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Both the Rightward and the Leftward Open Reading Frames within the *Bam*HI M DNA Fragment of Epstein-Barr Virus Act as *trans*-Activators of Gene Expression

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The *Bam*HI M DNA fragment of Epstein-Barr virus was shown to activate transcription of the cotransfected chloramphenicol acetyltransferase gene under the control of the simian virus 40 early promoter. Both the *Bam*HI-*Bgl*II and the *Hin*dIII-*Bam*HI subfragments of the *Bam*HI M fragment, corresponding to the rightward reading frame BMRF1 and the leftward reading frame BMLF1, respectively, had the ability to activate transcription from the simian virus 40 promoter. The *trans*-activating function was well correlated with the expression of nuclear early antigens, which suggests that early antigens encoded by BMRF1 and BMLF1 are responsible for *trans*-activation and possibly play a role in regulated expression of virus genomes.

Epstein-Barr virus (EBV) latency can be activated by a variety of compounds, such as halogenated pyrimidines (12, 16), tumor promoters (33, 38), *n*-butyrate (24), and anti-immunoglobulins (30, 34), and by infection with nontransforming P3HR-1 EBV (17, 18). The mechanism that maintains the latent state and promotes the lytic cycle still remains to be clarified.

The induction of viral replication in latently infected cells is accompanied by the synthesis of many new mRNAs and polypeptides (11, 20, 21, 25, 28, 36). The EBV-specific early antigen (EA) complex (17) is synthesized, even in the absence of EBV DNA replication, soon after induction (13), which suggests that EAs play an important role in promoting the shift from the latent state to the productive cycle. Recently, two regions of EBV DNA were shown to have the ability to activate transcription in *trans* (2, 5, 23, 32, 37). Countryman and Miller (5) reported that the rearranged DNA containing the *Bam*HI W and Z fragments from P3HR-1 virus can activate the expression of several polypeptides from the latent viral genome of D98-P3HR-1 hybrid cells. We demonstrated that the standard (nonrearranged) *Bam*HI Z fragment alone efficiently induces the latent EBV genome in Raji cells (32). It was also shown that the *Bam*HI Z fragment activates transcription of the cotransfected *Bam*HI Y, H, and fragment of EBV DNA in baby hamster kidney (BHK) cells. Chevallier-Greco et al. (2) subsequently reported that the open reading frame BZLF1 (1) is responsible for disruption of latency. They reported that BZLF1 does not activate transcription from the EBV promoter of the *Bam*HI B_1 region in EBV-negative cells. Lieberman et al. (23) and Wong and Levine (37), on the other hand, reported that an EA encoded by the leftward reading frame BMLF1 (1) within the *Bam*HI M fragment functions as a *trans*-activator of gene expression from the EBV, simian virus 40 (SV40), adenovirus, and herpes simplex virus promoters. These observations suggest that *trans*-activation by these genes, like activation by immediately early genes of herpes simplex virus (9, 19), may be important in regulating expression of virus genomes.

In the present study, the *Bam*HI M and Z fragments and their subfragments were tested for the ability to *trans*-activate the gene for chloramphenicol acetyltransferase (*cat*) (15) under the control of the SV40 early promoter, since this promoter has been extensively investigated and used to detect *trans*-activating function by immediately early gene products of herpes simplex virus (10).

To achieve the efficient expression of transfected genes, the *Bam*HI M and Z fragments of EBV DNA (Fig. 1A) were individually cloned into the plasmid vector pLTR (31, 32) (Fig. 1B), which contains two complete long terminal repeats (LTRs) from the avian myelocytomatosis virus (35) that are known to promote and enhance mRNA transcription in eucaryotic cells (6). The resultant plasmids and the plasmid pA10CAT (Fig. 1C) containing the *cat* gene, which is under the control of the SV40 early promoter, were cotransfected into BHK cells by the DEAE-dextran method, as described previously (8, 32). After 48 h of transfection, cells were harvested and assayed for CAT enzymatic activity. As a result, the *Bam*HI M fragment (30-fold increase in CAT activity, compared with the expression of the *cat* gene in BHK cells that were cotransfected with pLTR), but not the Z fragment (twofold), *trans*-activated transcription from the SV40 early promoter (Fig. 2). No induced CAT activity was observed on transfection of pA10CAT alone. pA10CAT contains the *cat* gene behind the SV40 promoter from which the 72-base-pair repeat enhancer element is deleted. In mammalian cells, *cat* expression in this plasmid requires the insertion of a *cis*-acting enhancer (14). CAT activity could not be detected on cotransfection with the *Bam*HI M DNA fragment cloned into pBR322 (pBR-M).

