The EMBO Journal vol.8 no.5 pp.1447 – 1453, 1989

The Epstein – Barr virus early protein EB1 activates transcription from different responsive elements including AP-1 binding sites

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Communicated by W.Keller

When expressed in Epstein – Barr virus (EBV) latently infected B cells, the EBV early protein EB1 *trans*-activates as many EBV early genes as does TPA. Several EB1 responsive elements (ZRE) have been identified in EBV early promoters and are located at relatively short distances from the TATA box. One of them (ZRE-M) overlaps with a consensus TPA responsive element (TRE) defined as an AP-1/c-*jun*/c-*fos* binding site and is located in an EBV promoter controlling the expression of the post-transcriptional activator EB2. Another (ZREZ) is located in the promoter controlling the expression of EB1 and does not respond to TPA. These two ZREs have no apparent sequence homology. Although EB1 activates transcription from the AP-1 enhancer sequence and from the ZREZ, the activation is severely impaired by distance, suggesting that EB1 is more likely to be a promoter factor than an enhancer factor. These properties also suggest that EB1 is not functionally related to c-*jun* and c-*fos*. However, since EB1 can activate transcription from AP-1 binding sites when properly positioned, the role of this factor in the oncogenic properties of EBV should be considered.

Key words: latency/viral *trans*-activators/AP-1 binding site

Introduction

For many mammalian DNA viruses, the expression of early gene products depends on the synthesis of viral proteins which act as *trans*-activators of early gene promoters (for a review, see McKnight and Tjian, 1986). The Epstein – Barr virus (EBV) follows the same rule. EBV is a human herpes virus which latently infects and immortalizes B lymphocytes. Recently, two EBV *trans*-activators of transcription, EB1 and R, have been identified (Countryman and Miller, 1985; Takada *et al.*, 1986; Chevallier-Greco *et al.*, 1986; Hardwick *et al.*, 1988). EB1 is expressed from two promoters Z and R, either as a 1 kb monocistronic mRNA or as 3 and 4 kb bicistronic mRNAs expressing both EB1 and R (Figure 1) (E.Manet *et al.*, in press). R activates an enhancer domain (Chavrier *et al.*, 1989; Chevallier-Greco *et al.*, 1989) in a promoter which overlaps with an origin of replication (Hammerschmidt and Sugden, 1988). EB1 was initially described as being able to induce the synthesis of several EBV early and late antigens detectable both by immunofluorescence and by immunoblotting, when expressed in latently infected B cells (Countryman and Miller, 1985; Countryman *et al.*, 1986, 1987). It has now been shown that EB1 is able to activate as many EVB early RNAs in latently infected Raji B cells (Chevallier-Greco *et al.*, 1986) as does the tumour-promoting phorbol ester 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA) (Zur Hausen *et al.*, 1978), and to induce transcription from two regions containing genes expressed during latency (Takada *et al.*, 1986). Moreover, a recent report suggests that EB1 shares sequence homologies with c-*jun* and c-*fos* (Farrell *et al.*, 1989). These polypeptides recognize and bind to a specific DNA sequence, the AP-1 binding site (Bohmann *et al.*, 1987; Lee *et al.*, 1987a,b; Angel *et al.*, 1988a; Chiu *et al.*, 1988; Rauscher *et al.*, 1988) which is a *cis*-acting enhancer element mediating a transcriptional response to TPA (Angel *et al.*, 1988a; Chiu *et al.*, 1988). It was therefore of interest to identify the EBV early promoters responding to EB1 and/or to TPA and to localize the sequences mediating these effects.

We have identified EB1 targets (ZREs) in several EBV early promoters. One target called ZRE-M coincides with a TPA responsive element (TRE) also described as the c-*jun*/c-*fos* consensus binding site AP-1, and is located in the promoter controlling the expression of the post-transcriptional *trans*-activator EB2 (Figure 1) (Kenney *et al.*, 1988). The second EB1 target is localized in the promoter controlling the expression of EB1 and is called ZRE-Z (Figure 1). ZRE-Z does not respond to TPA. The two EB1 targets have no clear sequence homology as was predicted by the observation that only ZRE-M/AP-1 responds to TPA. The EB1 responsive elements are only active when placed about −100 upstream from the β-globin CAP site, which

