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Epstein – Barr virus bicistronic mRNAs generated by facultative splicing code for two transcriptional *trans*-activators

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The Epstein – Barr virus (EBV) genome codes for several transcriptional *trans*-activators. One of them, the *BZLF1* open reading frame (ORF)-encoded product EB1, is able to induce the productive cycle in infected B cells. From the cloning and characterization of full-length cDNAs, we found that EB1 could be made from three overlapping messenger RNAs expressed under the control of two different promoters that we call P1 and P2. The first mRNA, 1 kb long, is made from the P1 promoter and codes for EB1 alone. The two other mRNAs, respectively 3 and 4 kb long and made by facultative splicing, are bicistronic mRNAs. They code not only for the *trans*-activator EB1 but also for a second EBV transcriptional *trans*-activator R, encoded by the *BRLF1* ORF. In effect, authentic EB1 and R proteins are expressed from the 3 and 4 kb long cDNAs as demonstrated by identification of the proteins with specific antisera. In addition, EB1 and R expressed from the 3 and 4 kb cDNAs activate transcription from their specific targets in the EBV early promoter DR.

Key words: bicistronic mRNAs/EBV/viral *trans*-activators

Introduction

The Epstein-Barr virus (EBV) is a human herpes virus that has the property of immortalizing peripheral B lymphocytes. Continuously growing EBV-infected B lymphoid cell lines can be established either by the infection of B lymphocytes with transforming EB virions or by cultivation of peripheral blood cells from EBV-seropositive humans. In such immortalized cells, the entire EBV genome is maintained as a plasmid at a constant copy number and its expression is reduced to the few genes that define latency: those encoding two small RNAs (EBERs), the products belonging to the Epstein – Barr nuclear antigen (EBNA) complex (six proteins so far characterized), the latent membrane protein (LMP), the leader protein (LP) (for a review see Miller, 1985), the *BHRF1*-encoded product (Bodescot and Perricaudet, 1986; Pfitzner *et al.*, 1987) and the terminal protein (TP), whose coding sequence is created by the joining of the ends of the linear virus (Laux *et al.*, 1988).

Since EBV is a lytic virus, the maintenance of latency is obviously a condition for persistence of immortalization. However, in particular cell lines, between 0.5 and 5% of the cells spontaneously produce virus. Moreover, various chemical agents, including the phorbol ester TPA (12-*O*-tetradecanoylphorbol-13-acetate), can cause the virus to switch from a latent to a lytic replicative cycle (zur Hausen *et al.*, 1976). The molecular mechanisms that lead to the onset of early gene expression in latently infected cells are poorly understood, but three *trans*-activators of gene expression encoded by the EBV genome have now been identified and shown to be involved in the activation of the lytic cycle (Countryman and Miller, 1985; Chevallier-Greco *et al.*, 1986; Lieberman *et al.*, 1986; Takada *et al.* 1986; Countryman *et al.*, 1987; Hardwick *et al.*, 1988). One of these which we call EB1, is encoded by the open reading frame (ORF) *BZLF1* and has been shown to induce the switch from latency to productive cycle (Figure 1A) (Countryman and Miller, 1985; Chevallier-Greco *et al.*, 1986; Countryman *et al.*, 1987). The other two *trans*-acting factors — R, encoded by the *BRLF1* ORF that is localized immediately upstream of the *BZLF1* ORF and EB2 encoded by the *BMLF1* ORF (Figure 1A) — have not, so far, been shown to induce a lytic cycle, when acting alone. It has therefore been postulated that latency could be due to the inactivity of the promoter controlling the expression of EB1. Once made, EB1 would induce the expression of the other EBV *trans*-acting factors which would then activate the expression of the early genes. It is therefore critical to characterize the structure of the EB1 coding mRNAs and to know more about the *cis*-acting elements controlling their expression. It has been shown that two mRNAs, 1 and 3 kb long, contained *BZLF1*-related sequences (Biggin *et al.*, 1987; Laux *et al.*, 1988). In order to define more exactly the structure of these mRNAs and to localize the promoter(s) that control their expression, we have constructed a cDNA library in the Okayama and Berg vector pcD (Okayama and Berg, 1983). We report here the cloning and characterization of full-length cDNAs representing four different mRNAs carrying the whole or part of the EB1 ORF. We show that EB1 can be made from different messenger RNAs, expressed under the control of two putative promoters. One promoter would control the expression of one class of mRNA containing only the EB1 ORF. The other promoter, located further upstream, would control the expression of three different mRNAs generated by facultative splicing. Two of these mRNAs are bicistronic: that they can express both EB1 and R has been demonstrated by identification of the proteins with specific antisera and by functional assays on their specific targets in the EBV early promoter DR.

Results

Construction and characterization of EB1-related cDNA clones

The two EBV *trans*-activators, EB1 and R, are encoded respectively by the ORFs *BZLF1* and *BRLF1*. These two ORFs are contained in ~3 kb of genomic DNA (Figure 1A).

