Characterization of the Epstein–Barr virus BRRF1 gene, located between early genes BZLF1 and BRLF1

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The switch from latency to a productive cycle in Epstein–Barr virus (EBV)-infected B cells proliferating in vitro is thought to be due to the transcriptional activation of two viral genes, BZLF1 and BRLF1, encoding two transcription factors called EB1 and R respectively. However, a third gene, BRRF1, is contained in the BZLF1/BRLF1 locus, overlapping with BRLF1 but in inverse orientation. We have characterized the 5’ end of the BRRF1 mRNA and the promoter, PNa, at which BRRF1 pre-mRNA is initiated. We show that although a single BRRF1 mRNA species is induced by 12-O-tetradecanoylphorbol 13-acetate/sodium butyrate in several EBV-infected B cell lines, in Akata cells treated with anti-IgG two BRRF1 mRNAs can be detected. Transcription initiated at the BRRF1 promoter was activated by EB1 but not by R, and EB1-binding sites which contribute to the EB1-activated transcription have been mapped to between positions −469 and +1. A 34 kDa protein could be translated from the BRRF1 mRNA both in vitro and in vivo, and was found predominantly in the nucleus of HeLa cells transfected with a BRRF1 expression vector. Thus there are three promoters in the region of the EBV chromatin containing the BZLF1/BRLF1 genes, two of which, PZ and PNa, potentially share regulatory elements.

Introduction

Epstein–Barr virus (EBV) is a human herpesvirus that persists latently for the lifetime of the infected host. EBV is also associated with malignancies such as Burkitt’s lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin’s disease (HD), and B and T cell lymphomas in immunocompromised individuals. EBV infects and immortalizes B lymphocytes in vitro, resulting in the establishment of a latent infection. In such infected cells, the entire EBV genome is maintained mainly as an episome and its expression is reduced to that of a few genes defining a type III latency. These genes are those for two small nuclear RNAs, the EBERs, six nuclear proteins, EBNA-1, EBNA-2, EBNA-3A, -3B, -3C and EBNA-LP, and three membrane proteins, LMP1, TP1 (LMP2a) and TP2 (LMP2b), (for a review see Kieff, 1996).

The hallmark of latent infection in vitro is the quasi-absence of cells producing virus (Schwarzmann et al., 1998). However, depending on the cell line, the number of cells producing virus can be dramatically increased by adding to the culture the tumour promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) (zur Hausen et al., 1978) or sodium butyrate (BA) (Luka et al., 1979) or anti-immunoglobulin (Takada & Ono, 1989). It is now well documented that these treatments induce, through different pathways, the expression of two EBV transcription factors: the BZLF1-encoded factor EB1 (also called Z, Zta or ZEBRA) (Countrryan & Miller, 1985) and the BRLF1-encoded factor, called R (or Rta) (Hartwick et al., 1988) (Fig. 1A). Once produced, EB1 and R activate the EBV early genes (Chevallier-Greco et al., 1986, 1989; Countrryan et al., 1986; Rooney et al., 1989; Urier et al., 1989; Flemington & Speck, 1990; Francis et al., 1999), probably by binding as homodimers to specific DNA-binding sites located in early EBV promoters (Farrell et al., 1989; Chang et al., 1990; Gruffat et al., 1990; Lieberman & Berk, 1990). Moreover, EB1 transactivates DNA replication from ORfllyt (Cho & Tran, 1993; Schepers & Hammerschmidt, 1993), the origin of replication active during the lytic cycle (Hammerschmidt & Sugden, 1988). Thus, the EB1 transcription factor is a key determinant for activation of the lytic cycle.

The precursors for EB1 and R mRNAs are transcribed from contiguous genes called BZLF1 and BRLF1 (Fig. 1A). The EB1 open reading frame (ORF) BZLF1 is found in mRNAs named Z24, Z13, Z15 and Z8 according to their full size cDNAs (Fig. 1A) (Manet et al., 1989). Z24 pre-mRNA is initiated at promoter PZ while Z13, Z15 and Z8 pre-mRNAs are initiated.