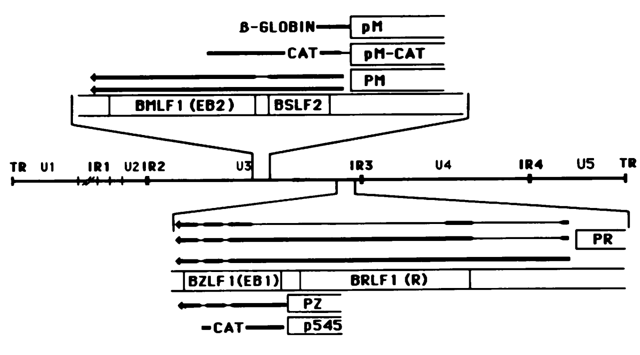

Fig. 1. Summary of the structure of EBV immediate early transcription units. EB1 is encoded by the ORF BZLF1 and expressed from two promoters PZ and PR. EB1 is translated either from a monocistronic 1 kb mRNA initiated from promoter PZ, or translated with R from two bicistronic mRNAs (3.5 and 4 kb long) generated by alternative splicing and initiated from promoter PR. p545 (Z545) is a plasmid carrying ~560 bp of PZ promoter cloned upstream of the CAT coding sequence. The BMLF1 ORF codes for EB2 which is translated from two mRNAs (1.5 and 1.6 kb long) initiated from promoter PM. About 2950 bp of promoter PM were cloned upstream of the CAT gene (plasmid pMCAT) or upstream of the rabbit β-globin gene (plasmid pM-β-globin). The different mRNAs have been isolated as cDNAs visualized by thick arrows over or under the ORFs. Thin lines in the cDNAs are intron sequences.

is their natural location in their homologous promoters. This is extremely well illustrated by the fact that the TRE-AP1 responds both to TPA and EB1 when placed at position −109 upstream of the β-globin CAP site, but responds only to TPA when placed at position −425. These results suggest that unlike the factors activating the AP1-TRE, EB1 is more likely to be a promoter factor than an enhancer factor.

Results

Identification of two EBV early promoters responding to EB1 and to TPA

We have previously shown that the expression of EB1 in latently infected Raji B cells activated as many early promoters as did treatment of these cells with TPA (Chevallier-Greco *et al.*, 1986). We therefore posed the question of how many EBV early promoters contain EB1 and/or TPA responsive elements, and then whether these elements are distinct or superimposed. To begin to answer this question, we first examined two important EBV early promoters (Figure 1). One promoter, PM, controls the expression of the BMLF1 open reading frame (ORF) (Figure 1). This ORF codes for an early protein, EB2, previously described as a transcriptional activator because in HeLa and Vero cells it increased the expression of the bacterial chloramphenicol acetyltransferase gene (CAT) linked to several heterologous promoters (Lieberman *et al.*, 1986; Wong and Levine, 1986). However, EB2 does not detectably activate any EVB early promoter when expressed in latently infected Raji B cells (Chevallier-Greco *et al.*, 1987). Moreover, EB2 is more likely to act at a post-transcriptional level (Kenney *et al.*, 1988). Another promoter, PZ, controls the expression of EB1, the EBV activator of the lytic cycle (Figure 1) (Chevallier-Greco *et al.*, 1986). These promoters were cloned upstream of the CAT reporter gene to generate plasmids pM-CAT and p545 (Figure 1). Since EBV replicates efficiently in epithelial cells (Lemon *et al.*, 1977; Sixbey *et al.*, 1983), we then determined whether these promoters were activated by EB1 and/or TPA in HeLa cells. Figure 2 shows that low CAT enzyme activity was present in HeLa cells transfected with plasmids p545 (lane 1) and pM-CAT (lane 5). However, co-transfection of p545 (lane 4) and pM-CAT (lane 8) with an EB1 expression vector (pSVZ1, see Materials and methods) led respectively to a 7- and 13-fold increase in the CAT enzyme activity. The p545 (lanes 2 and 3) and pM-CAT (lanes 6 and 7) constructions were also responsive to TPA treatment, and the TPA inducibility was seen after 6 h (lanes 2 and 6) or 24 h (lanes 3 and 7) of treatment.

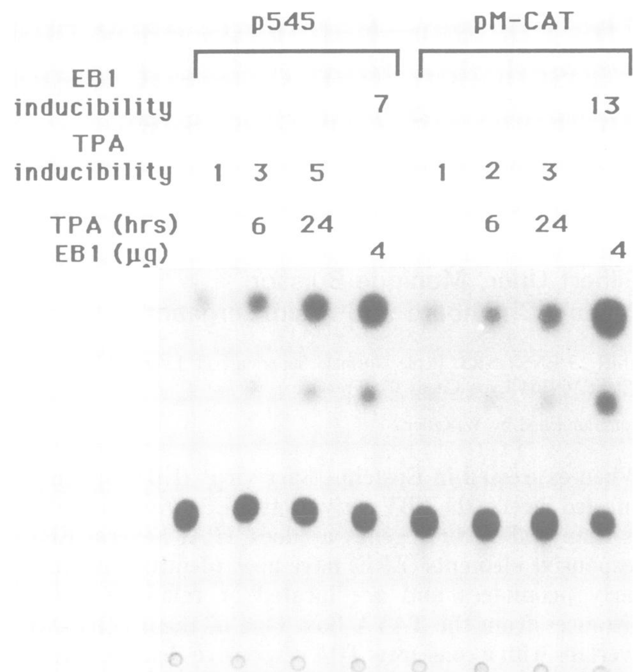

Fig. 2. Promoters Z and M respond to EB1 and TPA. Plasmids Z545 (**lanes 1–4**) and pMCAT (**lanes 5–8**) were transfected in HeLa cells (**lanes 1** and **5**), in HeLa cells treated 12 h (**lanes 2** and **6**) or 24 h (**lanes 3** and **7**) with TPA, and in HeLa cells co-transfected with the EB1 expressing vector pSVZ1 (**lanes 4** and **8**). The inducibility is expressed as the ratio of CAT enzyme activity in TPA treated or EB1 co-transfected HeLa cells to that in untreated cells.