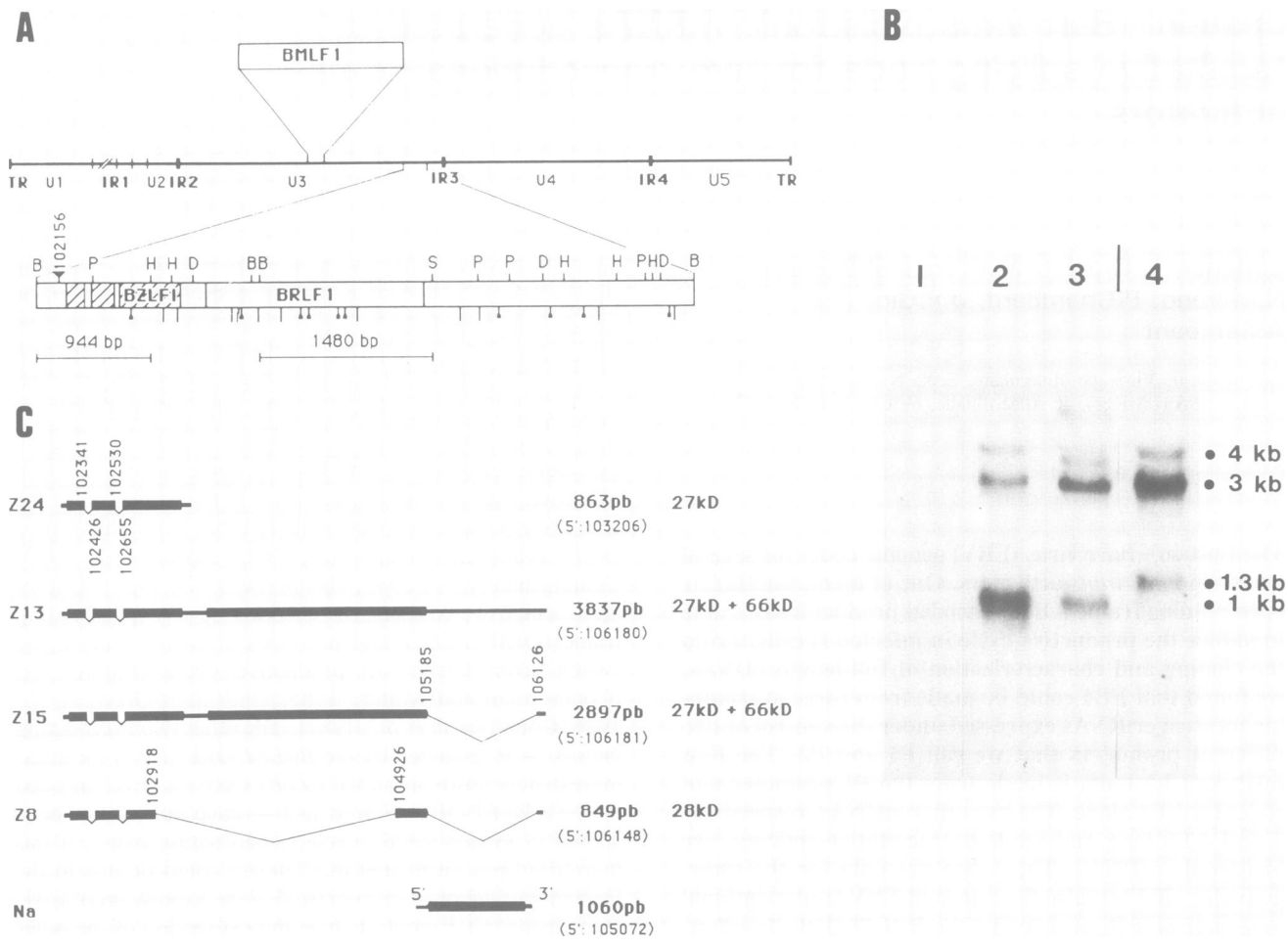

Fig. 1. Summary of the structure of cDNAs originating from the *Bam*HI Z/R region. (**A**) Schematic representation of the EBV genome region containing the *BZLF1* and *BRLF1* ORFs. The localization of this region is shown on the map of the viral genome as well as the location of the coding sequences for the EBV identified *trans*-activators. Positions of the major repeat elements (TR, IR1−IR4) and of the regions of unique sequence (U1−U5) are indicated on the viral genome map. Restriction endonuclease sites for *Bam*HI (B), *Pst*I (P), *Hin*dIII (H), *Dra*I (D), *Sal*I (S), *Ava*I (|) and *Ava*II (↓) are indicated as well as the 944 bp and 1480 bp fragments used for probing Northern blots. (**B**) Northern blots with poly(A)$^+$ RNA (4 μg per lane) from different cell lines. **Lane 1**, Raji cells, **lanes 2** and **4**, Raji cells treated by TPA/SB, **lane 3**, B95-8 cells treated by TPA/SB. The RNAs were separated on a 1% formaldehyde−agarose gel and transferred to nitrocellulose. The filters were probed with the multiprime ^{32}P-labelled 944 bp fragment (lanes 1, 2, 3) or the 1480 bp fragment (lane 4) indicated in A. The estimated sizes of the detected mRNAs are indicated. (**C**) Schematic structure of full length cDNAs corresponding to the different mRNAs detected in Northern blots. The cDNAs are named on the left part of the figure. Thin horizontal lines represent the exons and the heavier region the major ORF within these exons. The ends of the exons are indicated vertically following the Baer *et al.* numbering (Baer *et al.*, 1984). On the right of the figure, the sizes of the cDNAs and the position of their 5′ end (between brackets) as well as the theoretical size of the putative encoded proteins are notified.

We therefore examined the mRNAs expressed in this region. Poly(A)$^+$ RNAs were isolated from the EBV latently infected Raji cells and from Raji and B95-8 cells treated with the phorbol ester TPA to induce the viral cycle (zur Hausen *et al.*, 1978). These mRNAs were separated on a denaturing agarose gel and transferred to nitrocellulose. The Northern blots were then hybridized with ^{32}P-labelled DNA probes from the ORFs *BZLF1* (p944) and *BRLF1* (p1480) (Figure 1A). No RNAs could be detected in latently infected Raji cells (Figure 1B, lane 1). In Raji cells where latency was disrupted by TPA (Figure 1B, lane 2), the 944-bp *BZLF1* probe detected three mRNA species respectively 1, 3 and 4 kb in length. Since late gene expression does not occur in Raji cells, we considered that these mRNAs were early RNAs containing sequences related to the *BZLF1* ORF. These mRNAs are not unique to TPA-treated Raji cells, since we observed the same transcription pattern in other EBV-infected B cells such as B95-8 where the lytic cycle was induced by TPA (Figure 1B, lane 3). A probe from *BRLF1* detected the 4 and 3 kb RNAs but failed to detect the 1 kb species. Instead a new 1.3 kb mRNA was detected (Figure 1B, lane 4). Additional probes scanning 3 kb upstream of the *BRLF1* ORF also failed to detect the 1 kb mRNA species (not shown).

