzner et al. (43). Our Northern analysis detected several additional rightward RNAs which varied in size from 8 to 0.5 kb, indicating that the transcription pattern through this region is complicated. A splice acceptor consensus sequence (TTTTCTAG/) is located about 40 bp upstream from the initiating codon for EA-R. Utilization of this acceptor site would help explain the complex RNA pattern and suggest that EA-R could be expressed from a further upstream promoter. The only additional RNA observed with the bidirectional probes was a 2.3-kb species that was previously identified as an abundant transcript for the *Not*I repeats (24, 27). This leftward RNA is initiated by a promoter element located about 300 bp upstream of the AUG (29; M. Hardwick, unpublished data).

The potential EA-R promoter lies in an interesting region of the EBV genome. Located between the *Not*I and EA-R genes is a stretch of over 1,000 bp which is rich in direct and indirect repeats and palindromes. We refer to this region as a divergent promoter, because the TATA box for the leftward *Not*I transcript is located near the left end of this region and a potential TATA box for the rightward EA-R transcript is located near the right end. This stretch of repetitive sequences flanked by divergent transcriptional start sites may serve as a target for EBV transactivators.

Expression of EA-R from the divergent promoter in response to EBV transactivators. Thus far we have identified the EA-R gene and a potential promoter for this gene. If the divergent promoter is indeed the functional promoter for EA-R, then it should respond to EBV transactivators. The EA-R open reading frame under the regulation of the divergent promoter (pMH53) was cotransfected with potential transactivators. The *Bam*HI-Z DNA fragment (containing the Z transactivator, pPL17 [Fig. 3A]), as well as the *Hin*dIII I2 fragment (containing the R transactivator, pMH25 [Fig. 3B]), induced the expression of EA-R. A similar staining pattern was observed when the *Bam*HI Z and *Hin*dIII I2 DNA fragments were both present (Fig. 3C). EA-R was detected by immunofluorescent staining with a monoclonal antibody to EA-R (40). Neither the MS transactivator (pTS6) nor the *Bam*HI R fragment (pMH24) could induce EA-R expression (Fig. 3D). No activation by any transactivator was observed when the divergent promoter had a partial *Xma*I deletion which removed 1,133 bp, leaving the TATA box intact (pMH81; data not shown). Therefore, this palindrome-rich region must contain an element (or elements) which responds to EBV transactivators. Furthermore, the transactivators which activate this promoter are encoded by *Bam*HI-Z and *Hin*dIII I2.

To determine the subcellular localization of EA-R, we performed immunofluorescence assays on transfected NIH 3T3 cells because they spread out on the substratum, making

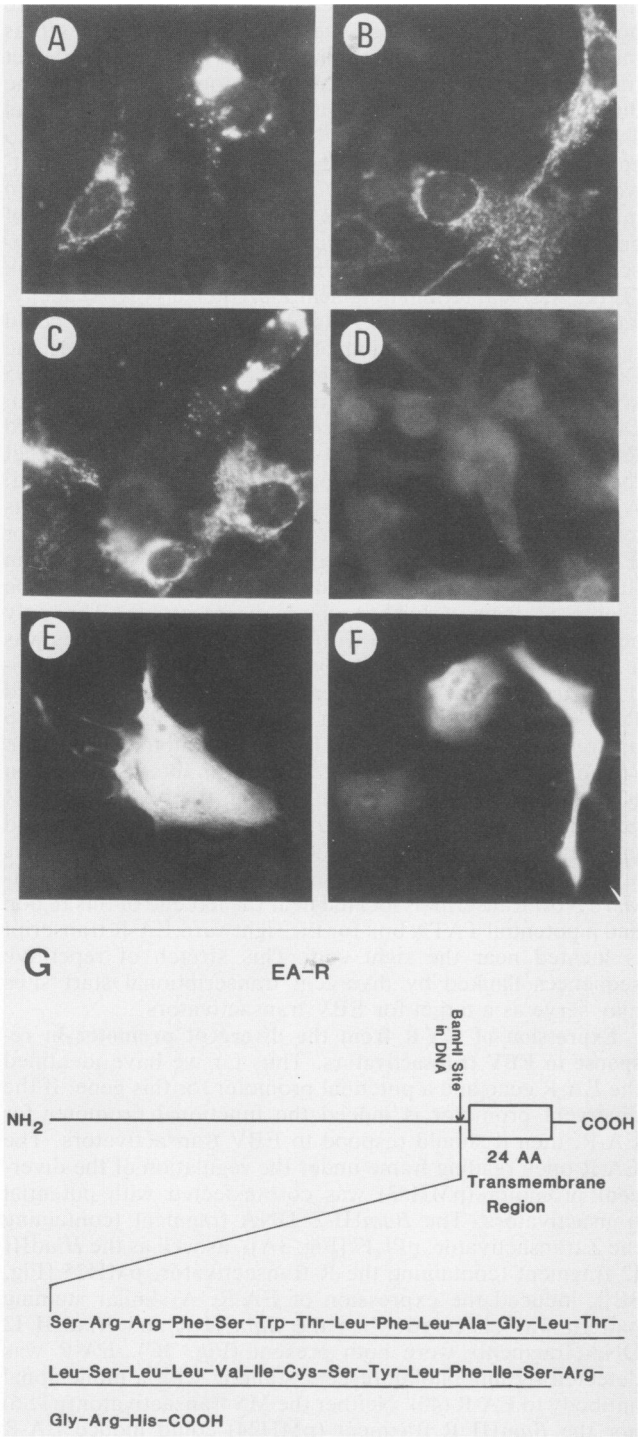

FIG. 3. Induction of EA-R expression by EBV transactivators. Transfected NIH 3T3 cells were fixed and stained with 5B11 monoclonal antibody specific for EA-R. The region of the EBV genome containing the rightward divergent promoter and the EA-R gene (pMH53) was cotransfected with *Bam*HI-Z under the regulation of the SV40 promoter (pPL17) (A), *Hin*dIII-I2 (pMH25) (B), both *Bam*HI-Z and *Hin*dIII-I2 (pPL17 and pMH25) (C), or the MS transactivator encoded by BMLF1 (pTS6) (D). The *Bam*HI H fragment in pBR322, pSL77, which lacks the 3′ end of the EA-R open reading frame, was cotransfected with *Bam*HI-Z (pPL17) (E) or *Hin*dIII-I2 (pMH25) (F). (G) Diagram of the EA-R gene product. The open box and the underlined amino acid (AA) residues correspond to the hydrophobic domain.