EB1 and TPA responsive elements are overlapping in the M promoter

Having shown that the CAT constructs containing the M and Z promoters were responsive to EB1 and TPA, we then wanted to know where the responsive elements for these activators were located and whether these elements were superimposed.

We therefore constructed a series of mutants with deletions extending in the 5′ to 3′ direction from the 5′ side of the two promoters. The M mutant promoters were fused to the rabbit β-globin gene (Figure 3A) and the Z mutant promoters were fused to the CAT gene (Figure 4A). The different constructs were transfected in HeLa cells to analyse their activity in the presence or absence of TPA, and their inducibility by EB1.

RNAs extracted from HeLa cells transfected with the M mutant promoters were analysed by S1 nuclease protection assay. The RNAs were hybridized with a 60 nucleotide (nt) 5′-^{32}P-labelled single-stranded β-globin DNA probe (Figure 3A). Two correctly initiated β-globin mRNAs were detected and protected fragments 43 and 49 nt in length. The results show that promoter M has a weak endogenous activity (Figure 3B, mutant M13) and that this basal activity was unaffected by deletion of M promoter sequences until nucleotide −216 before the major initiation site (Figure 3B, mutants M14, M10 and M19). The basal activity of all the mutants was increased by EB1 and, again, the EB1 induction was not affected by deletions extending until −216 (Figure 3B). In the region between −216 and the TATA box, there is an AP-1/c-*jun* binding site (TGAGTCA, Figure 3A) which has been shown to mediate the transcriptional response to TPA (Angel *et al.*, 1988a; Chiu *et al.*, 1988). Since it has also been shown that a fusion protein (protein A-EB1) can bind and protect against DNases a region containing the AP-1/c-*jun* binding site (Farrel *et al.*, 1989), this site could therefore be the target that mediates the EB1 transcriptional induction of the M promoter. In order to examine this possibility, we deleted the sequence TGACTCA in the M promoter and generated mutant M19Δ AP-1. This mutant was transfected in HeLa cells and its response to EB1 and TPA was compared to its M19 counterpart which still has an AP-1 binding site. As shown in Figure 3C, deletion of the sequence TGACTCA in mutant pM19Δ AP-1 abolished its basal activity as compared to mutant M19. Mutant M19 was responding to EB1, but EB1 did not induce the transcrip-

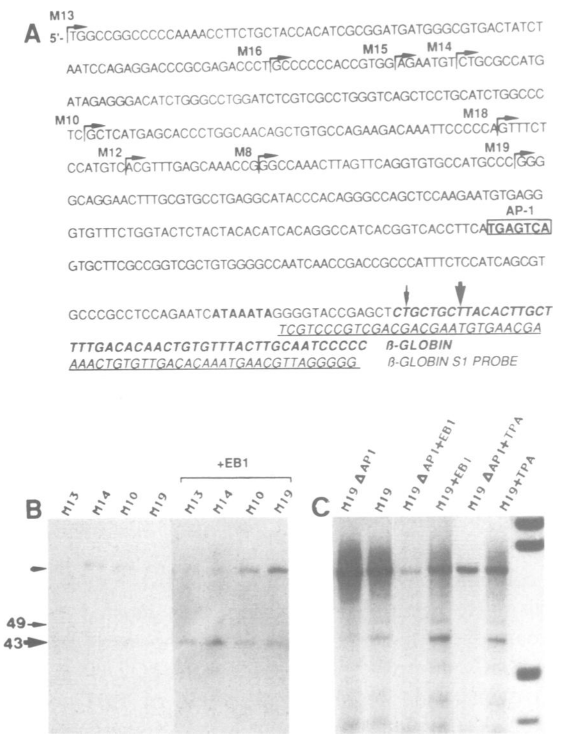

Fig. 3. In the PM promoter the TPA responsive element AP-1 is inducible by EB1. (**A**) Partial nucleotide sequence of the pM promoter β-globin chimeric gene. The end point of the deletions in the promoter are indicated by horizontal arrows and an M plus a number indicates its position. The TATA box and the AP-1 binding site are indicated. The major start site of transcription was indicated by a thick vertical arrow. The β-globin sequences cloned downstream of the PM TATA box are printed in bold letters. The β-globin probe used for S1 nuclease mapping is underlined. (**B**) HeLa cells were transfected with the different mutants as indicated on the top panel. TPA induction was for 4 h. 48 h after transfection, total cellular RNA was isolated and the amount of β-globin specific transcripts was measured by S1 nuclease protection analysis. Two correctly initiated β-globin transcripts protected 43 and 49 nt of the S1 probe.