In order to establish the structure of these messenger RNAs, we have cloned their cDNAs. For that purpose, an oligo(dT)-primed cDNA library was prepared in the Okayama and Berg vector pcD (Okayama and Berg, 1983). The cDNAs were synthesized from poly(A)$^+$ RNAs isolated from Raji cells where the EBV early gene expression was induced by TPA. The library was first screened with the 944 bp *BZLF1* probe (see Figure 1A). Thirty positive recombinants (pcDZ1−30) were analysed using restriction enzymes. Three cDNA clones (pcDZ24, pcDZ15 and pcDZ13) were selected for further studies as their sizes were close to that expected for full-length copies of the 1, 3 and

4 kb mRNAs detected in Northern blots. All cDNAs had a 3′ poly(dA.dT) tract varying in size from 25 to ~100 bp, located downstream of a consensus polyadenylation signal, and representing the poly(A) tract of the corresponding mRNAs. We will therefore give the size of the cDNAs without taking into account the size of the poly(dA.dT) tract. The schematic structure of these three cDNAs is shown in Figure 1C.

The cDNA clone in pcDZ24 is 863 bp long. It could thus correspond to the 1 kb mRNA detected in the Northern blot. Comparison of the cDNA sequence to the EBV genome sequence of the B95-8 virus strain revealed that the corresponding mRNA consisted of three exons. The 5′ end of this cDNA was found at position 103 206 on the B95-8 sequence following Baer *et al.* numbering (Baer *et al.*, 1984), which is 51 bp before the AUG of the *BZLF1* ORF. The 3′ limit of the first exon is at position 102 655. The 5′ and 3′ limits of the second exon are at positions 102 530 and 102 426 and the 5′ limit of the last exon is at position 102 341. Inside the exons, no difference was found between the sequence of this cDNA and the B95-8 genomic sequence. The splice junctions found are the same as those previously described in an incomplete cDNA (B95-8 strain) (Biggin *et al.*, 1987) and correspond to consensus splice sequences. A major consequence of these two splicing events is to change the end of the previously described *BZLF1* ORF (Baer *et al.*, 1984) by removing the in-frame termination codon and extending the ORF on the 3′ side. The expected protein would have a theoretical mol. wt of 27 kd. The polyadenylation site was found at position 102 136.

The two other cDNA clones, pcDZ15 (insert 2897 bp long) and pcDZ13 (insert 3837 bp long), are full-length copies of the two high mol. wt RNAs of 3 and 4 kb. Their structures have been deduced both by sequencing of the 3′ and 5′ ends and at the splice junctions and by endonuclease restriction analysis (for details see Materials and methods). Both cDNAs are colinear with the B95-8 genomic sequence from position 102 656 to position 105 185, 2 bp before the beginning of the *BRLF1* ORF. The longest cDNA Z13 is colinear with the genomic sequence until its 5′ end. The cDNA Z15 has intron sequences removed from position 105 186 to 106 125. The two cDNAs differ by 1 bp at the 5′ end: Z13 stopping at position 106 180 and Z15 at position 106 181. As previously reported (Baer *et al.*, 1984), a TATA-box-like sequence (GATAAAA) is found 26 bp upstream of the start of the cDNAs. Both cDNAs contain in addition to the *BZLF1* coding sequence, the *BRLF1* ORF, which could produce a protein with a theoretical mol. wt of 66 kd. It should also be noted that by sequencing the 3′ end of several other cDNAs, we observed some variation in the polyadenylation site of the cDNAs (positions 102 126 and 102 136 alternatively found), suggesting some flexibility in the 3′ processing of the RNAs. This flexibility may be particular to the Raji strain because of a deletion (99 126–102 118) that may remove 3′ sequences important for the precise location of the termination site (Birnstiel *et al.*, 1985).

A fourth related cDNA, Z8 (size 849 bp) was isolated from a λgt10 cDNA library that we constructed using the same mRNAs as for the Okayama–Berg library. This cDNA has also a 3′ poly(dA.dT) tract and the poly(A) site is located at position 102 136. Its sequence showed an unexpected structure for the corresponding mRNA (Figure 1C). This

