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The Epstein-Barr Virus (EBV) Early Protein EB2 Is a Posttranscriptional Activator Expressed under the Control of EBV Transcription Factors EB1 and R

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From the cloning and characterization of cDNAs, we found that the Epstein-Barr virus (EBV) open reading frame (ORF) BMLF1-BSLF2 coding for the early protein EB2 is present in several mRNAs generated by alternative splicing and expressed in the leftward direction from two promoters PM and PM1. The PM promoter controls the expression of two abundant mRNA species of 1.9 and 2 kilobases (kb), whereas the PM1 promoter controls the expression of at least three mRNAs 3.6, 4.0, and 4.4 kb long. The PM promoter probably overlaps with the PS promoter which controls the transcription of a 3.6-kb mRNA expressed in the rightward direction and containing the ORF BSRF1. Although it increases the amount of chloramphenicol acetyltransferase enzyme expressed from the chimeric pMCAT gene, EB2 is not a promiscuous *trans*-activator of gene expression and does not positively regulate its own expression from promoter PM. The EB2 activation is not promoter dependent but could possibly act by stabilizing mRNAs and increasing their translation. The PM promoter is, however, activated by the two EBV transcription *trans*-acting factors, EB1 and R, encoded by the EBV ORFs BZLF1 and BRLF1, respectively. EB1 activates the PM promoter from a consensus AP-1 binding site, and R activates the PM promoter from an enhancer.

The Epstein-Barr virus (EBV) is a human herpesvirus which infects and immortalizes peripheral B lymphocytes, resulting in the establishment of a latent infection. In such latently infected cells, the entire EBV genome is maintained mainly as a plasmid and its expression is reduced to a few genes: those encoding two small RNAs (20), the six nuclear proteins EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNALP (24), the BHRF1-encoded product (2), the latent membrane protein (15), and the terminal membrane protein, whose coding sequence is created by joining the ends of the linear virus (19, 27).

The latent EBV genome is spontaneously activated in particular cell lines, where between 0.5 and 5% of the cells produce viruses. It can also be activated by various chemical agents, including the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (34). In both cases, the activation seems to be linked to the expression of two EBV-encoded *trans*-activators of early gene promoters, EB1 and R (6, 7, 9, 10, 14, 28). EB1 is encoded by the open reading frame (ORF) BZLF1 and is expressed from two promoters, PZ and PR, either as a 1-kilobase (kb) monocistronic mRNA or as 3- and 4-kb mRNAs generated by alternative splicing and expressing both EB1 and R, the BRLF1 ORF-encoded factor (Fig. 1) (22a). Expression of EB1 alone (6, 7) or R alone (A. Chevallier-Greco, personal communication) in Raji cells activates as many early promoters as does TPA. However, although EB1 activates the expression of R in Raji cells (5), the activator R does not detectably activate the expression of EB1 in these cells (A. Chevallier-Greco, personal communication). These results suggest that many EBV early promoters must have EB1-responsive sequences, R-responsive sequences, or both. In effect, this seems to be the case. EB1 shares sequence homologies with c-*jun* and c-*fos* (3, 11) and activates transcription from different specific sequences located at about position −100 in three EBV early promoters: PZ, DR, and PM (Fig. 1) (4, 29). R activates an enhancer domain in the DR and DL promoters (Fig. 1) (5) which overlap with an origin of replication (13).

A third EBV *trans*-activator called EB2 (6), encoded by the BMLF1-BSLF2 ORF, has been shown to increase the expression of the bacterial gene chloramphenical acetyltransferase (CAT) linked to the EB2 PM promoter (17, 18, 21) and to several heterologous promoters (17, 18, 21, 32). However, EB2 does not detectably activate any EBV early promoter when expressed in latently infected Raji B cells (7). Moreover, EB2 is more likely to be acting at the posttranscriptional level (17, 18).

In order to study the function and regulation of expression of EB2, we have cloned and characterized cDNAs representing four mRNAs containing the BMLF1-BSLF2 ORF. These cDNAs correspond to mRNAs expressed under the control of the two promoters, PM and PM1 (Fig. 2). Promoter PM controls the expression of two mRNAs generated by facultative splicing. Promoter PM1, which is located further upstream, would control the expression of mRNAs also generated by facultative splicing containing ORF BMLF1-BSLF2 and a long leader sequence including the PM promoter and possibly also the ORF BSLF1. We have previously shown that the PM promoter is induced by EB1 and by TPA, and that EB1 and TPA inducibilities are superimposed and mediated by an AP-1/c-*jun*/c-*fos* binding site (30) located 73 base pairs (bp) upstream from the PM promoter TATA box (Fig. 1) (29). We show here that the PM promoter also contains an enhancer induced by the EBV *trans*-activator R, reinforcing the idea that many EBV early promoters could contain EB1-responsive elements, R-responsive elements, or both. Moreover, we also show that although EB2 increases the amount of CAT enzyme ex-

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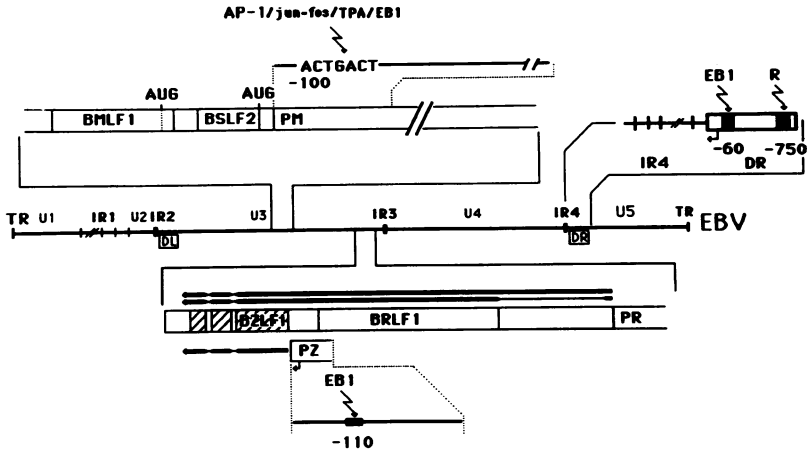

FIG. 1. Schematic structure of EBV *trans*-activators and their targets. Three EBV ORFs have been shown to code for *trans*-activators: BMLF1, BZLF1, and BRLF1. Three targets for EB1 (4, 5, 29) and one target for R (5) (both indicated with closed boxes) have been identified in three EBV early promoters.

pressed under the control of the PM promoter, this is not seen at the level of specific CAT mRNAs. Similarly, when the PM promoter was inserted upstream from the rabbit β-globin ORF or located upstream from the BMLF1-BSLF2 ORF, EB2 did not increase the amount of specifically initiated rabbit β-globin or EB2 mRNAs, although EB1 and R did. These results suggest that EB2 is not a transcriptional *trans*-activator but acts mainly at the posttranscriptional level.