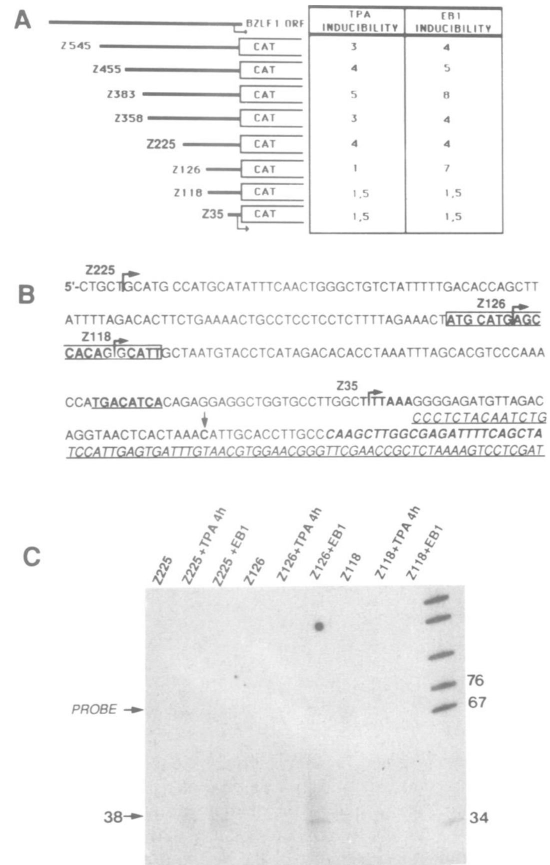

Fig. 4. EB1 and TPA responsive elements are separated on the PZ promoter. (**A**) Deletion mutants in the PZ promoter fused to the CAT gene were transfected in HeLa cells. To analyse inducibility by EB1, 5 μg of plasmids were co-transfected with 2 μg of EB1 expressing vector pSVZ1. To analyse inducibility by TPA, cells were transfected with 5 μg of plasmid and 6 h before the end of transfection TPA was added (20 ng/ml). The induction by TPA refers to the ratio of CAT enzyme activity in TPA-treated cells to that of untreated cells. EB1 inducibility refers to the ratio of CAT enzyme activity in HeLa cells co-transfected with deletion mutants and EB1 expressing vector to that of cells transfected solely with deletion mutants. (**B**) Nucleotide sequence of the shorter deletion mutants. The EB1 responsive element (ZREZ), the putative AP-1 binding site and the TATA box are indicated. The major start of transcription is indicated by a vertical arrow. The CAT gene sequence cloned downstream the PZ promoter is printed in bold letters. The S1 CAT-Z hybrid probe is underlined. (**C**) HeLa cells were transfected with the different mutants as indicated on the top panel. The amount of specific CAT transcripts was measured in total cellular RNAs as described in the legend to Figure 3.

tion of detectable amounts of β-globin transcripts from mutant M19Δ AP-1. Similarly, mutant M19 responded to TPA but not mutant M19Δ AP-1. The EB1 and TPA inductions of mutant M19 were of a similar magnitude but difficult to quantitate due to the weak activity of the M promoter. This was probably also due to the fact that only one copy of the sequence TGACTCA is present in the M promoter. However, the results suggested that TPA and EB1 inducibility could be superimposed and mediated by the AP-1/c-*jun* binding site. Moreover, deletion of the sequence TGACTCA decreased the low basal amount of specific β-globin transcripts expressed from the shorter mutant M19Δ AP-1, indicating that c-*jun*/c-*fos* related factors could contribute to the basal activity of the M promoter.

EB1 and TPA responsive elements are distinct in the Z promoter

As quantified by CAT assays (Figure 4A), the Z promoter in mutant Z545 had a weak endogenous activity and this activity could be induced both by TPA and EB1. Progressive deletions in the Z promoter (Figure 4A) abolished TPA and serum inducibility when sequences between −225 (mutant Z225) and −126 (mutant Z126) were deleted (Figure 4B). Mutant Z126 expression was still induced by EB1, but EB1 inducibility was impaired by the deletion of sequences located between positions −126 and −118 (mutant Z118). The results obtained by CAT assays were confirmed by S1 nuclease protection analysis of RNAs extracted from HeLa cells transfected with mutants Z225, Z126 and Z118 (Figure 4C). The RNAs were hybridized with a 70 nt 5′-^{32}P-labelled single-stranded DNA probe spanning the region of fusion between the PZ promoter and the CAT gene (Figure 4B). Correctly initiated CAT RNAs protected a fragment of 38 nt. The results confirmed the CAT assay results (Figure 4C) and suggested that TPA and EB1 induction of the Z promoter is mediated by distinct elements. We localized the EB1 responsive element in a 15 bp long DNA sequence that we have called ZRE-Z (Figure 4B).