The DNA sequence data of Baer et al. (1) indicated that the *Bam*HI M fragment of EBV DNA is composed of three open reading frames, i.e., BMRF1, BMRF2, and BMLF1 (Fig. 3). To determine reading frames responsible for *trans*-activation, we first constructed plasmids containing the open reading frame BMRF1 and then tested them for the ability to induce transcription from the SV40 early promoter. Plasmid pgptLTR-MBBg, containing the *Bam*HI-*Bgl*II subfragment of the *Bam*HI M fragment cloned into the pgptLTR vector (Fig. 1B), efficiently induced CAT activity (15-fold increase) (Fig. 3 and 4A). The *Bam*HI-*Bgl*II subfragment of the *Bam*HI M fragment cloned into the pSV2gpt vector failed to *trans*-activate *cat* expression, which suggests that transcrip-

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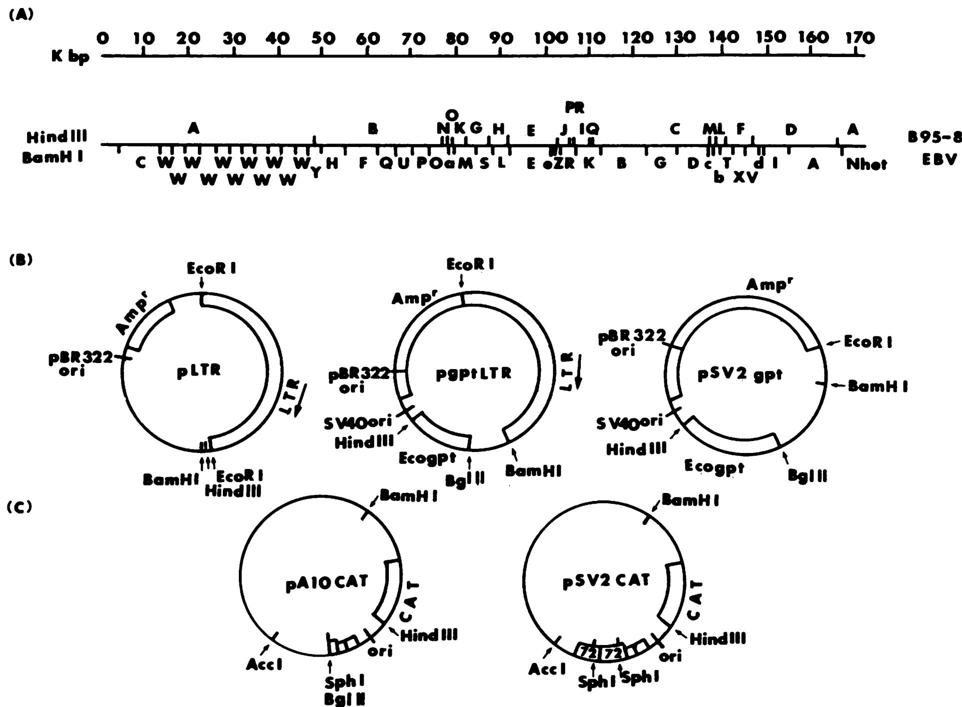

FIG. 1. (A) Restriction map of *Hin*dIII and *Bam*HI in B95-8 EBV DNA (1, 7, 29). (B) Construction of pLTR (31, 32) and pgptLTR vectors. The 2.0-kilobase-pair *Eco*RI-*Bam*HI fragment containing two complete tandem LTRs from avian retrovirus (MCV29) DNA (35) was inserted into the unique *Eco*RI site of pBR322 after the addition of the *Eco*RI linker. pgptLTR was constructed from pSV2gpt (26) by deleting the *Eco*RI-*Bam*HI fragment and replacing it with the *Eco*RI-*Bam*HI fragment containing LTRs from MCV29 DNA. (C) Construction of plasmid pA10CAT (22). pA10CAT was constructed from pSV2CAT (15) by deleting the 72-base-pair repeat of the SV40 enhancer from the *Sph*I site to the *Bam*HI site and by replacing it with the *Sph*I-*Bam*HI fragment of pA10 (a pBR322 derivative plasmid). Kbp, Kilobase pairs.