MATERIALS AND METHODS

Cell lines. The EBV genome-negative human lymphoid B cell line BJA-B was a gift from G. Lenoir. The EBV latently infected human B cell line Raji-tk$^-$ has been described elsewhere (6). These cells were grown at 37°C in RPMI 1640 (Boehringer Mannheim Biochemicals) containing 10% fetal calf serum. HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum.

Plasmid constructions. Plasmids pMCAT and pMβ, and mutants with deletions extending in the 5′ to 3′ direction from the 5′ side of the PM promoter were constructed as described elsewhere (29). The endpoint of each deletion mutant was determined by sequencing. Plasmid pM2CAT was constructed by inserting a double-stranded synthetic oligonucleotide between the PM promoter TATA box and the CAT gene in plasmid pMCAT. Plasmid pM2 was constructed by deleting the simian virus 40 (SV40) promoter from the cDNA clone pcDM80. The construction of the EB1 expression vector pSVZ1 and the R expression vector pKSVR have been described elsewhere (22a). The vector expressing EB2, pcDM42D, is a derivative of the cDNA

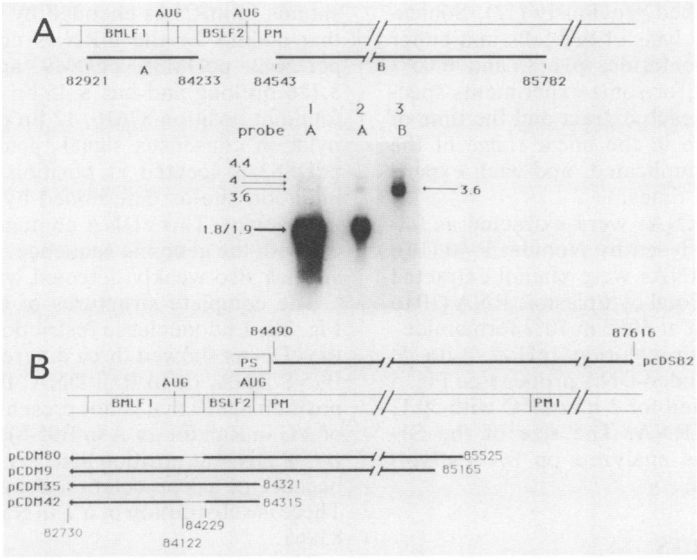

FIG. 2. At least four different RNAs expressed from two promoters could code for EB2. (A) Schematic structure and gel electrophoresis of Northern blots with poly(A$^+$) RNA (5 μg per lane) from Raji cells treated by TPA/SB. The RNAs were separated on a 1% formaldehyde-agarose gel and transferred to nitrocellulose. The filter was either hybridized with probe A (lanes 1 and 2) or probe B (lane 3). Lane 1 is a different sample of RNA than that in lanes 2 and 3. Sizes in kilobases of RNA species are given on either side of the gel. (B) Schematic structures of cDNAs corresponding to the different mRNAs detected in Northern blots. Thin horizontal lines represent the introns.

clone pcDM42 which contains only the BMLF1 ORF located between positions 84233 and 82730 (1), expressed under the control of the SV40 early promoter-enhancer sequences. Plasmids pGM5′ and pGM3′ were constructed by inserting a DNA fragment (positions 84628 to 84922 [1]) containing the PM promoter R-responsive sequences either 425 bp upstream in normal (plasmid pGM5′) or inverted (plasmid pGM5′In) orientations or 2,500 bp downstream (plasmid pGM3′) from the rabbit β-globin promoter. These constructions were verified by sequencing. Plasmid pSV2β was made by inserting the SV40 *Stu*I-*Pvu*II fragment containing the early promoter-enhancer sequences into plasmid pG (16) digested by *Sma*I and *Bam*HI. Plasmids pSCAT and pSCAT258$^+$ have been described elsewhere (4). Plasmid pSV2β expresses a chimeric SV40–β-globin RNA and was cotransfected as an internal control for transient expression experiments. Plasmid pNSTG was made by ligating human immunodeficiency virus (HIV) type 1 long terminal repeat (LTR) sequences to the β-globin ORF (see Fig. 5A). Plasmid pSV0 contains the SV40 *Hpa*II (map position 346)-to-*Hin*dIII (map position 5171) fragment cloned in pUC19 digested with *Hin*dIII and *Bam*HI. This plasmid is included in most transfections to keep the amount of SV40 early promoter sequences constant, since EB1, R, and EB2 are expressed under the control of the SV40 early promoter.

cDNA synthesis and screening of the cDNA library. The pcD cDNA library was prepared by a modification of the method of Okayama and Berg (25) and has been described elsewhere (22a). The cDNA library was screened by in situ hybridization with specific multiprimed ^{32}P-labeled probes A and B (Fig. 2A). The structure of the cDNAs has been established both by sequencing and by restriction endonuclease analysis.

Transfection procedure and CAT assays. One day before transfection, lymphoid B cells were suspended at a density of 5×10^5 cells per ml in fresh medium. These cells were transfected by the DEAE-dextran procedure (22). HeLa cells were seeded at 10^6 cells per 100-mm petri dish 4 h before transfection. The cells were transfected by the $CaPO_4$-DNA precipitate method (31). CAT assays were performed essentially as described previously (12). Sonication, however, was replaced by lysis of the cells in a buffer containing 0.25 M Tris hydrochloride, pH 8, and 0.05% sodium dodecyl sulfate. Except for some experiments specified in the text, the volume of each extract and the time of the reaction were chosen to be in the linear range of the assay. Each transfection was duplicated, and each experiment was repeated at least two times.