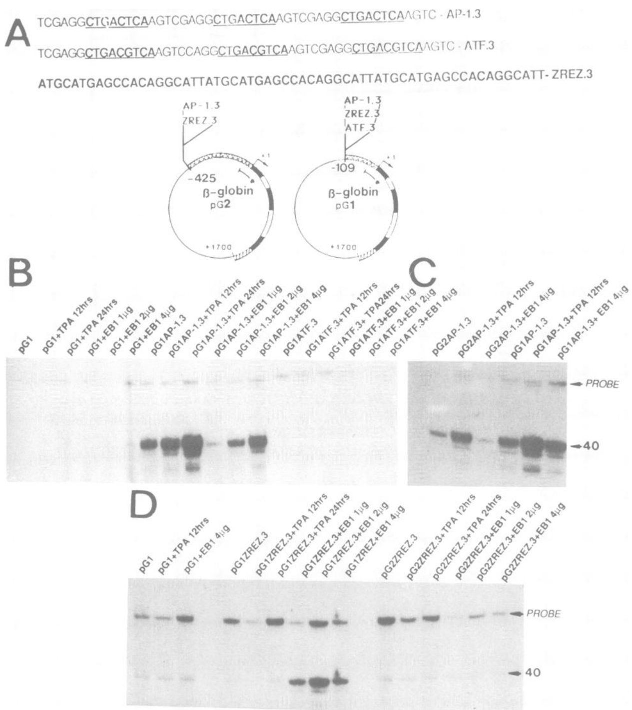

Fig. 5. The TRE AP-1 and the ZREZ confer EB1 inducibility to the β-globin promoter only when located as promoter elements. (**A**) The AP-1 binding site (AP-1.3), the ATF binding site (ATF.3) and the ZREZ (ZREZ.3) were cloned as trimers either 425 bp (plasmid pG2) or 109 bp (plasmid pG1) upstream of the β-globin promoter. (**B–D**) HeLa cells were transfected with the different constructions as indicated on the top panel. The amount of specific β-globin transcripts in total cellular RNAs was measured by S1 nuclear protection as described in the legend to Figure 3.

EB1 is not an enhancer factor

EB1 can activate transcription from at least two target sequences called ZREs (BZLF1 product *R*esponsive *E*lements), including an AP-1/c-*jun* binding site. The AP-1 binding site is a *cis*-acting element with enhancer properties (Angel *et al.*, 1987; Lee *et al.*, 1987b). We therefore wanted to know whether EB1 was an enhancer factor. Three copies of synthetic double-stranded oligonucleotides containing the ZRE-M (AP-1/c-*jun*) were cloned either 425 or 109 bp upstream of the β-globin promoter (Figure 5A). The different constructs were transfected in HeLa cells, and their responsiveness to either TPA or EB1 was determined by quantitative S1 analysis. As shown in Figure 5B, the β-globin promoter has a low basal activity (pG1), and this activity was not modified after treatment by TPA for 12 h (pG1+TPA 12 h) or 24 h (pG1+TPA 24 h), or after co-transfection with increasing amounts of EB1 (pG1+EB1 1 μg, 2 μg and 4 μg). Addition of three AP-1 binding sites 109 bp upstream of the β-globin CAP site enhanced the basal activity of the promoter (pG1AP-1.3), and this activity was further increased by 12 and 24 h of TPA treatment (pG1AP-1.3+TPA 12 h and 24 h) and by co-transfection of increasing amounts of EB1 (pG1AP-1.3+EB1 1 μg, 2 μg and 4 μg). As a control, we also placed three ATF binding sites (TGACGTCA), the cAMP-responsive element (CRE) (Montmigny *et al.*, 1986), 109 bp upstream of the β-globin CAP site (Figure 5A). The ATF binding site has an additional G in the AP-1 consensus sequence. As shown in Figure 5B, three ATF binding sites did not enhance the β-globin promoter basal activity (compare pG1 and pG1ATF.3), and these ATF binding sites did not respond to TPA (pG1ATF.3+TPA 12 h and 24 h) or to increasing amounts of EB1 (pG1ATF.3+EB1 1 μg, 2 μg and 4 μg), although c-*jun* has been shown to bind to the motif TGACGTCA *in vitro* (Nakabeppu *et al.*, 1988).