intracellular structures more discernable. The staining pattern observed in Fig. 3A to C suggests that the EA-R protein is localized in the *trans*-reticular Golgi network (64). Proteins associated with this vesicular network can be either in the lumen or anchored in the lipid bilayer. Integral membrane proteins are anchored by a stretch of uncharged amino acids which span the lipid bilayer (13, 20, 41). A deletion to the right of the *Bam*HI site in the EA-R gene results in a truncated protein that is missing a 24-amino-acid hydrophobic stretch near the carboxy terminus (Fig. 3G). Cotransfection of this mutant with either the Z transactivator (pPL17; Fig. 3E) or the *Hin*dIII I2 fragment (pMH25; Fig. 3F) produced a fluorescence pattern which was relocalized to the extramembranous cytoplasm. This result suggests that the hydrophobic stretch near the carboxy terminus serves as a transmembrane anchor. The amino acid sequence of the putative transmembrane region (Fig. 3G) is typical of membrane anchors. Of the 24 uncharged residues, 15 are nonpolar, and the sequence is leucine rich (30%).

Mapping the R transactivator gene. We have identified a divergent promoter which can respond to EBV transactivators and express the EA-R gene product in cotransfected cells. The *Hin*dIII I2 fragment appears to code for a transactivator that can work on this divergent promoter. Although the *Hin*dIII I2 fragment contains sufficient sequence to encode 25 amino-terminal residues of the Z transactivator, it seemed likely that an additional transactivator gene was present. The *Hin*dIII I2 fragment contains two head-to-head complete open reading frames, BRLF1 and BRRF1. To determine which of the two encodes the transactivator, we made deletion mutations in each open reading frame (Fig. 4C). The wild type and mutants were tested for their ability to activate the rightward divergent promoter (EBV-CAT) in a CAT assay. For this assay the divergent promoter was linked to the bacterial gene for CAT (pMH49) and was cotransfected with the potential transactivators into Vero cells. The results of this and several other experiments show that *Hin*dIII I2 can activate the rightward divergent promoter by three- to fivefold over the basal level (Fig. 4A). Similar results were obtained when the *Hin*dIII I2 fragment was placed in an SV40 expression vector (pMH48). A 606-bp deletion removing most of the BRRF1 coding sequence did not affect activity, whereas a 312-bp deletion near the 3′ end of BRLF1 reduced the activity to basal levels (see maps in Fig. 4C). The *Bam*HI R fragment, which does not contain an intact BRLF1, also did not activate the EBV promoter. Deletion of the 5′ 25 codons of the Z transactivator which are present in *Hin*dIII I2 did not affect transactivation (not shown). Therefore, we concluded that a new EBV transactivator, designated R, is encoded by BRLF1.

The same experiment described above was performed in EBV-positive Raji cells. The results obtained were the same with two exceptions. The *Hin*dIII I2 fragment did not have transacting activity unless it was placed in an SV40 vector in which the SV40 promoter was driving the leftward open reading frame, BRLF1. Therefore, a promoter driving the R transactivator (presumably contained in the upstream EBV sequences) is functioning in Vero cells but not in Raji cells. This difference between Raji and Vero cells is likely to be due to differences in cellular transcription factors. Second, and in the same vein, the divergent promoter has 50- to 500-fold-higher basal activity in Vero and BHK cells than in Raji cells (compare pBR322 controls in Fig. 4A and B). In Raji cells, in which the basal activity of the divergent promoter is low, R activated this promoter 20- to 70-fold.

Bidirectional response of divergent promoter to EBV tran-

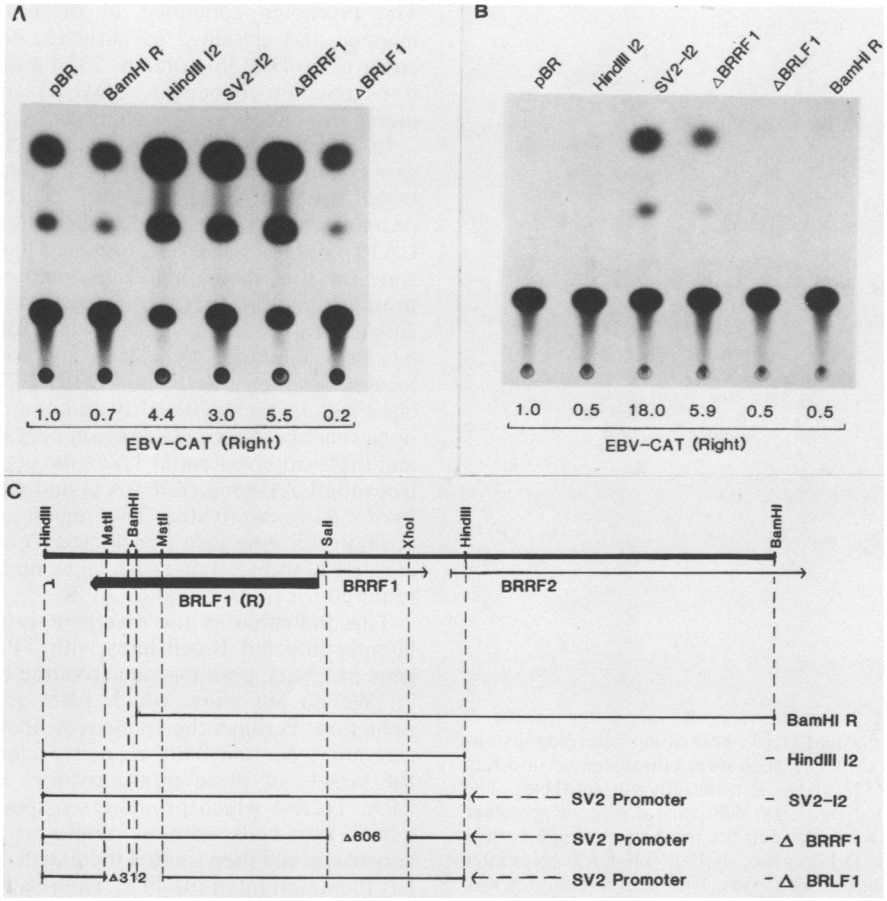

FIG. 4. Identification of the R transactivator gene by mutational analysis. The rightward divergent promoter linked to the *cat* gene (pMH49) was cotransfected into (A) Vero or (B) Raji cells with pBR322 (control), *Bam*HI-R (pMH24), *Hin*dIII-I2 (pMH25), *Hin*dIII-I2 driven by the SV40 promoter (pMH48), a mutant with a deletion in BRRF1 (pMH50), or a mutant with a deletion in BRLF1 (pMH58). Autoradiograms of the CAT assay results are shown. The chromatography plates were scraped and counted for quantitation. The fold increase in CAT activity compared with the pBR322 control is indicated and was assumed to reflect the extent of transactivation. Only 5 μl of Vero cell extracts was used in this analysis, compared with 50 μl of Raji cell extracts. (C) Map of overlapping *Hin*dIII I2-*Bam*HI R fragments of EBV. Long arrows indicate open reading frames and the direction of transcription. Dashed arrows indicate the direction of transcription from the SV40 early promoter. EBV sequences and restriction enzyme deletions of DNAs used in the CAT assay are indicated.