RNA analysis. Cytoplasmic RNAs were extracted as follows: cells were harvested and lysed by Nonidet P-40 (16), the nuclei were pelleted, and RNAs were phenol extracted from the cytoplasmic fraction. Total cytoplasmic RNA (10 to 40 μg) was hybridized overnight at 30°C in 50% formamide–0.3 M NaCl–0.01 M Tris hydrochloride (pH 7.4) to 5′ ^{32}P-labeled synthetic single-stranded DNA probes (see Fig. 5 and 6). The hybrids were digested for 2 h at 20°C with 50 U of S1 nuclease per 20 μg of RNA. The size of the S1-protected DNA fragments was analyzed on 8% (wt/vol) polyacrylamide-8.3 M urea gels.

RESULTS

The EB2 ORF found in several mRNAs generated by facultative splicing and expressed under the control of two separate promoters. It has been shown by Northern (RNA) blot and S1 nuclease mapping that two mRNAs of 2 and 1.9 kb contain the BMLF1-BSLF2 ORF (26), the 1.9-kb mRNA being a spliced version of the 2-kb species. These two transcripts appear as a single band on a Northern blot of poly(A)$^+$ RNAs isolated from Raji cells treated with TPA to induce the expression of the EBV early genes and hybridized with probe A located in the ORF BMLF1 (Fig. 2A, lanes 1 and 2). However, besides the 2-kb RNA species, three additional RNA species of about 3.6, 4.0, and 4.4 kb were detected with probe A. Another probe located further upstream, probe B (Fig. 2A), also detected these RNA species but failed to detect the 2- and 1.9-kb RNAs (Fig. 2A, lane 3). Instead, the 3.6-kb RNA species was detected. In order to establish the structure of these mRNAs, we have cloned their cDNAs. An oligo(dT)-primed cDNA library was prepared in the Okayama and Berg vector pcD (25; Manet et al., in press). The cDNAs were synthesized from poly(A$^+$) RNAs isolated from Raji cells in which the EBV early gene expression was induced by TPA (34). The library was first screened with probe A (Fig. 2A), and 28 positive clones were isolated. All cDNAs had a poly(dA-dT) tract starting at position 82370, corresponding to the polyadenylation site located downstream from the polyadenylation signal found at position 82747. About 50% of the cDNAs had an intron located between positions 84228 and 84122 excised (numbering is as that in reference 1). Two of the clones, pcDM42 and pcDM35, were selected, as their sizes were close to that expected for full-length copies of the 1.9- and 2-kb mRNAs detected in Northern blots. The schematic structures of these cDNAs are shown in Fig. 2B. Since long mRNAs were detected with probe B, the library was also screened with probe B and afterwards with probe A. A total of 21 cDNAs were isolated with probe B and only two of them also hybridized with probe A. These two cDNAs, pcDM80 and pcDM9, probably incomplete in size, could correspond to any of the 3.6-, 4.0-, and 4.4-kb RNA species (Fig. 2B). These two cDNAs also had a poly(dA-dT) tract at position 82370, and one of them, pcDM80, had the intron sequence (from 5′ position 84228 to 3′ position 84122) excised.

Finally, from the 19 cDNAs responding only to probe B, the longest one, cDNA pcDS82, was selected and corresponds to mRNAs encoded by the DNA strand opposite to that coding for the mRNAs corresponding to the cDNAs pcDM42, pcDM35, pcDM9, and pcDM80. This cDNA is 3,126 bp long and has a 45-bp poly(dA-dT) tract, which is found at position 87616, 12 bp downstream from a polyadenylation consensus signal (position 87599). The 5′ end of pcDS82 is located at position 84490, which is the major initiation site as determined by S1 nuclease mapping (data not shown). This cDNA contains the BSRF1 ORF, is colinear with the genomic sequence, and is likely to be the 3.6-kb species also weakly detected with probe A.

The complete structures of the cDNAs are indicated in Fig. 2B. Endonuclease restriction analysis and sequencing of the cDNAs showed three differences between Raji DNA and B95-8 DNA. (i) In Raji DNA, there is a *Pst*I site located at position 82819 that is not present in B95-8 DNA (substitution of a G in Raji for an A in B95-8). (ii) The *Xho*I site present in B95-8 DNA at position 83625 is not present in the Raji DNA because of a 3-bp deletion at positions 83626 to 83628). (iii) There is substitution of a T in Raji for a C in B95-8 at position 83965.

We have shown by cDNA cloning that the ORF coding for EB2 is found in different mRNAs generated by alternative splicing and is expressed under the control of two separate promoters called pM and pM1 (Fig. 2B). In addition, the PM promoter could overlap with the PS promoter controlling the

expression of the 3-kb mRNA species containing the ORF BSRF1.

EB1-, EB2-, and R-influenced increase in the amount of CAT enzyme expressed under the control of the PM promoter. EB2 was originally described as being able to increase the amount of CAT enzyme expressed from the PM promoter, the SV40 enhancerless promoter, the HIV promoter, and the adenovirus E3 promoter linked to the CAT gene (17, 18, 21, 32). In the case of the HIV-CAT and PM-CAT promoters, no direct link between the amount of CAT enzyme activity and the amount of CAT-specific mRNAs could be made, suggesting that EB2 activation of CAT enzyme activity was mainly posttranscriptional (17, 18). This was also already suggested by the fact that when EB2 was expressed in EBV latently infected Raji B cells, no transcriptional activation of EBV early genes was detected compared with TPA and EB1 (the BZLF1-encoded product) activation of early gene expression (7).