Addition of the three AP-1 binding sites 425 bp upstream of the β-globin CAP site also resulted in enhancement of the basal activity of the β-globin promoter (Figure 5C, pG2AP-1.3), although this was reduced as compared to that observed when the AP-1 binding sites were placed 109 bp upstream of the β-globin CAP site (Figure 5C, compare pG2AP-1.3 and pG1AP-1.3). Again, the basal activity induced by three AP-1 binding sites located −425 or −109 bp upstream of the β-globin CAP site could be increased by TPA (Figure 5C, compare pG2AP-1.3 with pG2AP-1.3+TPA 12 h, and pG1AP-1.3 with pG1AP-1.3+TPA 12 h). However, although three AP-1 binding sites located at −109 upstream of the β-globin CAP site responded to EB1 (Figure 5C, compare pG1AP-1.3 with pG1AP-1.3+EB1 4 μg), surprisingly, three AP-1 binding sites located −425 bp upstream of the β-globin CAP site did not respond to EB1 (Figure 5C, compare pG2AP-1.3 with pG2AP-1.3+EB1 4 μg) even when increasing amounts of EB1 were co-transfected (not shown). These results were reproduced several times, with different DNA preparations.

EB1 is a promoter factor

It seemed that EB1 could activate transcription from the AP-1 binding site only when this enhancer was localized close to the TATA box. However, this could be specific to the interaction between EB1 and the AP-1 site. In order to know if this observation could be extended to other EB1 responsive elements, we also placed three copies of a double-stranded synthetic oligonucleotide containing the ZRE-Z (ATGAGC-CACAGGCATT) 425 or 109 bp upstream of the β-globin CAP site (Figure 5A). These constructs were transfected in HeLa cells and their activation by EB1 and TPA analysed by quantitative S1 mapping.

As shown in Figure 5D, three copies of the ZRE-Z placed 109 bp upstream of the β-globin CAP site did not influence the basal activity of the β-globin promoter (compare pG1 and pG1ZREZ.3). The three copies of ZRE-Z did not respond to TPA (pG1ZREZ.3+TPA 12 h and 24 h), but responded to increasing amounts of EB1 (pG1ZREZ+EB1 1 μg, 2 μg and 4 μg). Three copies of the ZRE-Z cloned 425 bp upstream of the β-globin CAP site did not influence the basal activity of the β-globin promoter (compare pG1 with pG2ZREZ.3), did not respond to TPA (pG2ZREZ.3 +TPA 12 h and 24 h) and also no longer responded to increasing amounts of EB1 (pG2ZREZ.3+EB1 1 μg, 2 μg and 4 μg). These results have also been reproduced with different DNA preparations.

We have thus demonstrated that EB1 can activate transcription from two apparently unrelated targets, and that EB1 can only act as a positive factor for promoter function, even when activating a *cis*-acting element which can function as an enhancer.

Discussion

The main conclusions of our experiments are (i) the EBV *trans*-acting factor EB1 activates transcription from two apparently unrelated *cis*-acting elements, one being defined as an AP-1/c-*jun*/c-*fos* binding site; and (ii) this activation is confined to these *cis*-acting elements when they are positioned like promoter sequences.

Is EB1 interacting directly with the AP-1 binding site?

EB1 has been shown to bind to and to protect from DNases an AP-1 binding site, either as a fusion protein expressed in a bacterial host or as an *in vitro* translation product (Farrell *et al.*, 1989). However, nothing is known of the interaction between EB1 and the AP-1 binding site in nuclear crude extracts or *in vivo*. What is known is that *in vivo* the AP-1 binding site located at position −109 on plasmid pG1 or at position −425 on plasmid pG2 (Figure 5A) confers an endogenous activity to the β-globin promoter, probably through the binding of c-*jun*/c-*fos*, and what we have shown is that this endogenous activity is reduced in the presence of EB1 (see Figure 5B,C). Moreover, when EB1 activates from the AP-1 site located at position −109, the magnitude of activation is lower than that observed with TPA (see Figure 5B,C). These results suggest that EB1 interacts alone with the AP-1 binding site and that it is a weaker activator than c-*jun*/c-*fos* or related factors. Alternatively, EB1 might interact with the transcription factor(s) that bind to the AP-1 site, but this interaction will decrease the activation capacity of the protein complex formed. In this context, one must then consider the following question.

Are EB1, c-jun and c-fos functionally related?

EB1, c-*jun* and c-*fos* have been described to share some sequence homology (Farrell *et al.*, 1989). The c-*jun*/c-*fos* binding site AP-1 functions like an enhancer element (Angel *et al.*, 1987; Lee *et al.*, 1987b). However, although EB1 activates transcription from the AP-1 enhancer element placed at a relatively close proximity to the β-globin start site of transcription, this activation is not seen when the AP-1 enhancer is moved at relatively longer distances upstream. Moreover, TPA induction (through c-*jun*/c-*fos* or related factors) of the AP-1 enhancer is relatively insensitive to distance. These results strongly suggest that although EB1 can bind to an AP-1 site *in vitro*, EB1 does not seem to be an enhancer factor *in vivo*.