sactivators. Because the Z transactivator also has been shown to induce EA-R expression (58), we assayed all three lytic transactivators, MS, Z, and R, for their ability to transactivate in both the leftward and rightward directions. The left-CAT (pMH47) and right-CAT (pMH49) constructs contain identical promoter sequences that differ only in orientation. In Raji cells the Z transactivator worked in both directions, the MS transactivator did not work in either direction, and the R transactivator worked primarily in the rightward direction (Fig. 5). Combinations of transactivators did not produce a synergistic effect in this experiment. These results are consistent with the fluorescence assay in which both Z and R, but not MS, activated EA-R.

Response of heterologous promoters to the R transactivator. To determine the specificity of the R transactivator, we tested several heterologous promoters for their responsiveness to R in a CAT assay (Fig. 6). Both Raji and Vero cells were cotransfected with R (pMH48) and the heterologous promoter-CAT constructs. The results of the CAT assay indicate that in Raji cells R does not transactivate the promoter for the herpes simplex virus immediate-early gene IE175 (ICP4), the herpes simplex virus thymidine kinase promoter (TK-CAT), the human serum amyloid gene pro-

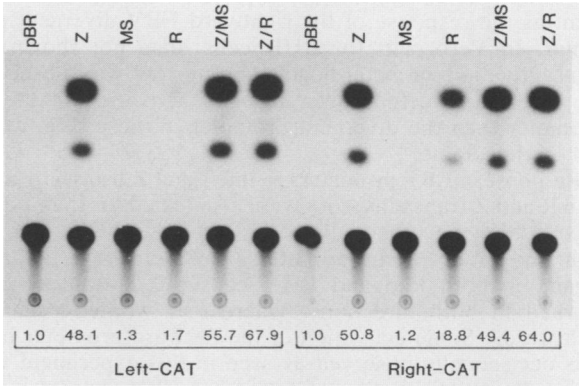

FIG. 5. Transactivation of the divergent promoter. The divergent promoter was linked to the CAT gene either in the leftward (pMH47) or rightward (pMH49) orientation. Lytic-cycle transactivator Z (pPL17), MS (pTS6), or R (pMH48) or combinations of transactivators were cotransfected into Raji cells with the target promoter-*cat* construct. The fold increase in transactivation compared with the pBR322 control is indicated.

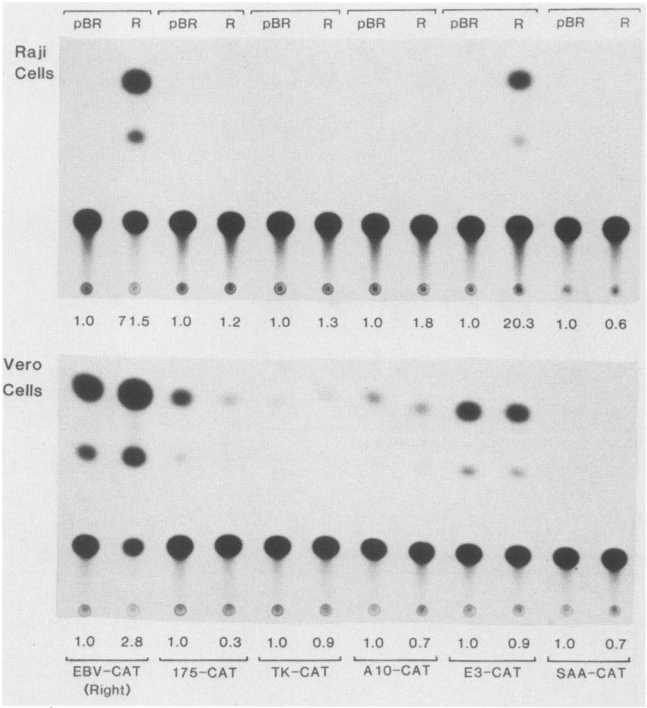

FIG. 6. Response of the adenovirus E3 promoter to the R transactivator. Several plasmid DNAs containing heterologous target promoters linked to the CAT gene were cotransfected into Raji or Vero cells with pBR322 or the R transactivator (pMH48). The target promoters included the EBV rightward divergent promoter (pMH49), 175-CAT (promoter region for the herpes simplex virus type 1 immediate-early IE175 gene; ICP4), TK-CAT (promoter region for the herpes simplex virus type 1 thymidine kinase gene), A10-CAT (the SV40 early promoter minus the 72-bp repeats), E3-CAT (adenovirus type 5 E3 promoter region), and SAA-CAT (serum amyloid gene promoter region). The fold increase in transactivation compared with the pBR322 control is indicated. CAT assays were performed with 50 μl of cell extract, except for pMH49 in Vero cells, for which only 0.5 μl was used.

moter (SAA-CAT), or the enhancerless SV40 promoter (A10CAT). In contrast, R activated the adenovirus E3 promoter 6- to 20-fold in Raji cells. R activation of the E3 promoter was consistently one-quarter to one-third as efficient as the response of the rightward EBV divergent promoter. In Vero cells (or BHK cells; data not shown) no transactivation of heterologous promoters was observed. However, a threefold-lower level of activation of the E3 promoter than the divergent promoter in these cells would not be detectable.

Response of EBV promoters to the R and Z transactivators. The R and Z transactivators were also tested for their ability to transactivate other EBV early promoters (Fig. 7). The right and left divergent promoters were included as controls. Again, in both Raji and BHK cells the Z transactivator worked in both directions, whereas R worked primarily rightwards. Some activation of the leftward promoter by R was occasionally observed as seen in this experiment with Raji cells. A stimulation of two other EBV promoter constructs, M-CAT and MS-CAT, was observed with the Z transactivator in Raji cells; the most striking was a 15- to 50-fold stimulation of the M promoter in BHK cells (and Vero cells; data not shown). The M-CAT construct (pMH1A) contains 754 bp of sequences upstream from the EA-D gene encoded by BMRF1 at the left end of *Bam*HI-M. The promoter contained in these sequences has been mapped and classified as early (3, 42, 51). The MS-CAT construct (pDH136) contains 737 bp upstream from the MS transactivator encoded by BMLF1 and BSLF2. This promoter was mapped in S1 analyses by Sample et al. (51).