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from a library of Raji cells in which expression of the EBV early genes had been activated by TPA/BA (Manet et al., 1989).

In this report, we present a characterization of the 5’ end of the BRRF1 mRNA and therefore of the promoter, PNa, at which the BRRF1 pre-mRNA is initiated. Transcription initiated at the BRRF1 promoter was activated by EB1 but not by R, both in the viral genome and in transient expression assays, and several EB1-binding sites have been detected between positions −469 and +1. A 34 kDa Na protein could be translated from the BRRF1 mRNA in rabbit reticulocyte lysate. A rabbit polyclonal antibody raised against the purified Na protein detected Na protein both in HeLa cells transfected with an BRRF1 expression vector and in Raji and Akata cells treated with TPA/BA or anti-IgG respectively. The Na protein was found predominantly in the nucleus of HeLa cells transfected with a BRRF1 expression vector. There are thus three promoters in the BZLF1/BRRF1 region of the EBV chromatin, two of which, PZ and PNa, could share regulatory elements.

**Methods**

### Cell culture and chemical induction

Lymphoblastoid cell lines B95-8, HH514, Akata and Raji, harbouring the EBV genome, as well as EBV genome-negative DG75 cells, were grown at 37 °C in RPMI 1640 (Boehringer Mannheim) containing 10% (v/v) foetal calf serum (FCS). Exponentially growing cells were treated with 20 ng/ml TPA (Sigma) and 3 mM BA (Sigma), or were treated with anti-human IgG (Sigma) at a final concentration of 0.1 mg/ml. HeLa cells were grown in DMEM (Gibco) supplemented with 10% (v/v) FCS and antibiotics.

### Plasmids

**Expression vectors.** The expression vectors for EB1 (pCMV-EB1), R (pCMV-R) have been described elsewhere (Manet et al., 1989). The FLAG-BRRF1 expression vector (pCMV-FLAG-BRRF1) was generated by PCR using the 5’ primer sequences coding both for FLAG peptide (IBI Flag system, Kodak) and for part of the N terminus of BRRF1 (5’ GCCCCGATCCACATGGATCAAAAGGAGCGAGGATGACAAGGTGACTAGTAAACAGAGG 3’) and the 3’ primer (5’ GCCCGATATCCAGATAGAGAGG 3’) complementary to the 3’ end of the BRRF1 cDNA. The PCR-amplified product was digested with BamHI/EcoRI, and cloned into plasmid pRC/CMV (Invitrogen) to generate pCMV-FLAG-BRRF1. Plasmid pRC/CMV contains the cytomegalovirus (CMV) immediate-early promoter, the bovine growth hormone cleavage-polyadenylation signal and the T7 promoter. The pGST-Δ1-36BRRF1 bacterial expression vector was constructed by inserting a SphI–BanII fragment from pDZ-Z13 (Manet et al., 1989) into the BanII site of pGEX-1T (Pharmacia). The GST-BRRF1 fusion protein expressed from this construction lacks amino acids 1 to 86.

**Reporter genes.** The pZ-CAT reporter gene construct containing the CAT gene under the control of the pZ promoter has been described elsewhere (Urier et al., 1989). The pNa 1300CAT reporter gene was generated by inserting the BanII–PstI fragment of pDZ-Z13 (Manet et al., 1989), containing 1300 bp of the Raji BRRF1 promoter, into the BanII–BglII site of plasmid p8LCAT4. The pNa 1300CAT construct was obtained by a partial XhoI and SphI digestion of pNa 1300CAT. The pNa 185CAT plasmid was constructed by inserting the CfoI–PstI fragment from pD-Z13 into the BanII–BglII site of p8LCAT4. The pNa 90CAT was obtained by PCR as follows. The 5’ primer contained the
EBV B95-8 DNA sequences between positions 104956 and 104973 (Baer et al., 1984) and a HindIII restriction site (5′ CCAGGAAGCTT-GGAGATTGTTGACGCCGTCGCG 3′). The 3′ primer (5′ GGAGAAGCTT-TCTGAGGCTAACTC 3′) was complementary to the EBV B95-8 DNA sequences between positions 105160 and 105138. The PCR-amplified product was digested with HindIII and PvuII, and cloned into the HindIII–BglII site of plasmid pBlCAT, to generate pNa 90CAT. In all the PNa promoter constructs, the promoter sequences are collinear at the 3′ end.