The pMCAT constructs used by others (17, 18, 21) contained different amounts of EBV sequences located between the PM promoter putative TATA box and the CAT sequence AUG. We, therefore, investigated whether sequences located 5′ to the PM promoter TATA box were required for the EB2 *trans*-acting function, as detected by CAT assays. We placed the PM promoter upstream from the CAT gene and generated plasmid pMCAT, which contains EBV sequences from +3 downstream from the PM promoter TATA box to the *Hin*dIII site located −2,935 bp upstream from the TATA box (Fig. 3A), and plasmid pM2CAT, which contains sequences from +61 to −2935 (Fig. 3A). The plasmids were transfected in different cell lines either alone or together with an EB2 expression vector, and the activity of the PM promoter was analyzed by quantitation of CAT enzyme activity. As a control, the different constructions were also cotransfected with an EB1-expressing vector.

The PM promoter in pMCAT and pM2CAT was not detectably active in Raji cells (Fig. 3B, lanes 1 and 5), and was very poorly active in HeLa cells (Fig. 3C, lanes 1, 2, 9, and 10). EB1 has been shown to activate specific transcription from the PM promoter, and the activation is mediated by the c-*jun*/c-*fos* binding site AP-1 (29, 30). As expected, cotransfection of pMCAT and pM2CAT with the EB1-expressing vector pSVZ1 resulted in an increase in the amount of CAT enzyme expressed in Raji cells (Fig. 3B, lanes 2 and 6) and HeLa cells (Fig. 3C, lanes 3, 4, 11, and 12). Finally, cotransfection of pMCAT and pM2CAT with the EB2 expression vector pcDM42d also resulted in an increase in the amount of CAT enzyme expressed in Raji cells (Fig. 3, lanes 3 and 7) and HeLa cells (Fig. 3C, lanes 7, 8, 15, and 16). Similar results were obtained in the EBV genome-negative B cell line, BJA-B, compared with the results obtained with HeLa cells (Fig. 3D, lanes 1 to 6). Our results confirmed that EB2 *trans*-activates the CAT enzyme expressed from the PM promoter linked to the CAT gene (17, 18, 21) and demonstrated that sequences located downstream from the PM promoter, between the TATA box and the BSLF2 sequence AUG, were not required for the EB2 *trans*-acting function, as determined by CAT assays.

In Raji cells (Fig. 3B, lanes 2 and 6), EB1 induced much more efficiently the amount of CAT enzyme expressed from plasmids pMCAT and pM2CAT, than in HeLa cells (Fig. 3C, lanes 3, 4, 11, and 12) or BJA-B cells (Fig. 3D, lanes 3 and 4). This could be because in Raji cells, EB1 activates the expression of the EBV-encoded enhancer factor R (5), suggesting that the PM promoter contains sequences responsive to R. This is indeed the case. Cotransfection of plasmids

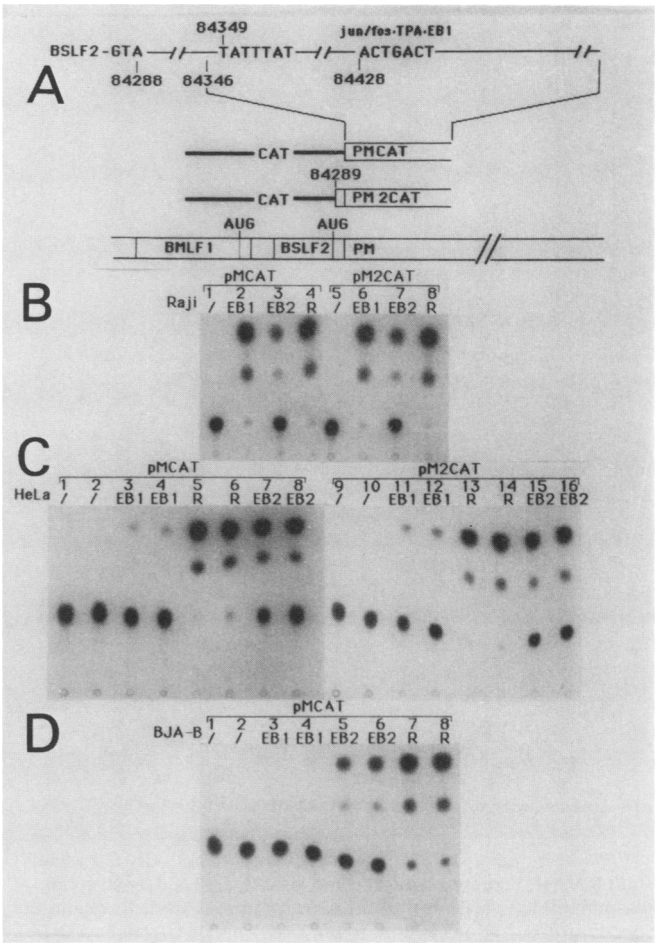

FIG. 3. EB2 increases the amount of CAT enzyme expressed from the PM promoter. (A) Schematic representation of the EBV genome region present in plasmids pMCAT and pM2CAT. (B through D) Visualization of the effects of EB1, EB2, and R on CAT enzyme activity present in Raji cells (B), in HeLa cells (C), or in BJA-B cells (D) transfected with plasmids pMCAT and pM2CAT. Some of the assays are out of the linear range in order to detect the basal activity of the PM promoter in the CAT constructs.

pMCAT and pM2CAT with the R-expressing vector, pKSVR, resulted in a strong increase of CAT enzyme expressed in Raji cells (Fig. 3B, lanes 4 and 8), in HeLa cells (Fig. 3C, lanes 7, 8, 15, and 16), and in BJA-B cells (Fig. 3D, lanes 7 and 8). These results suggested that the PM promoter contains an enhancer inducible by R.