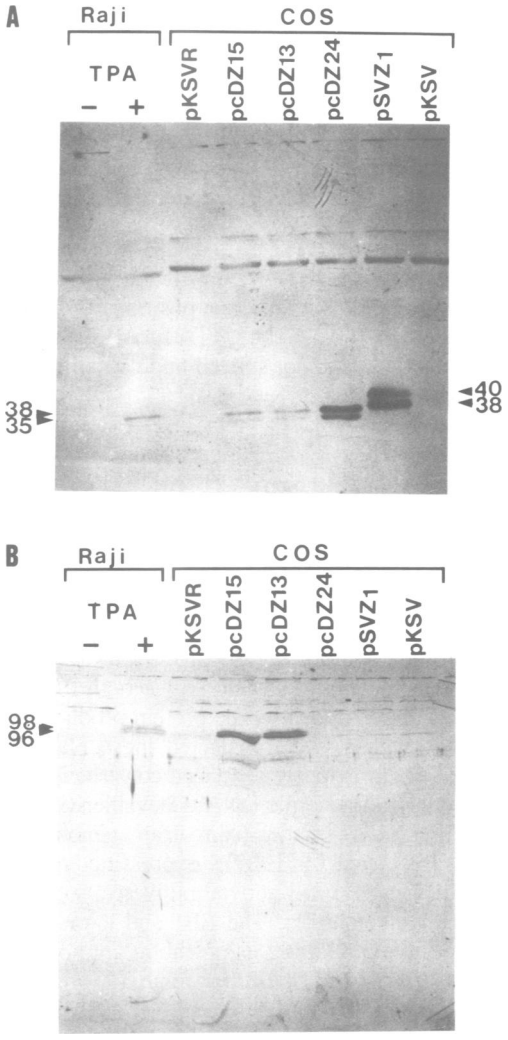

Fig. 2. Detection of the EB1 and R proteins with rabbit antisera raised to fusion proteins containing the carboxy end of each protein. The different constructions were transfected into COS-7 cells. Immunoblots of transfected COS-7 cell extracts and TPA treated Raji cell extracts were reacted with a 1:50 dilution of the rabbit anti-BZLF1 (**panel A**) or anti-BRLF1 (**panel B**) sera.

cDNA has the same structure as the Z15 cDNA except for the removal of a fourth intron (positions 104 925–102 919). The main consequence of this additional splice is to create a new ORF that is a fusion between the beginning of *BRLF1* and the end of *BZLF1*. The 5′ end of the cDNA is found at position 106 148. When compared with the sequence of B95-8, we found that there is a difference of a single base at position 104 917 (substitution of a G in Raji for an A in B95-8). This substitution is silent as far as the amino acid sequence is concerned. The protein that could be translated from the new ORF described above would have a theoretical mol. wt of 28 kd and would share its C-terminal domain with the EB1 protein and its N-terminal domain with the R protein. We have not been able to detect with certainty an mRNA of a corresponding size (~1 kb) with probes located in the right part of *BRLF1* (data not shown) and with the 944 bp probe. This is due to the weak representation of this mRNA and the proximity in size of the relatively more abundant EB1-encoding 1 kb mRNA. This newly described mRNA is likely to be expressed under the control of the same

promoter as the mRNAs represented by the cDNAs Z15 and Z13.

The complete transcriptional map of the region was completed by isolating a cDNA corresponding to the 1.3 kb mRNA detected only with the p1480 probe. This cDNA (Figure 1C), called Na, is 1060 bp long, has a poly(dA.dT) tract and the corresponding mRNA is encoded by the strand opposite to that which codes for the mRNAs corresponding to the cDNAs Z24, Z15, Z13 and Z8. Its poly(A) site is found at position 106 128, 16 bp after a polyadenylation consensus signal and its 5′ end is at position 105 072, 60 bp downstream of a TATA box (TATAAAT) at position 105 016. This cDNA is not spliced and contains the whole *BRRF1* ORF.

In conclusion, we have shown by cDNA cloning that EB1 can be expressed either alone from a 1 kb long monocistronic mRNA, or together with the *BRLF1*-encoded *trans*-activator R from potentially bicistronic mRNAs 3 and 4 kb long.

Authentic R and EB1 proteins are expressed from cDNAs Z13 and Z15 transfected in COS-7 cells

The pcDZ24 cDNA clone contains the coding sequence for the EB1 protein, while the pcDZ13 and pcDZ15 cDNA clones contain the coding sequences for both the EB1 and R *trans*-activators. To identify the protein products encoded by the three cDNAs, we transfected these plasmids into COS-7 cells. The previously described constructions pSVZ1 (made from p3HR-1 genomic DNA; Chevallier-Greco *et al.*, 1986) and pKSVR (made from Raji genomic DNA; Chevallier-Greco *et al.*, 1989), expressing respectively functional EB1 and R *trans*-activators, were also transfected in COS-7 cells as controls. The EB1 and R proteins were revealed by immunoblotting using rabbit antibodies raised against the carboxy end of *BZLF1* for EB1, or *BRLF1* for R (Seibl *et al.*, 1986).

As described previously (Marshall *et al.*, 1989), at least two EB1 polypeptides of apparent mol. wt 35 and 38 kd are made in Raji cells treated with TPA. In P3HR-1 cells, larger polypeptides of 38 and 40 kd are expressed, probably due to several amino acid changes (Jenson and Miller, 1988). Accordingly, as shown in Figure 2A, in COS-7 cells transfected with pSVZ1, pcDZ24, pcDZ15 and pcDZ13 clones, two EB1 polypeptides were detected. Those expressed from the Raji pcD cDNAs were identical in size to the proteins made in Raji cells treated with TPA. However, as expected, EB1 proteins expressed from the P3HR-1 genomic DNA fragment cloned in pSVZ1 migrate with apparent mol. wts of 38 and 40 kd.

For the *BRLF1*-encoded *trans*-acting factor R, two polypeptides of 94 and 98 kd are detected in Raji cells treated with TPA. Figure 2B shows that two polypeptides of 94 and 98 kd were detected in COS-7 cells transfected with the pKSVR construction and with the pcDZ13 and pcDZ15 cDNA clones. The size of the proteins detected conforms with the size of the protein made in Raji cells treated with TPA.