### Production of bacterial GST–A1–86Na. GST–A1–86Na and GST proteins were generated by standard techniques and purified as previously described (Manet et al., 1993).

### Production of polyclonal rabbit antiserum. New Zealand White rabbits were inoculated with 100 µg of purified GST–A1–86Na fusion protein in Freund’s complete adjuvant and were then given three similar immunizations but in incomplete Freund’s adjuvant, 2 weeks apart. The Na antibodies (AbNa) were purified on an agarose–GST column in similar immunizations but in incomplete Freund’s adjuvant, 2 weeks apart. The Na antibodies (AbNa) were purified on an agarose–GST column in order to eliminate antibodies raised against the GST protein.

### Transfections and immunoblotting. The plasmids were prepared by the alkaline lysis method and purified through two sequential ethidium bromide–caesium chloride gradients. HeLa cells were seeded at 1×10^5 cells per 100 mm culture plate 8 h before the transfection. Transfections were performed by the calcium precipitation method. Cells were mixed with the appropriate DNAs: typically 15 µg of DNA was used which included the expression vector, plasmids carrying the reporter genes and pUC19 up to 15 µg. EBV-infected B cells (Raji) and EBV-negative B cells (DG75) were resuspended 1 day before transfection at a density of 5×10^5 cells/ml in fresh medium. Transfections of B cells were performed by electroporation of 50 µg of reporter genes and 10 µg of EB1 or R expression vectors per 5×10^6 cells. Cells were shocked at 220 V, 950 µF with a Zapper electroporation unit (Bio-Rad) and then incubated in fresh medium with 10% FCS at the concentration of 0.5×10^6 cells/ml. Transfected cells were washed and collected 48 h after transfection. Immunoblots were performed and stained with the anti-EB1 antibody as described previously (Segouffin et al., 1993).

### CAT assays. CAT-ELISA was performed using the Boehringer Mannheim CAT-ELISA kit following the manufacturer’s instructions. After transfection, cells were lysed in 1 ml of lysis buffer, and the amount of CAT protein produced was calculated for the total protein extract.

### EMSA (electrophoretic mobility shift assay). The EB1 protein was produced in E. coli as a His-tagged protein and purified by standard techniques. For EMSA, the DNA probes used were the restriction fragments shown in Fig. 5(A). EMSA was performed by incubating 4×10^4 c.p.m. of 5′–32P-labelled double-stranded DNA probes with 10 µl of His-tagged EB1 for 30 min at room temperature in 20 mM HEPES (pH 7.9), 100 mM KCl, 1 mM MgCl₂, 0.5 mM DTT, 10% glycerol and 0.1 µg poly(dI–dC) in a final volume of 20 µl. After incubation, the mixture was loaded onto a 4.5% (w/v) polyacrylamide gel (29:1 cross-linked), 0.2× TBE, and run at room temperature at 10 V/cm for 3 h. The protein–DNA complexes were visualized by autoradiography.

### RNA extraction, poly(A)^+ isolation and Northern blotting. Total RNA was extracted by denaturation in guanidium isothiocyanate followed by pelleting through a CsCl cushion. Poly(A)^+ RNA was isolated by using oligo(dT) Dynabeads (Dynal) as instructed by the supplier. Poly(A)^+ RNA was then size fractionated by electrophoresis in denaturing agarose gels and transferred onto reinforced nitrocellulose membrane (Schleicher & Schuell). The immobilized RNAs were hybridized for 18 h at 42 °C with 32P-labelled DNA fragments in 50% formamide, 1% SDS, 10% dextran sulphate, 1 M NaCl and 150 µg/ml herring sperm DNA. The filters were washed twice with 2× SSPE at room temperature, twice with 0.5× SSPE–0.1% SDS at 65 °C and once with 0.1× SSPE–0.1% SDS at 65 °C.