The influence of R, EB1, and EB2 on the increase in the accumulation of specific transcripts expressed from the PM promoter linked to the CAT ORF, the rabbit β-globin ORF, or the BMLF1-BSL2 ORF. CAT enzyme activity induced by EB2 from pMCAT and pM2CAT was comparable in Raji cells, in BJA-B cells, and in HeLa cells. Others have demonstrated that CAT enzyme induction from a construct similar to pMCAT was not detected at the level of CAT mRNAs in Raji cells (17). This was also true for HeLa cells. Plasmid pM2CAT was transfected in HeLa cells either alone or together with an EB1, EB2, or R expression vector. At the level of CAT enzyme, the activation was similar to that shown in lanes 9 through 16 of Fig. 3C. The amount of specific CAT mRNA was quantitated by hybridizing total cellular RNA to a ^{32}P-labeled single-stranded RNA probe

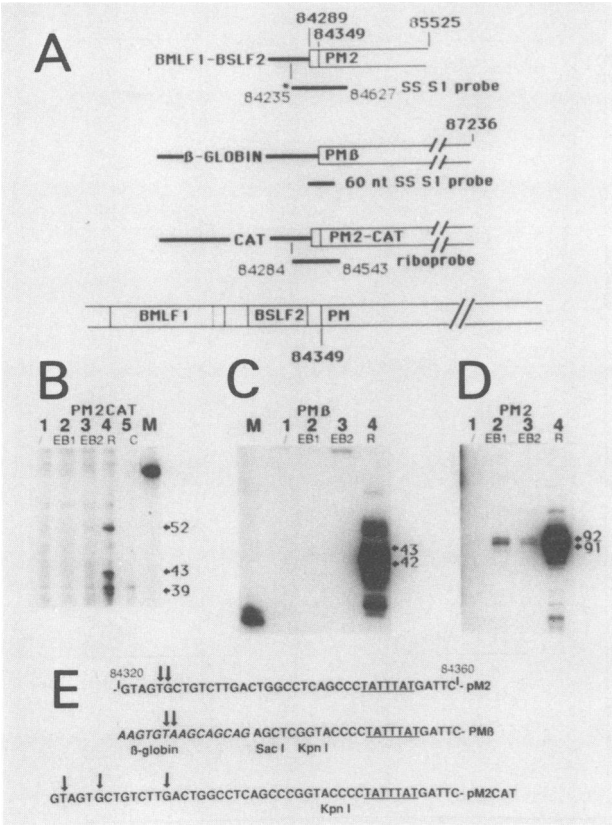

FIG. 4. Effect of EB2 on CAT, β-globin, and BSLF2 steady-state RNAs expressed from the PM promoter. (A) Construction of plasmids pM2, pMβ, and pM2CAT and probes used to quantitate specific transcription. Plasmids pM2 (B), pMβ (C), and pM2CAT (D) were transfected in HeLa cells either alone (/) (lanes 1), or with a vector expressing EB1 (lanes 2), EB2 (lanes 3), or R (lanes 4). Lane C, Plasmid as internal control. M, Molecular weight marker. (E) Schematic representation of the TATA box (underlined) downstream sequences in plasmids pM2, pMβ, and pM2CAT, and localization of the major initiation sites (arrows) as detected by RNase and S1 mapping.

(Fig. 4A) and digesting the hybrids with ribonuclease. Only R (Fig. 4B, lane 4) detectably increased the amount of CAT mRNA, and it was only with R that a similar degree of stimulation at both the mRNA level and CAT enzyme level was detected (data not shown). Our results, therefore, confirm that EB2 is not a transcription transacting factor, at least not in an assay where the PM promoter is linked to the CAT gene.

No investigation has been made, however, to test the effect of EB2 on the accumulation of specific RNAs expressed from the PM promoter when linked to the homologous ORF BMLF1-BSLF2 or to a gene known to encode a stable RNA, the rabbit β-globin ORF. We therefore constructed the plasmids pM2 and pMβ described in Fig. 4A. Plasmids pMβ and pM2 were transfected in HeLa cells, and their inducibility by EB1, EB2, and R were analyzed by a S1 nuclease protection assay. Very few specific β-globin transcripts were expressed under the control of the PM promoter (Fig. 4C, lane 1). As expected, EB1 increased by about three times the amount of β-globin RNA expressed (Fig. 4C, lane 2) and EB2 had no detectable effect on PM promoter activity (Fig. 4C, lane 3), whereas R strongly increased β-globin transcription (Fig. 4C, lane 4). Almost identical results were observed when the PM promoter was linked to the BMLF1-BSLF2 ORF (Fig. 4D). The S1 probe located in the BSLF2 ORF could detect RNAs expressed from plasmid pM2 but not from plasmid pcD42d, where EB2 is expressed from a truncated cDNA lacking the BSLF2 ORF. Very few specific BSLF2 transcripts were expressed from the PM promoter (Fig. 4D, lane 1). EB1 increased by about five times the specific BSLF2 transcripts (Fig. 4D, lane 2), and surprisingly, EB2 increased about by three times the amount of BSLF2 RNA (Fig. 4D, lane 3). As expected, R induced a high level of specifically initiated BSLF2 transcripts (Fig. 4D, lane 4). The PM promoter TATA box, the 5′ flanking sequence in plasmids pM2, pMβ, and pM2CAT, and the specific initiation sites detected by S1 mapping and RNase mapping are shown in Fig. 4E. Thus, specific initiation occurs at about the same position in all three constructs.

EB2 did not increase the amount of specifically initiated CAT and β-globin RNA expressed from the PM promoter, compared with EB1 and R, and is not, therefore, a transcription factor per se. EB2 increased the amount of BSLF2-specific RNAs, however, although less than did EB1. These results have been reproduced several times with different DNA preparations.

EB2-influenced increase in the accumulation of specific RNAs expressed from the SV40 early promoter linked to the CAT ORF but not from the SV40 early promoter linked to the β-globin ORF. From our results and from the results published by others (17, 18), it could be proposed that EB2 influences the translation of CAT proteins rather than stabilizing CAT RNAs expressed from the PM promoter. However, we also observed (i) that EB2 increased the amount of BSLF2-specific RNA (Fig. 4D, lane 3) and (ii) that EB2 increased the amount of CAT enzyme and of specific CAT RNAs expressed from the enhancerless SV40 early promoter linked to the CAT ORF (plasmid pSCAT) from plasmid pSCAT linked to the DR enhancer (plasmid pSCAT258$^+$) (4). In the latter case, the increase in CAT activity was almost proportional to the increase in CAT mRNA. Again, if the increase in CAT RNA was promoter dependent, then EB2 should be expected to increase the amount of specifically initiated RNAs when the SV40 enhancer-promoter is linked to part of the β-globin ORF (plasmid pSV2β) (Fig. 5A). Specifically initiated RNA were expressed from pSV2β (Fig. 5B, lane 1). As published elsewhere (4), EB1 had no effect on the activity of the SV40 early promoter (Fig. 5B, lane 2). EB2 also did not increase the amount of specific RNAs expressed from pSV2β (Fig. 5B, lanes 3 and 4).