The c-*jun* protein can bind *in vitro* as a homodimer to its cognate enhancer sequence TGACTCA, but c-*jun*/c-*fos* heterodimers bind 25 times more efficiently (Halazonetis *et al.*, 1988), and also more efficiently activate *in vivo* transcription from the site TGACTCA (Chiu *et al.*, 1988; Sassone-Corsi *et al.*, 1988). Nothing is known of the possible interactions between EB1, c-*jun*, c-*fos* and the AP-1 site. Experiments are in progress in our laboratory to answer this question both by *in vitro* binding studies and by co-transfections in HeLa cells. Nevertheless, even if this interaction turns out to be possible *in vitro*, it is not followed *in vivo* by transcriptional activation when the AP-1 site is located far upstream from the CAP site. This suggests that EB1 and c-*jun*/c-*fos* activate transcription by different mechanisms.

Other EB1 responsive elements are also promoter elements

In contrast to the AP-1 binding site, the ZRE-Z is a *cis*-acting regulator which behaves like a promoter element, and does not alone confer a higher basal activity on the β-globin promoter. We have identified other EB1 responsive elements in two EBV early promoters (Chavrier *et al.*, 1989). They have no apparent sequence homology to AP-1 and ZRE-Z and are located at about −100 from the TATA box, and have no detectable endogenous activity. These results suggest that unlike the AP-1 binding site, these EB1 responsive elements are not recognized by cellular factors. It has been reported that some specific *trans*-acting transcription factors can activate gene expression by binding to their cognate sequences located close to the TATA box or far upstream, suggesting that enhancers and promoters may stimulate initiation of transcription by quite similar mechanisms (Wirth and Baltimore, 1988). However, although EB1 has been described as being able to bind *in vitro* to defined enhancer sequences (Farrell *et al.*, 1989), it is not a functional enhancer factor. Moreover, it seems that several EBV early promoters are similarly organized and in addition to an EB1 inducible promoter they also contain an enhancer which is activated by another EBV encoded *trans*-acting factor called R (Chevallier-Greco *et al.*, 1989; M.Buisson, unpublished results). Interestingly, EB1 activates many EBV early promoters when expressed alone in latently infected Raji B cells, including the R promoter (Chevallier-Greco *et al.*, 1986). However, the activator R activates as many EBV early promoters as EB1 in Raji cells but does not activate the expression of EB1 in these cells (A.Chevallier-Greco, personal communication). These results suggest that if R is confined to enhancers, then many EBV promoters must have such R responsive enhancer sequences.

Regulation of EBV early gene expression

EBV is the first mammalian DNA virus for which it has been shown that the virally encoded *trans*-acting transcription factors are sequence specific and probably function specific, and several EBV promoters have already been characterized and shown to contain both EB1 and R responsive elements (Chavrier *et al.*, 1989; Chevallier-Greco *et al.*, 1989; M. Buisson, personal communication).

The results are also of importance for TPA induction of the EBV early promoters in latently infected B cells. This induction could be mediated by the activation of the Z and M promoters through their TPA responsive elements (Figure 1). The M promoter controls the expression of a *trans*-acting post-transcriptional activator (Kenney *et al.*, 1987) called EB2 (Chevallier-Greco *et al.*, 1986). It could be that EB1 is expressed at a very low level in infected B cells and is activated post-transcriptionally by TPA, either directly through phosphorylation by protein kinase C or indirectly by EB2. In any case, EB1 would positively autoregulate its own promoter, and then activate the expression of the R promoter. The R promoter controls the expression of bicistronic mRNAs encoding both EB1 (the promoter factor) and R (the enhancer factor) (Figure 1 and E.Manet *et al.*, submitted). Once made, EB1 and R will efficiently activate all the EBV early promoters.

Our results are also of importance with regard to understanding the mechanisms which lead to reactivation of the latent genome *in vivo*. Positive autoregulation of the Z promoter resembles the autoregulation of the c-*jun* promoter (Angel *et al.*, 1988b), and may reflect a common mechanism where an initial activating signal will raise an inefficient low level of EB1 expression to a high permanent level of expression. This activation could then be followed by efficient expression of EB2 and R. In addition, the sequence TGACATCA which mediates the activation of the c-*jun* promoter by Jun/AP-1 and TPA is also found 22 bp upstream of the Z promoter TATA box (Figure 4B). Experiments are in progress to determine the contribution of this sequence to Z promoter activity and to examine its response to TPA and EB1. In this respect, it must be emphasized that EBV immortalizes B cells *in vivo* and *in vitro*, and that EB1 can activate transcription from the AP-1/c-*jun*/c-*fos* binding site. Since virally transduced forms of Fos (v-*fos*, Curran *et al.*, 1982) and Jun (v-*jun*, Maki *et al.*, 1987) are oncogenic, it will be of interest to examine the possible role of EB1 in the oncogenic properties of EBV.