This set of experiments shows that polypeptides of the correct size are expressed from the cloned cDNAs and demonstrates that the two bicistronic cDNAs express both EB1 and R proteins. We then investigated whether the proteins detected by immunoblotting were functional. For this purpose we used the DR promoter (Laux *et al.*, 1985) as a reporter gene to test the *trans*-activation properties of

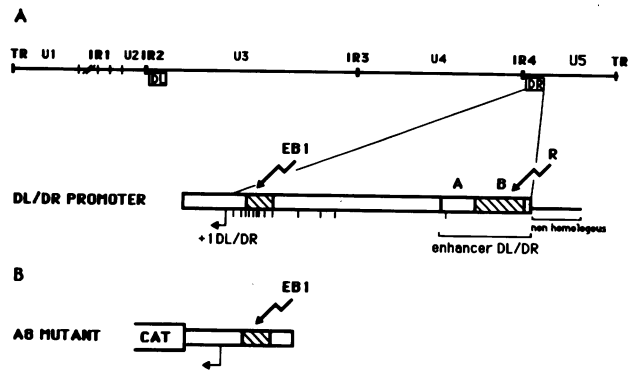

Fig. 3. Organization of the DR/DL promoter. (**A**) Organization of the IR2-DL and IR4-DR partially homologous regions and localization on the viral genome: a jagged arrow indicates the start of transcription. The dashed regions correspond to the *trans*-activator's responsive elements. The respective targets for EB1 and R are indicated by arrows. (**B**) representation of the A8 enhancerless mutant of the DR promoter cloned upstream of the CAT gene.

EB1 and R expressed from pcDZ15 and pcDZ13. The DR promoter (Figure 3A) is an EBV early promoter that contains an upstream responsive element specifically activated by EB1 (Chavrier *et al.*, 1989) and an enhancer containing an element specifically activated by the R protein (Chevallier-Greco *et al.*, 1989). The *trans*-activation by EB1 and R has been shown at the transcriptional level by S1-mapping experiments. The EB1- and R-specific targets are active in B cells and in HeLa cells and were used to test the activity of EB1 and R expressed from pcDZ15 and pcDZ13.

Plasmids pcDZ15 and pcDZ13 express a functional R protein that trans-activates the B domain of the DR enhancer

We have shown previously that the *trans*-activator R specifically activates the B domain of the DR enhancer (Figure 3A) cloned in inverted orientation and downstream of the CAT gene expressed under the control of the HSV-1 tk promoter in the plasmid pBLCAT2 (plasmid pBLCAT2 B3′) (Chevallier-Greco *et al.*, 1989). We therefore investigated whether a product made from plasmids pcDZ13 and pcDZ15 would activate the B domain of the DR enhancer. As shown in Figure 4, the HSV-tk promoter in plasmid pBLCAT2 had a constitutive activity in HeLa (lane 1), and the B domain had a 2-fold enhancing effect on the tk promoter activity when cloned 3′ to the CAT gene in pBLCAT2 (plasmid pBLCAT2 B3′) (lane 2). EB1, expressed from pcDZ24, had no effect on the activity of the tk promoter in plasmids pBLCAT2 (lane 3) and pBLCAT2 B3′ (lane 4). Cotransfection of plasmid pBLCAT2 with plasmid pcDZ13 (lane 5) or plasmid pcDZ15 (lane 7) had no detectable effect on the tk promoter activity as compared to the tk promoter activity in plasmid pBLCAT2 alone (lane 1). However, cotransfections of plasmid pBLCAT2 B3′ with plasmid pcDZ13 (lane 6) or with plasmid pcDZ15 (lane 8) resulted in a strong stimulation of CAT expression. When plasmid pKSVR expressing only R was cotransfected with pBLCAT2, a non-significant increase in CAT activity was observed (lane 9), but R expressed from pKSVR strongly increased the promoter activity in plasmid pBLCAT2-B3′. These results clearly show that a functional *trans*-activator R is expressed from plasmids pcDZ13 and pcDZ15 and

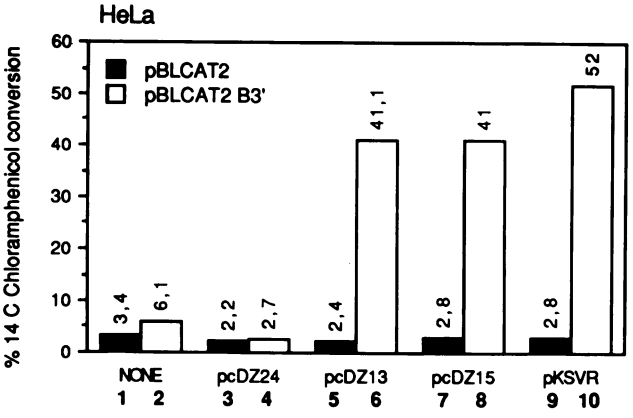

Fig. 4. Expression of functional R *trans*-activator from pcDZ15 and pcDZ13 cDNA clones. CAT expression from plasmid pBLCAT2 or plasmid pBLCAT-B3′ cotransfected in HeLa cells with the pcDZ24 (**lanes 3** and **4**), pcDZ13 (**lanes 5** and **6**) and pcDZ15 (**lanes 7** and **8**) cDNA clones or the pKSVR construction (**lanes 9** and **10**). 4 μg of pBLCAT2 or pBLCAT-B3′ were used for 2 μg of each *trans*-activator. The CAT activity is expressed as percentage of chloramphenicol conversion. CAT assays were performed in the following conditions: half the extracts were incubated in a 1 h time reaction.

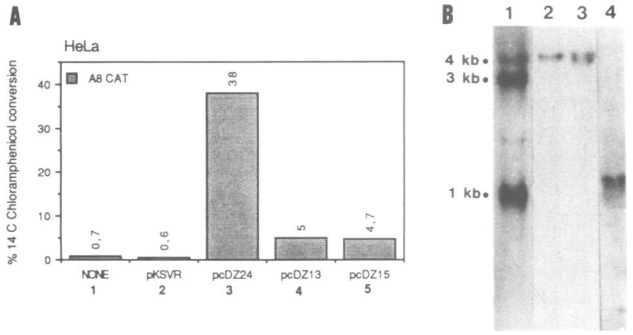

Fig. 5. Expression of functional EB1 from pcDZ24, pcDZ13 and pcDZ15 cDNA clones. (**A**) CAT expression from plasmid A8CAT cotransfected in HeLa cells with the pcDZ24 (**lane 3**), pcDZ13 (**lane 4**) and pcDZ15 (**lane 5**) cDNA clones and the pKSVR construction (**lane 2**). 4 μg of A8CAT were used while equivalent molar amounts of the four cotransfecting plasmids on the basis of 1 μg of pcDZ24 were used. The CAT activity is expressed as percentage of chloramphenicol conversion. CAT assays were performed in the following conditions: half the extracts were incubated in a 2 h time reaction. (**B**) Northern blot with total RNA from Raji cells treated with TPA (**lane 1**) and HeLa cells transfected with pcDZ15 (**lane 2**), pcDZ13 (**lane 3**) or pcDZ24 (**lane 4**). The Northern blot was probed with the multiprime ^{32}P-labelled p944 probe.