Moreover, in the case of the HIV LTR-tar-CAT construct, in addition to CAT activity enhancement (29-fold increase), it has been shown that EB2 also has an effect on the amount of specific CAT RNAs (four-fold increase), probably because of the stabilization of CAT mRNA (18). This, however, was not seen when the HIV LTR-tar was linked to the β-globin ORF (Fig. 5C). Very few specific β-globin transcripts were expressed from plasmid pNSTG in HeLa cells (Fig. 5D, lane 2). The HIV tat protein expressed from plasmid pTATB strongly increased the amount of β-globin RNA expressed from the HIV LTR-tar promoter (Fig. 5D, lane 3). However, EB2 had no effect on the accumulation of β-globin transcripts expressed from plasmid pNSTG (Fig. 5D, lanes 4 and 5). Plasmid pSV2β was included in each transfection as an internal control for efficiency of transfection and expression (Fig. 5D, lanes 6 to 7).

The PM promoter containing an enhancer inducible by R. The PM promoter is strongly activated by R in Raji, BJA-B,

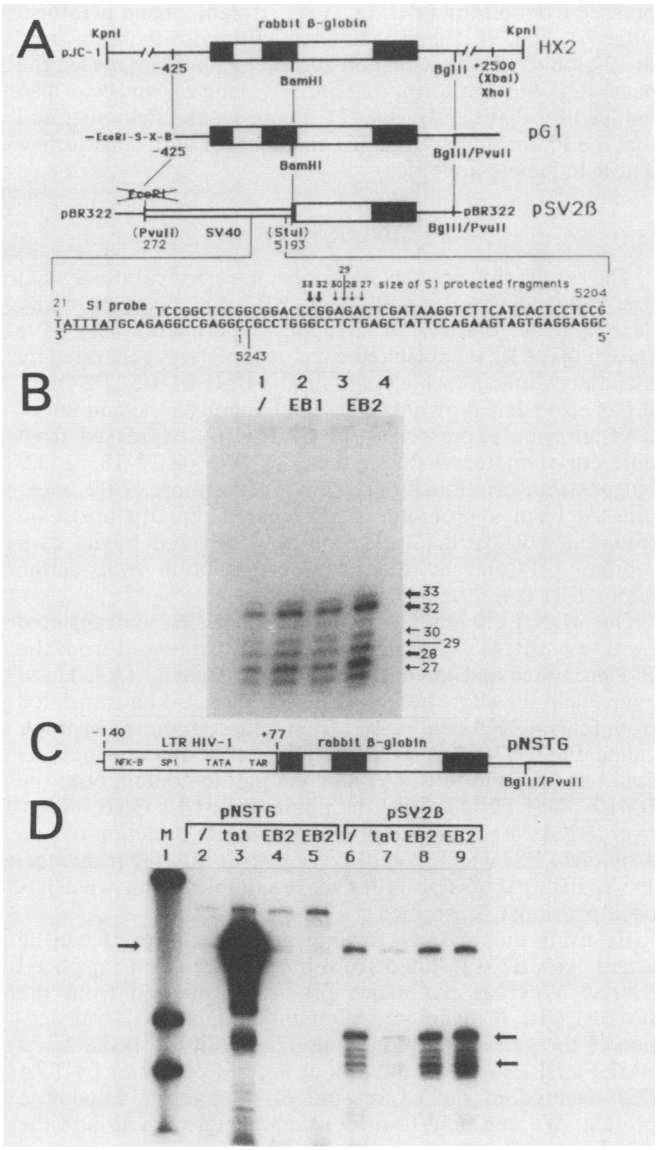

FIG. 5. EB2 has no effect on the activity of the SV40 early promoter and on the HIV LTR. (A) Construction of plasmid pSV2β, location of the single-stranded DNA S1 probe and specific initiation sites (arrows) as determined by S1 nuclease digestion. The SV40 early promoter TATA box is underlined. (B) Quantitative S1 nuclease analysis of steady-state β-globin RNA expressed in HeLa cells from the PM promoter linked to part of the β-globin ORF. Plasmid pSV2 was transfected in HeLa cells either alone (/) (lane 1) or with a vector expressing EB1 (lane 2), or EB2 (lanes 3 and 4) [In transfections 3 and 4, two different preparations of EB2 were used]). Sizes in kilobases are indicated at the right. (C) Construction of plasmid pNSTG. (D) Quantitative S1 nuclease analysis of β-globin RNA expressed from the HIV LTR linked to the β-globin ORF. The single-stranded DNA probe is the one described in Fig. 6B. Plasmid pNSTG was transfected in HeLa cells either alone (/) (lane 2), with the tat-expressing vector pTAT-B (tat) (lane 3), or with two different DNA preparations of the EB2-expressing vector pcD42d (lanes 4 and 5). S1 nuclease mapping of RNA expressed from plasmid pSV2β was cotransfected as an internal control alone (/) (lane 6) or with indicated vectors (represented in lanes 7 through 9 as in lanes 3 through 5, respectively). The S1 probe sequence is shown in panel A. M, Molecular weight markers.