In addition to the divergent promoter, R stimulated expression from the MS promoter fivefold in all cell types tested. In BHK or Vero cells R also activated other EBV-promoter constructs: M-CAT, RR-CAT (pPL23A), and R/Z-CAT (pMH44). RR-CAT contains 320 bp upstream from the gene for the ribonucleotide reductase small subunit. This promoter and the RNA transcript were identified and classified as early (3, 51). A TATA box for the R transactivator has been identified by cDNA analysis as the CATAAAA located between BRRF1 and BRRF2 (5). This transcript was classified as immediate early and contains both the R and Z open reading frames. The region containing the CATAAAA and three other potential TATA boxes was placed upstream from the CAT gene (Z/R-CAT) and was stimulated twofold by the R transactivator. This region apparently serves as a promoter driving R in transfected Vero and BHK cells (see *Hin*dIII-I2 in Fig. 3 and 4A), since no SV40 sequences were required for transactivation by R.

TPA induction of the divergent promoter. Treatment of latently infected B-cell lines with TPA induces lytic-cycle gene products, perhaps via activation of protein kinase C (4, 7). We do not know which EBV genes respond to TPA induction. Perhaps the sequences upstream from the transactivators contain TPA response elements. Alternatively, the targets of these transactivators may be activated by TPA. To test which promoters respond to TPA, we transfected Raji cells with a variety of EBV promoter-CAT constructs and then treated them with 40 ng of TPA per ml or left them untreated for 40 h. The results indicated that none of the EBV promoters tested was activated in the absence of TPA (Fig. 8). However, in the presence of TPA the divergent promoter was stimulated 3.5-fold in both the rightward and leftward directions. This level of induction is similar to that of a number of other TPA-responsive promoters (15, 25). TPA induction of the other EBV promoters may occur, but was not detectable in this assay. Induction of the divergent promoter by TPA was also observed in BJAB cells, which are EBV negative (not shown); therefore, induction was not mediated by another EBV gene product. This evidence suggests that the divergent promoter contains a TPA response element. Transactivation of the rightward divergent promoter by Z and R was included as a control to demonstrate that the system was operating as expected.

DISCUSSION

We have identified a new EBV transactivator, designated R, which is encoded by the BRLF1 open reading frame in the *Bam*HI R/Z fragments. This transactivator activates the expression of a cytoplasmic early antigen (EA-R) encoded by BHRF1 which crosses the *Bam*HI H-F boundary. R appears to activate EA-R expression via upstream promoter sequences found in *Bam*HI-H. In the *Bam*HI H fragment of EBV is a region of over 1,000 bp which has a sequence of unusual complexity. It contains two large palindromes of 18 and 20 bp, plus several others of 16, 14, and 12 bp. There are also several direct and indirect repeats of similar complexity. In addition, this region contains sequence motifs (detailed below) which resemble the binding sites of regulatory proteins. This suggests that this region and its duplicated equivalent, which is located upstream from the *Pst*I repeats

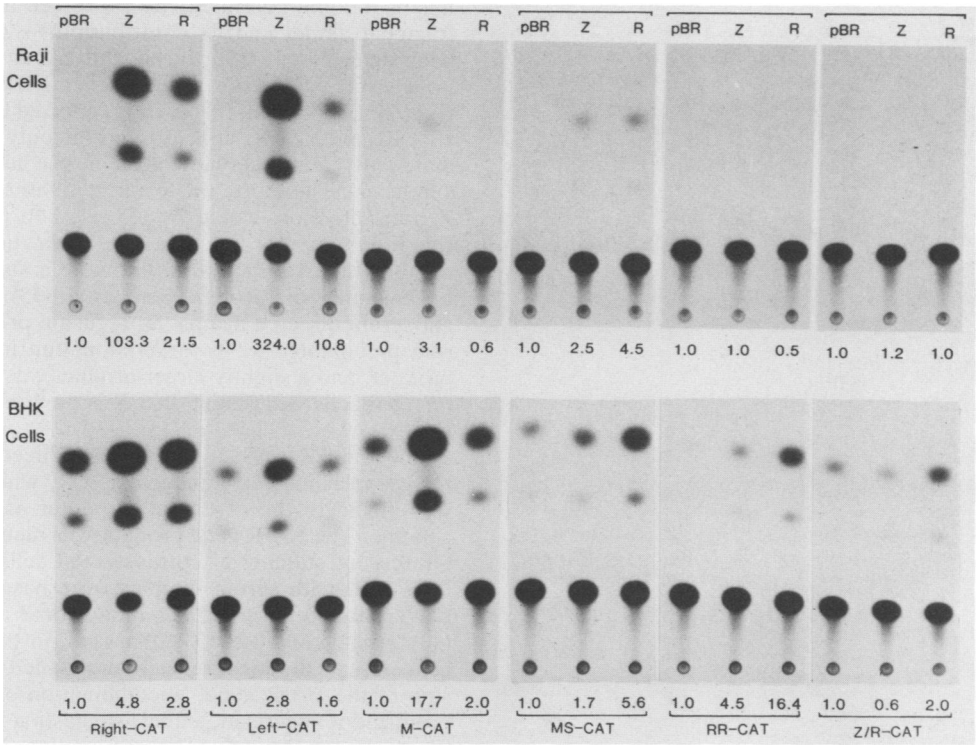

FIG. 7. Response of EBV promoters to the R and Z transactivators. Raji or BHK cells were cotransfected with plasmids containing EBV promoters linked to CAT, plus Z (pPL17), R (pMH48), or pBR322 as a control. The target promoters included rightward (pMH49) and leftward (pMH47) divergent promoters, M-CAT (promoter region for BMRF1 [pMH1A]), MS-CAT (promoter region for BMLF1 [pDH136]), RR-CAT (promoter region for ribonucleotide reductase small subunit [pPL23A]), and Z/R-CAT (promoter region upstream from the R transactivator [pMH44]). CAT assays were performed on 50 μl of cell extract with the following exceptions: pMH49 and pMH47 in BHK cells (0.5 μl) and MS-CAT in BHK cells (10 μl). The fold increase in transactivation compared with the pBR322 control is indicated.