indicate that the corresponding mRNAs could code for the same factor in EBV-infected cells.

Plasmids pcDZ15 and pcDZ13 also express EB1

We have shown above that R is expressed from the cDNAs cloned in plasmids pcDZ15 and pcDZ13, indicating that the corresponding mRNAs could code for the same factor in EBV-infected cells. These mRNAs also code for a functional EB1. We have previously shown that the EB1-responsive sequences are located between nucleotides −69 and −112 in the DR promoter (Chavrier *et al.*, 1989). We therefore used a DR promoter mutant, A8 (Figure 3B), containing these EB1-responsive sequences, cloned upstream of the CAT coding sequences to test for functional expression of EB1 from pcDZ15 and pcDZ13. The results are shown in Figure 5A. The A8 construction was weakly active when transfected alone in HeLa cells (lane 1). However, as expected, EB1 expressed from pcDZ24 strongly activated the DR minimal promoter sequences in the mutant A8 (lane 3), while R expressed from pKSVR did not (lane 2). When plasmids pcDZ15 (lane 4) or pcDZ13 (lane 5) were cotransfected with mutant A8, the CAT expression was increased, suggesting that EB1 was expressed from pcDZ15 and pcDZ13. Complementary experiments in which increasing amounts of the pKSVR, pcDZ13, pcDZ15 or pcDZ24 were cotransfected with the A8CAT construction confirmed that activation of the DR promoter sequences in plasmid A8CAT was specific for EB1. In effect, increasing amounts of transfected pKSVR did not change the basic level of expression of plasmid A8CAT, whereas increasing amounts of pcDZ13, pcDZ15 as well as pcDZ24 increased the amount of CAT activity detected (not shown). To reach the level of CAT expression obtained upon cotransfection of plasmid A8CAT with pcDZ24, twice the amount, molarwise, of pcDZ13 and pcDZ15 seemed to be needed, suggesting that EB1 was translated less efficiently from the pcDZ13 or pcDZ15 constructions than from pcDZ24 (not shown). This was already noticeable on the Western blot described in Figure 2, where less EB1 protein was expressed from pcDZ13 and pcDZ15 as compared to pcDZ24. These experiments, together with the immune detection of proteins, suggest that the long cDNAs express functional EB1 and R proteins.

Z13 and Z15 cDNAs, however, both contain a promoter region for the EB1 1 kb messenger RNA (Urier *et al.*, 1989). Plasmids pcDZ15 and pcDZ13 thus could express EB1 from a shorter RNA. In order to ensure that EB1 is made from a messenger RNA containing both *BRLF1* and *BZLF1*, HeLa cells were transfected with pcDZ24, pcDZ13 and pcDZ15 and the sizes of the mRNAs expressed from these plasmids were examined 48 h after transfection. Figure 5B shows that only one species of RNA is made from each construction. If the mRNAs transcribed are colinear with their coding cDNAs and if the putative promoter for the 1 kb RNA is not functioning, the sizes of the expected mRNAs expressed from pcDZ24, pcDZ13 and pcDZ15 should be respectively 1.2, 4.2 and 3.3 kb. These expected sizes are those observed in HeLa cells except for pcDZ13 (lane 2), which is found to give an RNA of the same size as the one transcribed from pcDZ15 (lane 1), suggesting that the 5′ intron still present in the cDNA is removed from the RNA expressed from pcDZ13. Moreover, no small size RNAs were detectably expressed from pcDZ13 and pcDZ15, suggesting that the potential promoter for EB1 was not active in these conditions and that the additional intron described for the Z8 cDNA was not removed.

We therefore conclude that the *trans*-activator EB1 can be translated from the longer mRNAs expressed from pcDZ13 and pcDZ15, together with another *trans*-acting factor called R, encoded by the *BRLF1* ORF.

Discussion

From a cDNA library constructed from poly(A)$^+$ mRNA extracted from TPA-treated Raji cells, we have isolated and characterized full-length cDNAs corresponding to five overlapping EBV mRNAs. These transcripts, which are

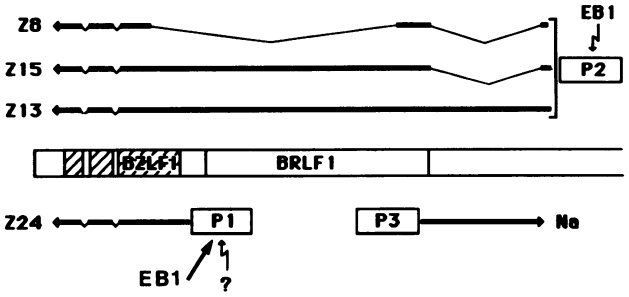

Fig. 6. Schematic representation of the different transcriptional units in the *BZLF1* and *BRLF1* encoding region. The respective positions of the *BZLF1* and *BRLF1* ORF are indicated on the linear genomic DNA. The mRNAs identified in this region are represented and named from their corresponding cDNAs. The open boxes localize the three different promoters identified in this region.

respectively 4, 3, 1.3 and 1 kb long, are located in a region previously shown to code for two EBV *trans*-activators, EB1 and R (Chevallier-Greco *et al.*, 1986; Countryman *et al.*, 1985, 1987; Hardwick *et al.*, 1988). The precise mapping of these mRNAs on the genomic DNA indicates that two leftward promoters (P1 and P2) and one rightward promoter (P3) control their expression (Figure 6). Promoter P1 would direct the transcription of a 1 kb mRNA corresponding to the Z24 cDNA, while promoter P2 would direct the expression of the 4, 3 and 1 kb long mRNAs corresponding to the Z13, Z15 and Z8 cDNAs. The rightward promoter, P3, which directs the synthesis of the 1.3 kb mRNA, could overlap with the leftward promoter P1.