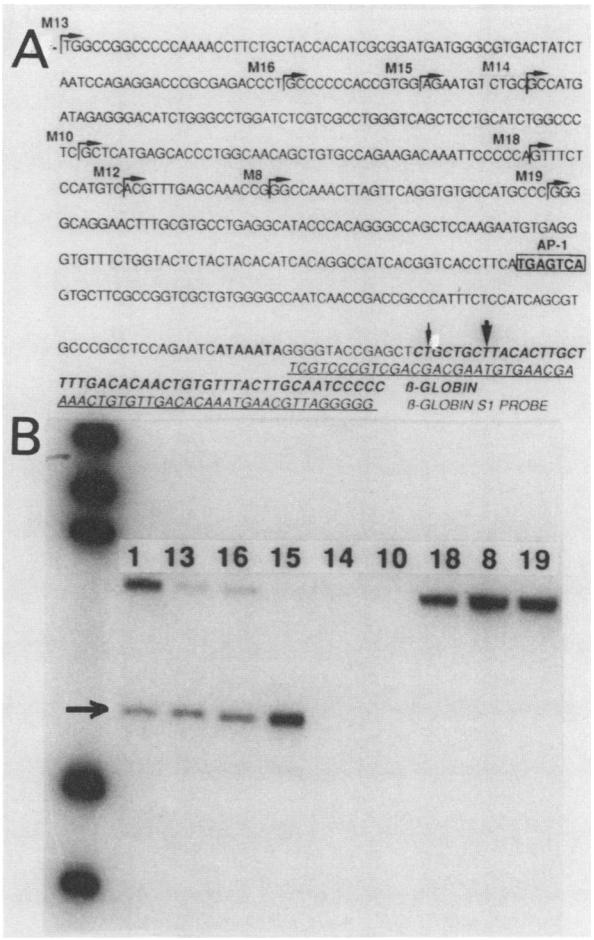

FIG. 6. The PM promoter contains an R-responsive element. (A) Progressive BAL 31 deletion mutants were made in the PM promoter, and their activation by R was determined by using a single-stranded DNA probe. Downward arrows indicate major initiation sites. (B) Gel electrophoresis showing activation of the PM promoter deletion mutants by R as determined by quantitative S1 nuclease analysis. Horizontal arrow indicates specific S1-protected DNA fragment. Left lane, molecular weight markers.

and HeLa cells. R has been shown to be an enhancer factor (5). In order to localize the R-responsive element in the PM promoter and to determine if it has enhancer properties, we constructed a series of mutants with deletions extending in the 5′-to-3′ direction from the 5′ side of the PM promoter. The PM mutants were fused to the β-globin gene (Fig. 6A) (29), and the different constructs were transfected in HeLa cells for analysis of their activity in the presence of R.

RNAs extracted from HeLa cells transfected with the PM promoter mutants were analyzed with the S1 nuclease protection assay. Two correctly initiated β-globin mRNAs were detected and protected fragments of 43 and 42 nucleotides. The results, shown in Fig. 6B, demonstrate that induction of the PM promoter by R is severely impaired by deletion of sequences located between −398 (mutant M15) and −387 (mutant M14) before the major initiation site. A longer exposure of the gel showed that a second region located between −387 and −322 (mutant 10) was also responding to R (data not shown).

To test whether this R-responsive element has enhancer properties, we placed a DNA fragment from between positions −300 and −595 containing the entire R-responsive

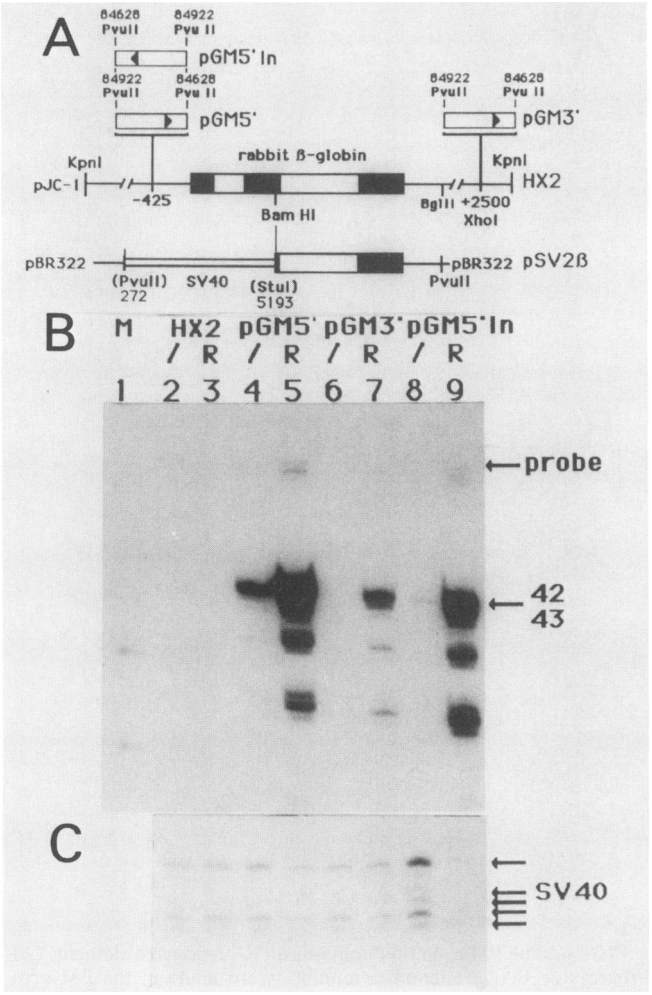

FIG. 7. The PM promoter R-responsive element has enhancer properties. (A) Construction of plasmids pGM5′, pGM5′In, and pGM3′. (B) Electrophoretic analysis of β-globin RNA by quantitative S1 nuclease mapping. Plasmids HX2 (lanes 2 and 3), pGM5′ (lanes 4 and 5), pGM3′ (lanes 6 and 7), and pGM5′In (lanes 8 and 9) were transfected in HeLa cells either alone (/) (lanes 2, 4, 6, and 8) or with an R expression vector (R) (lanes 3, 5, 7, and 9). M, Molecular weight markers. Probe and 42- and 43-nucleotide fragments are indicated at the right. (C) Plasmid pSV2β was included in each transfection as an internal control, and pSV2β-specific transcription was quantitated by using the S1 probe described in Fig. 5A. SV40 fragment is indicated on the right.

region, either 5′ in normal (plasmid pGM5′) or inverted (plasmid pGM5′In) orientations, or 3′ (plasmid pGM3′) to the β-globin promoter in plasmid HX2 (Fig. 7A). These constructions were transfected in HeLa cells (together with plasmid pSV2β expressing a SV40-β-globin chimeric RNA as an internal control of transfection efficiency), and their responsiveness to R was tested. The amount of SV40-β-globin chimeric RNA found in each transfection was comparable, suggesting that the same amount of DNA entered the cells (Fig. 7C). The β-globin promoter had a very weak basal activity (Fig. 7B, lane 2), and this basal activity was not influenced by R (lane 3). Addition of the PM promoter R-responsive sequences 5′ to the β-globin promoter either in normal (plasmid pGM5′; Fig. 7B, lane 4) or in inverted (plasmid pGM5′In; Fig. 7B, lane 8) orientations increased the basal activity of the promoter. However, R strongly enhanced the amount of the β-globin RNA expressed from plasmid pGM5′ (Fig. 7B, lane 5) and pGM5′In (Fig. 7B, lane 9). When located 2,500 bp downstream from the β-globin +1, the R-responsive element still increased the basal β-globin transcription (Fig. 7B, lane 6), and was also induced by R (Fig. 7B, lane 7). These results demonstrated that the PM promoter contains an enhancer with constitutive and R-inducible activities.