(deleted in B95-8), may be important regions for the regulation of EBV gene expression. Although this promoter region does not appear to initiate transcription during latency, it does appear to be important in the lytic cycle and possibly in the initial stages of primary infection of B cells (52). This structurally complex region in *Bam*HI-H is situated appropriately to regulate the expression of two flanking genes. There is a promoter at its left end which drives the leftward *Not*I repeat gene transcript (27, 29). The *Not*I mRNA is one of the most abundant early transcripts found in induced EBV-positive cells (24). Although this gene has maintained an open reading frame in all three reading frames, efforts to identify a protein product thus far have been unsuccessful. The gene to the right of this structurally complex region (BHRF1) extends into *Bam*HI-F and encodes an EA-R cytoplasmic antigen. A putative TATA box for EA-R is located near the right end of this complex region; hence, we refer to the entire region as the divergent promoter. The *Pst*I repeat promoter reported by Chevallier-Greco et al. (8) contains sequences which are homologous to the leftward divergent promoter.

Transcription in the rightward direction from this divergent promoter appears complicated, since a number of poly(A)$^+$ species were identified by Northern analysis (8.0, 6.3, 4.5, 1.9, and 1.4 kb; Fig. 2). It is possible that one or more of these species extends or is spliced beyond the putative EA-R adenylation signal in *Bam*HI-F. Hudson et al. (23) identified transcripts (ca. 10 and 6.5 kb) containing *Bam*HI-F open reading frames but having 5′ ends to the left of fragment F, possibly in fragment H. In addition, the EA-R transcripts may have alternate 5′ ends. Pfitzner et al. (43) recently identified four cDNAs of EA-R transcripts ranging in size from 1.5 to 2.8 kb. Although all of these RNA transcripts had 3′ termini near the expected adenylation signal, their 5′ ends (all in *Bam*HI-H) are not located near a promoter element, and their significance is uncertain. Pearson et al. (40) recently reported two major groups of EA-R transcripts, based on cDNA data. One group of RNAs was spliced from *Bam*HI-W and *Bam*HI-Y, similarly but not identically to the latent EBNA mRNAs. The other group of RNAs had 5′ ends just downstream from the TATTAA near the right end of the divergent promoter of *Bam*HI-H. They also identified a spliced version of this RNA which has a 440-base intron in the leader sequences upstream of the EA-R gene. These RNAs probably correspond to the 1.9- and 1.4-kb transcripts on our Northern blots. Bodescot and Perricaudet (6) have also identified a highly spliced EA-R transcript which contains *Bam*HI-C as well as *Bam*HI-W and *Bam*HI-Y sequences. This long-range splicing is reminiscent of the latent EBNA RNA transcripts and suggests that EA-R may be regulated by both latent-cycle and lytic-cycle promoters. It is intriguing that EA-R shares some amino acid sequence homology with *bcl-2* (B-cell lymphoma/leukemia 2), a putative *onc* gene found at the breakpoints of chromosome translocations with the immunoglobulin heavy-chain locus (11, 60). The possibility that *bcl-2* and EA-R could have functional similarity is interesting in light of the fact that EA-R may be expressed in the latent cycle as well as the lytic cycle.

Additional evidence for a rightward lytic-cycle promoter in *Bam*HI-H is derived from the CAT assays. Expression from lytic-cycle promoters appears to require induction by a

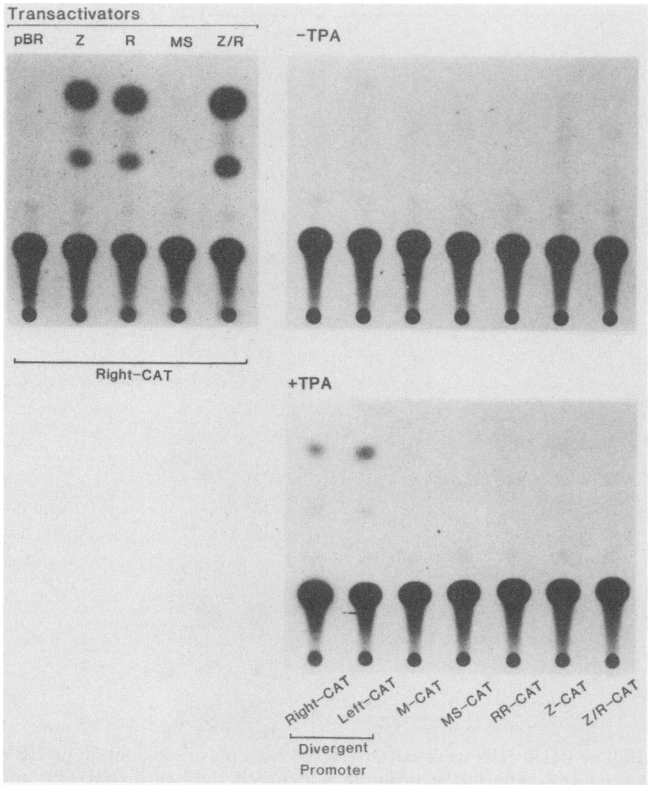

FIG. 8. TPA induction of the divergent promoter in Raji cells. A series of EBV promoter-CAT constructs were transfected into Raji cells and incubated in the presence or absence of TPA. The promoter targets tested in this CAT assay included the rightward (pMH49) and leftward (pMH47) divergent promoters, M-CAT (promoter for BMLF1 [pMH1A]), MS-CAT (promoter for MS transactivator [pDH136]), RR-CAT (promoter for ribonucleotide reductase small subunit [pPL23A]), Z-CAT (promoter in *Bam*HI-Z for the Z transactivator [pMH17]), and Z/R-CAT (promoter for the R transactivator in *Bam*HI-R [MH44]). As a positive control the rightward divergent promoter-CAT construct (pMH49) was cotransfected with EBV transactivators as described for Fig. 4.

positive regulator (or release from a negative regulator), since they are not normally expressed in B cells. When the divergent promoter was excised from *Bam*HI-H and placed upstream from the CAT gene, it was not expressed in Raji cells, but responded to EBV transactivators. This suggests that no other EBV sequences are required for inducibility and that the divergent promoter is behaving as would be expected for a lytic-cycle promoter. Also, the basal activity of this promoter varied greatly depending on the cell type in which it was placed. The divergent promoter was still inducible in BHK and Vero cells, but its basal activity was 50- to 500-fold higher than in Raji cells. Although lytic-cycle promoters in epithelial cells, in which EBV infection is lytic rather than latent, have not been studied, these results suggest that cellular transcription factors play a key role in the biology of EBV.