The three mRNAs corresponding to cDNAs Z13, Z15 and Z8 differ from each other on the basis of whether intron sequences are removed or not (Figure 6). We believe that these mRNAs are mature messenger RNAs for the following reasons: (i) they are polyadenylated as confirmed by the presence in the corresponding cDNAs of long poly(dA.dT) tracts added near a polyadenylation signal previously mapped by sequence analysis (Baer *et al.*, 1984) and mutagenesis (Chevallier-Greco *et al.*, 1986); (ii) they all have at least two intron sequences excised at their 3′ end as shown by sequencing of the corresponding cDNAs; (iii) they are highly represented in the mRNA pool, which would be unlikely if they were mRNA precursors; (iv) all four mRNAs have a comparable half-life in the presence of actinomycin D, and during the actinomycin D treatment there is no accumulation of the smaller mRNA species and disappearance of the larger mRNA species (not shown). The existence of such mRNAs implies that they are generated by facultative splicing. The biological significance of such a regulation is not clear. The removal of the rightmost intron sequence in Z15 as compared with Z13 does not appear to affect the products expressed from these RNAs since the level of translation of the proteins was not affected (see Figure 2). On the contrary, the removal of an additional intron in Z8 as compared with Z15 had important consequences on the translation product as it creates an hybrid protein between EB1 and R. This protein possesses the DNA binding domain of EB1 (Farrell *et al.*, 1989), but it does not activate or repress any of the EB1- or R-specific targets we have tested (not shown). Another alternative could be that the Z8 mRNA is the result of faulty splicing as we found only one cDNA of this kind, in which case it would not fulfil any particular function.

Except for the mRNA corresponding to Z8, which has the potential to code for a protein that contains the C terminus of EB1 and the N terminus of R, the other three cDNAs contain either one or two ORFs that have been shown to code for the *trans*-activators EB1 and R. The 1 kb mRNA is a monocistronic mRNA coding for EB1 while the 3- and 4-kb mRNAs are bicistronic mRNAs containing both *BZLF1* and *BRLF1* ORFs (Figure 6). It should be emphasized that no mRNA containing the *BRLF1* ORF alone has been detected, and the R protein can therefore only be made from these bicistronic mRNAs. Moreover, there is no polyadenylation signal between the *BRLF1* and *BZLF1* ORFs. The existence of minicistrons preceding major coding sequences has been reported several times, exclusively in viruses. Usually, however, the leader peptide is so short that it is unlikely to have a biological function (for a review see Kozak, 1986). The data presented here provide evidence that the Z13 and Z15 cDNAs correspond to bicistronic mRNAs expressing two *trans*-activators in EBV-infected cells. One of the very rare examples of bicistronic and probably bifunctional messenger RNAs is also found in EBV with the messenger RNA encoding two nuclear proteins: the EBNA2 and the so-called leader protein (LP) (Sample *et al.*, 1986; Wang *et al.*, 1987).

The existence of bifunctional mRNAs coding for the two *trans*-activators EB1 and R has a biological relevance. In effect, several EBV early promoters contain both EB1- and R-responsive elements (Chavrier *et al.*, 1989; Chevallier-Greco *et al.*, 1989; Urier *et al.*, 1989). EB1 behaves like an activator of proximal upstream elements, whereas R behaves like an enhancer factor. In addition, when EB1 or R are expressed in EBV latently infected B cells they activate as many early genes as does TPA (A.Chevallier-Greco, personal communication). Simultaneous expression of EB1 and R would therefore ensure efficient activation of the EBV early promoters.

The fact that the *trans*-acting factor EB1 can be made from mRNAs expressed from two different promoters poses the following question: are these two promoters simultaneously or sequentially active in EBV-infected cells? One possible model would be that EB1 is first expressed from the P1 promoter (Figure 6). This could be due to autoregulation since EB1 activates its own promoter (Urier *et al.*, 1989). In this model EB1 will be expressed, but at a very low level in latently infected B cells. TPA or other inducers will increase the activity or the amount of EB1, which will then increase its own synthesis by interacting with the EB1 promoter. Such a regulation pathway has also been suggested for autoregulation of c-*jun* (Angel *et al.*, 1988). EB1 would then activate many EBV early promoters including the P2 promoter, which would direct the expression of both EB1 and R. Activation of the P2 promoter would result in the inactivation of the P1 promoter as transcription from the P2 promoter would interfere with the activity of the P1 promoter (Figure 6). Finally, EB1 and R in concert could activate many EBV early promoters. Several experimental results support this model. Firstly, EB1 can induce the lytic cycle when expressed in EBV latently infected B cells (Chevallier-Greco *et al.*, 1986; Countryman *et al.*, 1985, 1987). Secondly, EB1 can induce the expression of R in these cells, whereas the converse is not true (A.Chevallier-Greco personal communication). Thirdly, in the plasmids pcDZ13 and pcDZ15, the SV40 early promoter directs the expression of the long mRNAs coding for EB1 and R (Figures 2 and

5). The P1 promoter is present in pcDZ13 and pcDZ15 but is not active, since no small RNA is detectably expressed from these cDNA clones upon transfection in HeLa cells (Figure 5). However, deletion of the SV40 early promoter is followed by activation of the P1 promoter (G.Urier, personal communication).