Materials and methods

Plasmid constructions

The construction of reporter plasmids carrying AP-1, ATF and ZRE-Z sequences was achieved by inserting gel-purified double-stranded oligonucleotides into the appropriate restriction sites of the polylinker located upstream of the β-globin promoter in plasmid pG-425 or pG-109 (Figure 5A). All clones were verified by DNA sequencing. The construction of the EB1 expression vector pSVZ1 has been described elsewhere (Chevallier-Greco *et al.*, 1986). Plasmid pMCAT was constructed as follows. First, a DNA fragment extending from position +3 downstream of the M promoter TATA box (Figure 3A) to the *Hin*dIII site located 2935 bp upstream of the TATA box was ligated into pUC18 cut by *Sma*I and *Hin*dIII, generating plasmid pUCM. Plasmid pMCAT was then prepared by ligating the CAT gene contained in the pSV2CAT *Hin*dIII–*Bam*HI DNA fragment, into plasmid pUCM cut by *Eco*RI. Plasmid Z545 was constructed as follows. First a DNA fragment extending from position +14 (*Nae*I site) to position −545 in the Z promoter (Figure 4B) was ligated in pUC18 cut by *Sma*I to generate plasmid pUCZ. Plasmid Z545 was then prepared by ligating the CAT gene contained in the pSV2CAT *Hin*dIII–*Bam*HI DNA fragment, into plasmid pUCZ cut by *Eco*RI.

Construction of 5′ deletion mutants

To generate mutants with deletions extending in a 5′ to 3′ direction from the 5′ side of the M and Z promoters, plasmid pM-CAT was opened at the *Hin*dIII site and plasmid p545 was opened at the *Sal*I site. Both plasmids were digested for various times with *Bal*31 exonuclease. The extent of digestion of the DNAs for each time point was determined by restriction enzyme analysis. The selected DNAs were treated with T4 DNA polymerase and redigested with *Bam*HI for the p545 derivatives and with *Sac*I for the pM-CAT derivatives. DNA fragments, containing different lengths of Z promoter sequences linked to the CAT gene, were isolated by polyacrylamide gel electrophoresis and ligated to pUC18 cut by *Sma*I and *Bam*HI. DNA fragments, containing different lengths of M promoter sequences were isolated as described above, and ligated to plasmid pGMo cut by *Sma*I and *Sac*I. Plasmid pGMo was generated by digestion of plasmid pG-425 (Figure 5A) with *Pvu*II and *Xho*I and religated, deleting the β-globin promoter. The end point of each deletion mutant was determined by sequencing.

The plasmid pM19Δ AP-1 was generated by ligation of gel-purified double-stranded oligonucleotides in plasmid pGMo cut by *Sac*I and *Sma*I. These oligonucleotides contain the M promoter sequences present in pM19 (Figure 3A) with the exception of the sequence TGACTCA. The clone has been verified by sequencing.

Cell lines, transfection procedure, TPA induction

HeLa cells were grown in Dulbecco's modified Eagle's medium (Gibco Diagnostics) supplemented with 5% (w/v) foetal calf serum. Four hours before transfection, the cells were seeded at a density of 10^6/100 mm Petri dish. The cells were transfected by the $CaPO_4$ method with 15 μg of DNA including 5 μg of reporter plasmid, and eventually 2 μg of *trans*-activator plasmid. Twenty hours after addition, the $CaPO_4$–DNA precipitate was removed and the cells were grown in 0.5% foetal calf serum for 24 h. When required, after 24 h in low serum, TPA was added directly (20 ng/ml), and left 4, 12 or 24 h on the cells before RNA analysis or CAT activity measurement. All transfections contained the same amount of SV40 early promoter sequences. Protein extracts and CAT assays were performed as described previously (Chevallier-Greco *et al.*, 1989).

RNA analysis

Cytoplasmic RNAs were extracted as follows. Cells were harvested and lysed by Nonidet P-40 as described elsewhere (Jalinot and Kedinger, 1986). Nuclei were pelleted, and RNAs were phenol extracted from the cytoplasmic fraction. Total cytoplasmic RNA (10–40 μg) were hybridized overnight at 30°C in 50% formamide, 0.3 M NaCl, 0.01 M Tris–HCl, pH 7.4, to 5′-^{32}P-labelled synthetic single-stranded DNA probes which are described in Figures 3 and 4. The hybrids were digested for 2 h at 20°C with 5 U of S1 nuclease per 10 μg of RNA. The size of the S1-protected DNA fragments was analysed on 8% (w/v) polyacrylamide–8.3 M urea gels.

Acknowledgements

We wish to thank I.Crenon for the constructions pG1AP-1.3 and pG1ATF.3, and R.Buckland for helpful discussion and for reading the manuscript. This work was financially supported by the Federation Nationale des Centres de Lutte contre le Cancer (FNCLCC), by the Institute National de la Santé et de la Recherche Médicale (INSERM, contract no. 871015), and by the Association pour la Recherche sur le Cancer. G.U. is a recipient of a fellowship from the Ligue Nationale Française Contre le Cancer.

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Received on February 2, 1989