The *Bam*HI Z fragment, which contains the Z transactivator (BZLF1), stimulated the divergent promoter in both the left and right directions, whereas the MS transactivator (BMLF1) did not activate in either direction. The rightward divergent promoter was also activated by a new transactivator, designated R, encoded by BRLF1 (Fig. 4). The R transactivator gene lies in the leftward orientation just upstream from the Z transactivator gene. The carboxy terminus of R could potentially be expressed from the *Bam*HI Z fragment alone, and its contribution to Z activity has not been determined. Both the R and Z transactivators stimulated expression from several other EBV early promoters (Fig. 7). Biggin et al. (5) recently reported that transcripts for BRLF1 and BZLF1 appear to be the only lytic-cycle immediate-early RNAs (synthesized in the absence of protein synthesis). Therefore, the R transactivator, in addition to the Z transactivator, would be expected to play an important role in initiation or reactivation of the lytic cycle. The open reading frame for the R transactivator is sufficient to code for a 605-amino-acid protein. Seibl et al. (53) produced a rabbit antiserum directed against an R fusion protein. This antiserum precipitated a 93- to 96-kilodalton in vitro translation product, and a slightly larger product was precipitated from infected cells, suggesting that R is posttranslationally modified.

The R and Z transactivators also induced the expression of the cytoplasmic early antigen, EA-R, when the EA-R gene was linked to the divergent promoter as it is in the EBV genome. The EA-R gene product was identified by immunofluorescent staining of cotransfected cells (BHK, Vero, or NIH 3T3) with serum samples from nasopharyngeal carcinoma patients or the 5B11 monoclonal antibody to EA-R (40). Analogous to the CAT assays, in the immunofluorescence assay the MS transactivator failed to induce expression of the EA-R gene. A combination of R and Z usually produced a synergistic effect, suggesting that Z and R may function cooperatively. The immunofluorescent staining pattern suggested that the subcellular localization of EA-R is the *trans*-reticular Golgi network (64). Thus, EA-R may be translocated across the endoplasmic reticulum, perhaps modified in the Golgi apparatus, and then sorted into vesicles which can be targeted for secretion, plasma membrane insertion, endosomes, or lysosomes (18). Proteins which pass through this network can be either membrane anchored (i.e., membrane glycoproteins) or free (i.e., secreted proteins). Near the carboxy terminus of EA-R is a stretch of 24 uncharged amino acids which could serve as a transmembrane anchor region. When the carboxy-terminal 30 amino acids, containing the hydrophobic stretch, were deleted, EA-R was relocalized within the cell. The mutant EA-R appeared to be free in the extramembranous cytoplasm, indicating that the carboxy-terminal hydrophobic region is a functional transmembrane anchor. Furthermore, if the carboxy-terminal mutant of EA-R is truly free in the cytoplasm and not within the endoplasmic reticulum, this would suggest that the transmembrane anchor also serves as a functional signal sequence (21, 54). Additional experimental results are required to clearly identify the EA-R translocation signal and subcellular localization.

Because EBV lytic-cycle genes can be induced by TPA, it was of interest to determine which EBV promoters respond to TPA treatment. Since the Z transactivator has been shown to activate latent virus, it is possible that TPA activates Z gene expression and no other EBV genes. However, we show here that the divergent promoter region of *Bam*HI-H can be activated by TPA treatment in both the leftward and rightward directions. Similar results were obtained with both Raji and BJAB cells. TPA induction in BJAB cells was only just above the lower limits of detection. The increased level in Raji cells could be due to a greater transfection efficiency, a higher basal level of expression, or a boosting of the response by the endogenous Z. The promoter for Z in *Bam*HI-Z and the promoter for R in *Bam*HI-R did not respond to TPA in our experiments.

Longer exposures of the CAT assays suggested that the Z promoter may be TPA responsive, but this was not measurable in our assay.

Several TPA-inducible genes have been identified, including human collagenase (1), human metallothionein IIA (2), interleukin-2 (15), and SV40 early antigens (25). All of their upstream regulatory sequences contain the motif TGACT in either a 5′ or a 3′ orientation. This motif binds the transcription factor AP-1, which apparently mediates one of many pathways for induction by TPA. When purified AP-1 was footprinted on the SV40 early promoter enhancer sequence, three binding sites were observed (31). One of these binding sites has a slight variation in sequence: CTGACACAC. A closely related sequence, TGACACAC occurs between −50 and −150 (from the TATA box) in the EBV divergent promoter; once upstream from the rightward promoter and twice upstream from the leftward promoter. It is possible that these elements confer TPA inducibility in EBV. Another cellular factor, E4F1, binds a similar sequence upstream from the adenovirus E4 promoter (30). This binding-site consensus sequence GT(G/T)ACGT also occurs upstream from the E1A, E2, and E3 promoters and is in a functionally important region for induction by E1A. The same sequence (GTGACGT) occurs upstream from the rightward divergent promoter. Since the R transactivator activates both the adenovirus E3 promoter and the rightward divergent promoter, it is possible that this E1A response element is the same as the R response element.