In order to understand more about the regulation of EBV early gene expression, we are now characterizing the *cis*-acting elements in the P1 and P2 promoters and the viral and cellular factors required for their activity.

Materials and methods

Cell culture

Lymphoblastoid cell lines producing EBV (B95-8) or containing a latent EBV genome (Raji), were grown at 37°C in RPMI 1640 (Boehringer Mannheim) containing 10% fetal calf serum (FCS). HeLa cells and COS-7 cells were grown in DMEM medium supplemented with 5% FCS.

RNA preparation and Northern blotting

Briefly, the cells were lysed by the addition of 50 mM Tris−HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 2% (v/v) *N*-lauryl-sarcosine and 200 μg/ml proteinase K. The lysate was incubated for 10 min at 30°C and then vortexed thoroughly to reduce the viscosity. The cleared lysate was mixed with CsCl (1 g/ml of lysate) and underlayed with 2.5 ml of 5 M CsCl (Glisin *et al.*, 1975). After 18 h at 27 000 r.p.m. in a Beckman SW40 rotor (20°C), the RNA was recovered as a DNA-free pellet, dissolved in 200 mM sodium acetate and precipitated with three volumes of ethanol. Poly(A)$^+$ RNAs were isolated by chromatography on oligo(dT)−cellulose (Madjar *et al.*, 1982), size fractionated by electrophoresis in formaldehyde−agarose gels (Lehrach *et al.*, 1977) and transferred onto nitrocellulose membrane. The immobilized RNAs were hybridized for 18 h at 42°C with multiprime labelled DNA fragments (Amersham multiprime DNA labelling system) in 50% (v/v) formamide, 10% (w/v) dextran sulphate, 10 × Denhardt's solution (Denhardt, 1966), 50 mM Tris−HCl, pH 7.5, 0.1% sodium pyrophosphate and 0.1 % SDS. The filters were washed once with 2 × SSC, 0.1% SDS at 22°C and three times in 0.1 × SSC, 0.1% SDS at 65°C.

cDNA synthesis and screening of the cDNA libraries

The λgt10 library was prepared from poly(A)$^+$ RNA isolated from the EBV-transformed Raji cell line treated for 48 h with 20 ng/ml of TPA by a modification of the method of Gubler and Hoffman (1983). *Eco*RI linkers were ligated to double-stranded cDNA, which was then size selected (>0.5 kb) after separation on a Bio-Gel A50M column (Bio-Rad Laboratories), ligated to *Eco*RI-digested λgt10 DNA, and packaged with Gigapack extracts (Stratagene) to yield infectious virus. Recombinant bacteriophages were plated on the C600 hfl$^-$ strain of *Escherichia coli*.

The pcD cDNA library was prepared by a modification of the method of Okayama and Berg (1982). Briefly, 10 μg poly(A)$^+$ RNA isolated from TPA-treated Raji cells was heat denatured and quickly cooled on wet ice. The reaction mixture (final total volume 40 μl) was then adjusted to contain 50 mM Tris−HCl, pH 8.3, 8 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, 750 U/ml RNAguard (Pharmacia) 2 mM each of dATP, dTTP, dGTP, dCTP, 40 μCi [^{32}P]dCTP and 2 μg oligo(dT)-tailed plasmid primer pcDV-1 (Pharmacia). The cDNA synthesis was started by the addition of 40 U of AMV reverse transcriptase (P.H.Stehelin & Cie AG). The mixture was incubated at 42°C for 60 min. All the subsequent steps were carried out according to Okayama and Berg (1982). The plasmid cDNAs were transfected into the SCS1 strain of *E.coli* (Stratagene), and the library was amplified before screening. The λgt10 cDNA library and the pcDV cDNA library were both screened by *in situ* hybridization with a specific multiprime ^{32}P-labelled probe.

Nucleic acid sequencing

DNA fragments to be sequenced were end labelled with polynucleotide kinase and then sequenced by the method of Maxam and Gilbert (1980).

Transfections and CAT assays

HeLa cells and COS-7 cells were transfected by the calcium phosphate coprecipitation method (Wigler *et al.*, 1978). CAT assays were performed essentially as described previously (Gorman *et al.*, 1982). Sonication, however, was replaced by lysis of the cells in a buffer containing 0.25 M Tris−HCl, pH 8, and 0.05% SDS.

Comparative restriction endonuclease analysis

A plasmid containing the EBV *Bam*HI Z, g and R fragments as well as the plasmids pcDZ15 and pcDZ13 were cut first by *Bam*HI and *Hin*dIII and the resulting DNA fragments analysed both on agarose and polyacrylamide gels. For more detailed analysis, a plasmid containing the *Bam*HI Z genomic fragment was cut by *Hin*dIII and the 660 bp right end fragment was purified. The equivalent fragments in pcDZ13 and pcDZ15 were also purified. A plasmid containing the *Bam*HI R fragment was cut by *Bam*HI and *Dra*I and the 2358 bp left end fragment was purified. The respective equivalent fragments in pcDZ13 and pcDZ15 were also purified. The purified fragments were then cut by *Pst*I, *Ava*I and *Ava*II, then analysed comparatively on a polyacrylamide electrophoresis gel.

Immunoblots

The equivalent of 5 × 10^6 COS-7 cells was collected 72 h following the transfection in 80 μl of SDS sample buffer and boiled for 2 min. Half of each extract was electrophoresed on a SDS−10% polyacrylamide gel and transferred to a nitrocellulose filter before incubation with antibody (Burnette, 1981).

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