DISCUSSION

The results presented here represent a clarification of what has been previously published. EB2 has been primarily described as follows. S1 mapping experiments (26) have shown that EB2 is translated from two mRNAs generated by facultative splicing which contain the BMLF1-BSLF2 ORF. It has also been shown that EB2 can increase the amount of CAT enzyme expressed from CAT RNA expressed from different promoters linked to the CAT ORF (6, 17, 18, 21, 32) but posttranscriptionally (17, 18). Furthermore, EB2 is expressed from a promoter, PM, located directly upstream from the ORF BSLF2 (21, 26) autoactivated by its own product (21) and by the EBV transcription *trans*-acting factor EB1 (11, 29).

Our cDNA cloning has confirmed that EB2 is translated from two mRNAs, 1.8 and 1.9 kb long, expressed from the PM promoter and made by facultative splicing (26). However, what we show here is that EB2 can also be translated from longer RNAs expressed from a more remote promoter called PM1. The incomplete cDNAs pcDM80 and pcDM9 could correspond to RNAs long enough to contain both the BMLF1-BSLF2 ORF and the putative BSLF1 ORF. Moreover, RNAs are transcribed in the opposite direction from a promoter, PS, which could overlap with the PM promoter. Thus, EB2 expression is far more complex than what has been previously described.

By using our cDNAs, several specific questions can be asked. Is EB2 translated from both spliced and unspliced RNAs? Whereas the major product expressed from the BMLF1 ORF is a 48- to 50-kilodalton antigen (33), transfection of the genomic region containing both the BSLF2 and BMLF1 ORFs (8) or induction of the PM promoter by TPA (23) resulted in the expression of a major 60-kilodalton protein. We can now transfect the spliced cDNAs and the unspliced cDNAs in which mutations impair the splicing without interrupting the ORF and ask which proteins are made. Additional mutations would indicate which AUG sequence is used for initiation of different translation products. Another question can be asked: is the alternative splicing specific to B cells, or does it occur in every cell type? By transfecting the unspliced cDNAs in different cells types, we could determine whether splicing occurs and, if so, in which proportion, compared with RNAs expressed in EBV-infected cells.

Although EB2 increases the amount of CAT enzyme expressed from different promoter-CAT constructs, it is not a transcription *trans*-activator but probably acts by stabilizing or increasing the translation of CAT mRNA. However, there are no direct proofs that EB2 has these properties but only indirect evidence. Our results confirm and extend the observation that EB2 could act at the posttranscriptional level (17, 18), but these results had been obtained by using SV40-CAT, HIV-CAT and PM-CAT constructs in EBV-negative cell lines. Since we show here that EB2 has no effect on the amount of specific RNAs expressed from the SV40, the PM, and the HIV promoters linked to the β-globin

ORF, it could be that the EB2 *trans*-acting function is restricted to CAT mRNA. However, it could still be that EB2 increases translation of β-globin or EB2 proteins, and this is under investigation.

What, then, is the function of EB2 in EBV-infected B cells? Transfection of an EB2 expression vector in the latently infected B cell line Raji has no effect on the activation or accumulation of EBV early mRNAs (7). However, it could be that EB2 stabilizes or increases translation from some of the EBV mRNAs, in spontaneously producing B cells or in B cells where the EBV lytic cycle has been induced by TPA. Indeed, we show here that EB2 increased twofold the amount of specific EBV BSLF2 RNAs (Fig. 4D) expressed from the PM promoter (Fig. 4B), and preliminary results indicate that the EBV *Pst*I repeat RNA is also stabilized by EB2 (A. Chevallier-Greco, personal communication). Obviously, more experiments are required to establish clearly at which posttranscriptional step(s) EB2 is acting and to understand the function of EB2 in EBV-infected cells.

What we have postulated is that many EBV early promoters must have EB1-responsive elements, R-responsive elements, or both. In effect, EB1 or R, when expressed in Raji cells, activates as many promoters as does TPA, but R does not activate the expression of EB1 (22a). The PM promoter has an EB1-responsive element which overlaps with the TPA-responsive element AP-1 where the proto-oncogenes c-*jun* and c-*fos* bind (29), and EB1 also binds to the AP-1 site in vitro (11; G. Urier, personal communication). In addition, the PM promoter contains an R-responsive element with enhancer properties. This R-responsive element is probably complex. Deletion of the region located between positions 84726 to 84715, containing the sequence AGAATGTCTGC, severely impaired R induction of the PM promoter (Fig. 6). However, a 38-bp oligonucleotide (M38) containing this region, **AGAATGTCTGC**GCCATGATAGAGGGACATCT GGGCCTG, and located −425 upstream from the β-globin promoter did not render this promoter responsive to R (H. Gruffat, unpublished results). Another R-responsive enhancer is present in the EBV DR promoter (5), and an R-responsive element has been defined by bidirectional mutagenesis. It is contained in the following sequence, called BO: CTGTGCC**TTGTCCCGTGGACA**ATGTCCC. However, although the element **TCTCC/T(X)$_n$GGACA** is conserved in both enhancers, BO conferred R responsiveness to the β-globin promoter (5) and M38 did not (data not shown). It is also not known if R interacts directly with R-responsive elements or not. These experiments are in progress. A positive answer would confirm that EBV is a mammalian DNA virus encoding sequence- and function-specific DNA-binding transcription factors expressed from bicistronic mRNAs (22a, 29).

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