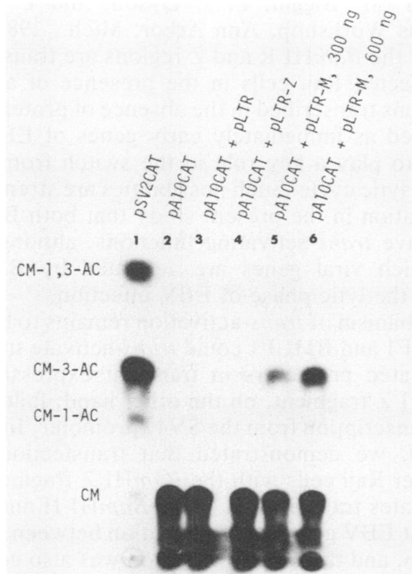

FIG. 2. Activation of *cat* expression under control of the SV40 promoter by the *Bam*HI M fragment of EBV DNA. Plasmid pA10CAT was cotransfected with each of the indicated plasmids into BHK cells. After 48 h of transfection, cells were harvested for CAT assays (14, 15). For quantitative comparisons of CAT activity, all three acetylated chloramphenicol forms were scraped into ACS II (Amersham International, Buckinghamshire, England) and counted in a liquid scintillation counter. CM-1,3-AC, 1,3-diacetate chloramphenicol; CM-3-AC, 3-acetate chloramphenicol; CM-1-AC, 1-acetate chloramphenicol; CM, chloramphenicol.

tional activation by the LTR sequence is required for efficient expression of the BMRF1 region.

The *Hin*dIII-*Bam*HI subfragment of the *Bam*HI M fragment containing BMLF1 was cloned into the pgptLTR vector so that BMLF1 was under the control of the LTR. The resultant plasmid, pgptLTR-MHB, was cotransfected with pA10CAT into BHK cells and tested for *trans*-activating function. pgptLTR-MHB *trans*-activated gene

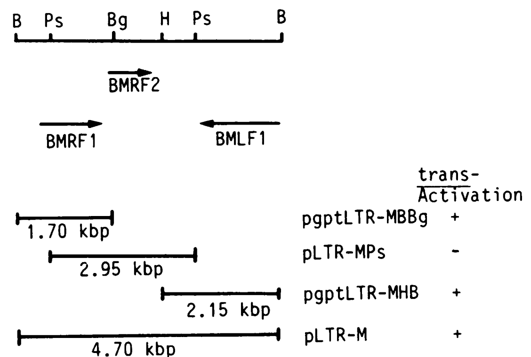

FIG. 3. Map of the *Bam*HI M fragment, illustrating the open reading frames (arrows) and restriction sties deduced from the DNA sequence data (1). The structures of the subclones used in the present study are also indicated. BMRF1 and BMLF1 regions are responsible for *trans*-activating ability. B, *Bam*HI; Ps, *Pst*I; Bg, *Bgl*II; *Hin*dIII; kbp, kilobase pairs.

expression from the SV40 promoter (eightfold increase in CAT activity) (Fig. 3 and 4B). The *Hin*dIII-*Bam*HI subfragment of *Bam*HI M cloned into the pSV2gpt vector (pgpt-MHB, which lacks LTR sequences) failed to activate *cat* expression.

The *Pst*I subfragment of the *Bam*HI M fragment, which contains a complete BMRF2 and an incomplete BMRF1 (deleted of its approximately 100 base pairs), was cloned into the pLTR and pBR322 vectors after the addition of the *Bam*HI linker. The resultant pLTR-MPs and pBR-MPs were tested for *trans*-activating functions (Fig. 4C). Both plasmids failed to enhance transcription of the cotransfected *cat* gene under our experimental conditions.

Cho et al. (3, 4) reported that the *Bam*HI M fragment codes for two components of the EA complex; BMRF1 codes for a 48- to 50-kilodalton polypeptide, and BMLF1 codes for a 60-kilodalton polypeptide. In parallel with the CAT assay, transfected cells were tested for the expression of EBV-specific antigens by indirect immunofluorescence methods. The results are shown in Table 1. Plasmids containing BMRF1 and BMLF1, when placed behind a strong heterologous promoter, induced nuclear antigens recognized by EBV-immune human sera. These induced antigens belonged to EA, since only EA antibody-positive sera, not EA antibody-negative sera, reacted with these antigens. The expression of nuclear antigens was well correlated with the expression of CAT activity in cotransfected cells, which suggests that these nuclear antigens are responsible for a *trans*-activating function. BMRF2, which lacks *trans*-activating function on pA10CAT, did not induce any EBV-specific antigens.

It has been demonstrated that the BMLF1 antigen has the ability to activate transcription from the heterologous promoters (23, 37). The present results confirm this observation. In addition, we found that BMRF1 has a *trans*-activating function. BMRF1 provided a *trans*-activating function in cotransfection experiments only when it was placed behind a strong heterologous promoter. Although Wong and Levine (37) reported that BMRF1 could not provide gene activation in *trans*, different target cells and different expression vectors used in their experiment may explain the discrepancy.