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LITERATURE CITED

1. **Angel, P., I. Baumann, B. Stein, H. Delius, H. J. Rahmsdorf, and P. Herrlich.** 1987. 12-*O*-Tetradecanoyl-phorbol-13-acetate induction of a human collagenase gene is mediated by an inducible enhancer element located in the 5′-flanking region. Mol. Cell. Biol. **7**:2256–2266.
2. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin.** 1987. Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. Cell **49**:729–739.
3. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.
4. **Bell, R. M.** 1986. Protein kinase C activation by diacylglycerol second messengers. Cell **45**:631–632.
5. **Biggin, M., M. Bodescot, M. Perricaudet, and P. Farrell.** 1987. Epstein-Barr virus gene expression in P3HR1-superinfected Raji cells. J. Virol. **61**:3120–3132.
6. **Bodescot, M., and M. Perricaudet.** 1986. Epstein-Barr virus mRNAs produced by alternative splicing. Nucleic Acids Res. **14**:7103–7114.
7. **Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka.** 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. **257**:7847–7851.
8. **Chevallier-Greco, A., E. Manet, P. Chavrier, C. Mosnier, J. Daillie, and A. Sergeant.** 1986. Both Epstein-Barr virus (EBV)-encoded *trans*-acting factors, EB1 and EB2, are required to activate transcription from the EBV early promoter. EMBO J. **5**:3243–3249.
9. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
10. **Cho, M.-S., K.-T. Jeang, and S. D. Hayward.** 1985. Localization of the coding region for an Epstein-Barr virus early antigen and inducible expression of this 60-kilodalton nuclear protein in transfected fibroblast cell lines. J. Virol. **56**:852–859.
11. **Cleary, M. L., S. D. Smith, and J. Sklar.** 1986. Cloning and structural analysis of cDNAs for *bcl*-2 and a hybrid *bcl*-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell **47**:19–28.
12. **Dambaugh, T., K. Hennessy, S. Fennewald, and E. Kieff.** 1986. The virus genome and its expression in latent infection, p. 13–45. *In* M. Epstein and B. Achong (ed.), The Epstein-Barr virus: recent advances. John Wiley & Sons, Inc., New York.
13. **Davis, N. G., and P. Model.** 1985. An artificial anchor domain: hydrophobicity suffices to stop transfer. Cell **41**:607–614.
14. **Dillner, J., B. Kallin, H. Alexander, I. Ernberg, M. Uno, Y. Ono, G. Klein, and R. A. Lerner.** 1986. An Epstein-Barr virus (EBV)-determined nuclear antigen (EBNA5) partly encoded by the transformation-associated *Bam* WYH region of EBV DNA: preferential expression in lymphoblastoid cell lines. Proc. Natl. Acad. Sci. USA **83**:6641–6645.
15. **Fujita, T., H. Shibuya, T. Ohashi, K. Yamanishi, and T. Taniguchi.** 1986. Regulation of human interleukin-2 gene: functional DNA sequences in the 5′ flanking region for the gene expression in activated T lymphocytes. Cell **46**:401–407.
16. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
17. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
18. **Griffiths, G., and K. Simons.** 1986. The *trans* Golgi network: sorting at the exit site of the Golgi complex. Science **234**:438–443.
19. **Grogan, E., H. Jenson, J. Countryman, L. Heston, L. Gradoville, and G. Miller.** 1987. Transfection of a rearranged viral DNA fragment, WZhet, stably converts latent Epstein-Barr viral infection to productive infection in lymphoid cells. Proc. Natl. Acad. Sci. USA **84**:1332–1336.
20. **Guan, J. L., and J. K. Rose.** 1984. Conversion of a secondary protein into a transmembrane protein results in its transport to the Golgi complex but not to the cell surface. Cell **37**:779–787.
21. **Hardwick, J. M., K. E. S. Shaw, J. W. Wills, and E. Hunter.** 1986. Amino-terminal deletion mutants of the Rous sarcoma virus glycoprotein do not block signal peptide cleavage but can block intracellular transport. J. Cell. Biol. **103**:829–838.
22. **Hayward, S. D., S. G. Lazarowitz, and G. S. Hayward.** 1982. Organization of the Epstein-Barr virus DNA molecule. II. Fine mapping of the boundaries of the internal repeat cluster of B95-8 and identification of additional small tandem repeats adjacent to the HR-1 deletion. J. Virol. **43**:201–212.
23. **Hudson, G. S., T. J. Gibson, and B. G. Barrell.** 1985. The *Bam*HI F region of the B95-8 Epstein-Barr virus genome. Virology **147**:99–109.
24. **Hummel, M., and E. Kieff.** 1982. Epstein-Barr virus RNA. VIII. Viral RNA in permissively infected B95-8 cells. J. Virol. **43**:262–272.
25. **Imbra, R. J., and M. Karin.** 1986. Phorbol ester induces the transcriptional stimulatory activity of the SV40 enhancer. Nature (London) **323**:555–558.
26. **Ito, Y.** 1985. Vegetable activators of the viral genome and the causation of Burkitt's lymphoma and nasopharyngeal carcinoma, p. 207–236. *In* M. Epstein and B. Achong (ed.), The Epstein-Barr virus: recent advances. John Wiley & Sons, Inc., New York.
27. **Jeang, K.-T., and S. D. Hayward.** 1983. Organization of the Epstein-Barr virus DNA molecule. III. Location of the P3HR-1 deletion junction and characterization of the *Not*I repeat units that form part of the template for an abundant 12-*O*-tetradecanoylphorbol-13-acetate-induced mRNA transcript. J. Virol.