TABLE 1. Correlation of *trans*-activating ability and expression of nuclear EA in cotransfected BHK cells[a]

Cotransfected plasmid DNA	CAT activity (%)[b]	Antigen-positive cells (%)
pLTR	0.3	ND[c]
pBR-M	1.0	<0.1
pLTR-M	20.4	6.4
pgpt-MBBg	1.0	0.1
pgptLTR-MBBg	19.0	6.9
pgpt-MHB	0.6	<0.1
pgptLTR-MHB	8.7	6.1
pBR-MPs	0.1	ND
pLTR-MPs	0.3	ND
pSV2CAT	83.0	ND

[a] Test (600 ng) and CAT (pA10CAT; 300 ng) plasmids were cotransfected into BHK cells (6×10^6) by the DEAE-dextran method. After 48 h of transfection, cells were harvested for CAT assay (14, 15) and detection of EBV-specific antigens (17, 31).

[b] The percentage of substrate acetylated is indicated.

[c] ND, No antigen-positive cells were detected.

During lytic infection with EBV, over 50 mRNAs are identified (21, 28, 36). Sample et al. (27) reported that the *Bam*HI A, F, H, and M fragments encode poly(A) RNAs that are transcribed in Raji cells superinfected with P3HR-1 EBV in the presence of cycloheximide. One of these, the *Bam*HI M fragment, encodes the earliest detectable poly(A) RNAs present in the cell cytoplasm. On the other hand, researchers (M. Biggin, P. J. Dyson, and P. J. Farrell, Herpesvirus Workshop, Ann Arbor, Mich., 1985) have reported that the *Bam*HI R and Z regions are transcribed first in superinfected Raji cells in the presence of anisomycin. These regions transcribed in the absence of protein synthesis are classified as immediately early genes of EBV and are suspected to play a key role in the switch from the latent state to the lytic cycle. Such possibilities are strengthened by the observation in the present study that both BMRF1 and BMLF1 have *trans*-activating functions, although it is not known which viral genes are activated by BMRF1 and BMLF1 in the lytic phase of EBV infection.

The mechanism of *trans*-activation remains to be clarified. Both BMRF1 and BMLF1 could *trans*-activate transcription from unrelated promoters in transient expression assays. The *Bam*HI Z fragment, on the other hand, failed to *trans*-activate transcription from the SV40 promoter. In a previous report (32), we demonstrated that transfection of EBV-nonproducer Raji cells with the *Bam*HI Z fragment of EBV DNA activates transcription of the *Bam*HI H and F regions of the latent EBV genome. Cooperation between the *Bam*HI H, F region, and the *Bam*HI Z region was also confirmed in BHK cells that were cotransfected with both fragments. The differences in specificities of the *trans*-activator functions may imply different mechanisms involved in transcriptional regulation.

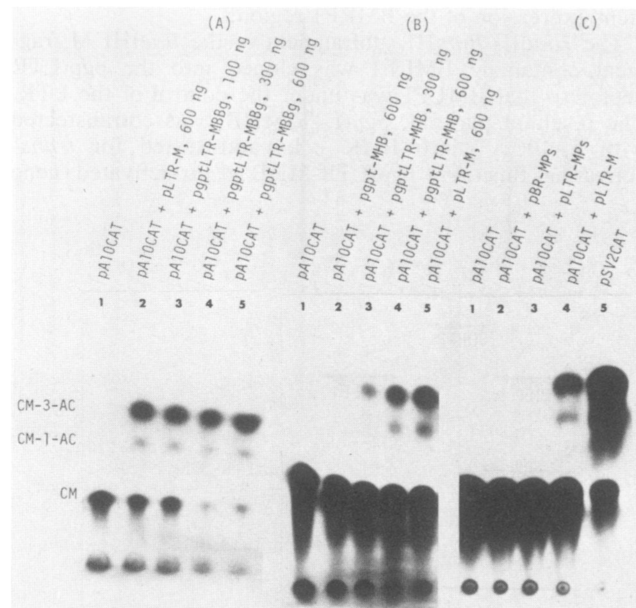

FIG. 4. Identification of regions within the *Bam*HI M fragment that are responsible for *trans*-activation. Plasmid pA10CAT was cotransfected with each of the indicated plasmids into BHK cells. After 48 h of transfection, cells were harvested for CAT assays. CM-3-AC, 3-acetate chloramphenicol; CM-1-AC, 1-acetate chloramphenicol; CM, chloramphenicol.

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