48:135–148.
28. **Klein, G., and D. T. Purtilo (ed.).** 1981. Symposium on Epstein-Barr virus-induced lymphoproliferative diseases in immunodeficient patients. Cancer Res. **41**:4208–4303.
29. **Laux, G., U. K. Freese, and G. W. Bornkamm.** 1985. Structure and evolution of two related transcription units of Epstein-Barr virus carrying small tandem repeats. J. Virol. **56**:987–995.
30. **Lee, K. A. W., and M. R. Green.** 1987. A cellular transcription factor E4F1 interacts with an E1a-inducible enhancer and mediates constitutive enhancer function *in vitro*. EMBO J. **6**:1345–1353.
31. **Lee, W., P. Mitchell, and R. Tjian.** 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell **49**:741–752.
32. **Lieberman, P. M., P. O'Hare, G. S. Hayward, and S. D. Hayward.** 1986. Promiscuous *trans*-activation of gene expression by an Epstein-Barr virus-encoded early nuclear protein. J. Virol. **60**:140–148.
33. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **McCutchan, J. H., and J. S. Pagano.** 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. J. Natl. Cancer Inst. **41**:351–356.
35. **Mueller-Lantzsch, N., G. M. Lenoir, M. Sauter, K. Takaki, J.-M. Bechet, C. Kuklik-Roos, D. Wunderlich, and G. W. Bornkamm.** 1985. Identification of the coding region for a second Epstein-Barr virus nuclear antigen (EBNA 2) by transfection of cloned DNA fragments. EMBO J. **4**:1805–1811.
36. **Nilsson, K., and G. Klein.** 1982. Phenotypic and cytogenetic characteristics of human B-lymphoid cell lines and their relevance for the etiology of Burkitt's lymphoma. Adv. Cancer Res. **37**:319–380.
37. **O'Hare, P., and G. S. Hayward.** 1984. Expression of recombinant genes containing herpes simplex virus delayed-early and immediate-early regulatory regions and *trans* activation by herpesvirus infection. J. Virol. **52**:522–531.
38. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751–760.
39. **Parker, B. A., and G. R. Stark.** 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infections by virus or viral DNA. J. Virol. **31**:360–369.
40. **Pearson, G. R., J. Luka, L. Petti, J. Sample, M. Birkenbach, D. Braun, and E. Kieff.** 1987. Identification of an Epstein-Barr virus early gene encoding a second component of the restricted early antigen complex. Virology **160**:151–161.
41. **Perez, L. G., G. L. Davis, and E. Hunter.** 1987. Mutants of the Rous sarcoma virus envelope glycoprotein that lack the transmembrane anchor and cytoplasmic domains: analysis of intracellular transport and assembly into virions. J. Virol. **61**:2981–2988.
42. **Pfitzner, A. J., J. L. Strominger, and S. H. Speck.** 1987. Characterization of a cDNA clone corresponding to a transcript from the Epstein-Barr virus *Bam*HI M fragment: evidence for overlapping mRNAs. J. Virol. **61**:2943–2946.
43. **Pfitzner, A. J., E. C. Tsai, J. L. Strominger, and S. H. Speck.** 1987. Isolation and characterization of cDNA clones corresponding to transcripts from the *Bam*HI H and F regions of the Epstein-Barr virus genome. J. Virol. **61**:2902–2909.
44. **Polack, A., G. Hartl, U. Zimber, U.-K. Freese, G. Laux, K. Takaki, B. Hohn, L. Gissmann, and G. W. Bornkamm.** 1984. A complete set of overlapping cosmid clones of M-ABA virus derived from nasopharyngeal carcinoma and its similarity to other Epstein-Barr virus isolates. Gene **27**:279–288.
45. **Pope, J. H., M. K. Horne, and W. Scott.** 1968. Transformation of foetal human leukocytes *in vitro* by filtrates of a human leukaemic cell line containing herpes-like virus. Int. J. Cancer **3**:857–866.
46. **Purtilo, D. T., G. Manolov, Y. Manolova, S. Harada, H. Lipscomb, and E. Tatsumi.** 1985. Role of Epstein-Barr virus in the etiology of Burkitt's lymphoma, p. 231–247. *In* G. Lenoir, G. O'Connor, and C. Olweny (ed.), Burkitt's lymphoma: a human cancer model. International Agency for Research on Cancer, Lyon, France.
47. **Reisman, D., and B. Sugden.** 1986. *trans* activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. Mol. Cell. Biol. **6**:3838–3846.
48. **Rosen, A., P. Gergely, M. Jondal, G. Klein, and S. Britton.** 1977. Polyclonal Ig production after Epstein-Barr virus infection of human lymphocytes *in vitro*. Nature (London) **267**:52–54.
49. **Rowe, D. T., P. J. Farrell, and G. Miller.** 1987. Novel nuclear antigens recognized by human sera in lymphocytes latently infected by Epstein-Barr virus. Virology **156**:153–162.
50. **Sample, J., M. Hummel, D. Braun, M. Birkenbach, and E. Kieff.** 1986. Nucleotide sequences of mRNAs encoding the Epstein-Barr virus nuclear proteins: a probable transcriptional initiation site. Proc. Natl. Acad. Sci. USA **83**:5096–5100.
51. **Sample, J., G. Lancz, and M. Nonoyama.** 1986. Mapping of genes in *Bam*HI fragment M of Epstein-Barr virus DNA that may determine the fate of viral infection. J. Virol. **57**:145–154.
52. **Sample, J., A. Tanaka, G. Lancz, and M. Nonoyama.** 1984. Identification of Epstein-Barr virus genes expressed during the early phase of virus replication and during lymphocyte immortalization. Virology **139**:1–10.
53. **Seibl, R., M. Motz, and H. Wolf.** 1986. Strain-specific transcription and translation of the *Bam*HI Z area of Epstein-Barr virus. J. Virol. **60**:902–909.
54. **Silhavy, T. J., S. A. Benson, and S. D. Emr.** 1983. Mechanisms of protein localization. Microbiol. Rev. **47**:313–344.
55. **Sixbey, J. W., D. S. Davis, L. S. Young, L. Hutt-Fletcher, T. F. Tedder, and A. B. Rickinson.** 1987. Human epithelial cell expression of an Epstein-Barr virus receptor. J. Gen. Virol. **68**:805–811.
56. **Sixbey, J. W., S. M. Lemon, and J. S. Pagano.** 1986. A second site for Epstein-Barr virus shedding: the uterine cervix. Lancet **ii**:1122–1124.
57. **Sixbey, J. W., J. G. Nedrud, N. Raab-Traub, R. A. Hanes, and J. S. Pagano.** 1984. Epstein-Barr virus replication in oropharyngeal epithelial cells. N. Engl. J. Med. **310**:1225–1230.
58. **Takada, K., N. Shimizu, S. Sakuma, and Y. Ono.** 1986. *trans* activation of the latent Epstein-Barr virus (EBV) genome after transfection of the EBV DNA fragment. J. Virol. **57**:1016–1022.
59. **Takaki, K., A. Polack, and G. W. Bornkamm.** 1984. Expression of a nuclear and cytoplasmic Epstein-Barr virus early antigen after DNA transfer: cooperation of two distant parts of the genome for expression of the cytoplasmic antigen. Proc. Natl. Acad. Sci. USA **81**:4568–4572.
60. **Tsujimoto, Y., M. M. Bashir, I. Givol, J. Cossman, E. Jaffe, and C. M. Croce.** 1987. DNA rearrangements in human follicular lymphoma can involve the 5′ or the 3′ region of the bcl-2 gene. Proc. Natl. Acad. Sci. USA **84**:1329–1331.
61. **Wang, F., C. D. Gregory, M. Rowe, A. B. Rickinson, D. Wang, M. Birkenbach, H. Kikutani, T. Kishimoto, and E. Kieff.** 1987. Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. Proc. Natl. Acad. Sci. USA **84**:3452–3456.
62. **Weeks, D. L., and N. C. Jones.** 1983. E1A control of gene expression is mediated by sequences 5′ to the transcriptional starts of the early viral genes. Mol. Cell. Biol. **3**:1222–1234.
63. **Whittle, H. C., J. Brown, K. Marsh, B. M. Greenwood, P. Seidelin, H. Tighe, and L. Wedderburn.** 1984. T-cell control of Epstein-Barr virus-infected B cells is lost during *P. falciparum* malaria. Nature (London) **312**:449–450.
64. **Willingham, M. C., and I. Pastan.** 1985. An atlas of immunofluorescence in cultured cells, p. 100–108. Academic Press, Inc., Orlando. Fla.
65. **zur Hausen, H., F. J. O'Neill, U. K. Freese, and E. Hecker.** 1978. Persisting oncogenic herpesvirus induced by the tumor promoter TPA. Nature (London) **272**:373–375.