### Mapping of the BRRF1 mRNA 5′-end by S1 nuclease protection assay. RNAs (50 µg) were resuspended in 20 µl 10 mM Tris–HCl (pH 7.4), 300 mM NaCl, 0.2 mM EDTA, 80% (v/v) formamide, and mixed with an excess of double-stranded HindIII–PvuII DNA fragment (S1 DNA probe, positions 104956 and 105120 on the B95-8 sequence (Baer et al., 1984)). The S1 DNA probe was asymmetrically 32P-labelled at the 5′ end of the PvuII site with polynucleotide kinase (see Fig. 3B). After heating at 80 °C for 5 min, probe and RNAs were allowed to hybridize for 18 h at 43 °C. 2 °C greater than the melting point of the DNA probe as determined experimentally in 80% formamide (data not shown).

### Mapping of the BRRF1 mRNA 5′ end by reverse transcription (RT). The single-stranded DNA primer used for RT had the following sequence: 5′-CTGCGGCTTCTTGCTCTGACTAC 3′ (positions 105097 to 105120 (Baer et al., 1984)) (see Fig. 3B). The asterisk shows the 32P-labelled end. The 5′ end of the RT and S1 DNA probes were at the same position, 105120 (see Fig. 3B). The complementary radioactive cDNA products were analysed on 8% (w/v) acrylamide–8.3 M urea sequencing gels.

### Rapid amplification of cDNA ends (RACE). Poly(A)^+ RNA (2 µg) purified from Raji cells treated with TPA/BA was reverse transcribed using Mo-MuLV reverse transcriptase (New England Biolabs) and the primer 5′ GGAAGAAGCTTGGCTACTAATCGT 3′ complementary to the 5′ end of the BRRF1 cDNA [EBV B95-8 DNA sequences 105160 to 105138 (Baer et al., 1984)]. The cDNA was purified on a green SPIN-X column (Dutscher). For tailing, 4 µl of 2 mM dATP, 4 µl of 5× tailing buffer and 20 units of Terminal Transferase (Promega) were added, and the mixture was incubated for 10 min at 37 °C and then heated at 70 °C for 15 min. The reaction mixture was diluted to 500 µl in water and 10 µl aliquots were used for amplification. For the first round of PCR, primer T0T1 (5′ GACITCGATGCTACTAATCGTAC 3′) and the primer 5′ CTGGCGCTCTTGCTCTGACTAC 3′; EBV B95-8 DNA sequences 105097 to 105120 (Baer et al., 1984)) were used. For the second PCR, primer T0 (5′ GACITCGATGCTACTAATCGTAC 3′) replaced T0T1. The PCR product was cloned directly into pGEM-T (Promega).

### Immunofluorescence staining. Cells were plated on sterile coverslips and transfected as described above. At 36 h after transfection, cells were washed with PBS and fixed for 15 min with 4% paraformaldehyde. The fixed cells were incubated twice (10 min each time) in PBS–0.1 M glycine and once for 5 min in PBS–0.1% Triton X-100. The coverslips were then washed for 10 min in PBS–0.2% gelatine and incubated for 30 min at room temperature with an anti-FLAG monoclonal antibody (mAbM2) (IBI FLAG System, Kodak). After three washes in PBS, the coverslips were finally incubated with a fluorescein isothiocyanate-conjugated donkey anti-mouse IgG antibody (Jackson; 1:200 dilution), washed extensively with PBS, and mounted on microscope slides with Mowiol and PBS.

### Results

**BRRF1 mRNA is detected in a variety of EBV-infected cells after induction of the lytic cycle.**

To evaluate whether the BRRF1 gene behaves like an EBV early gene, BRRF1 transcription was investigated by Northern
containing mRNAs called Z13, Z15 and Z24 according to the name of their corresponding full-length cDNAs (Fig. 1A), were detected at 10 h post-induction and accumulated up to 48 h post-induction (Fig. 2A, panel a, lanes 3, 4 and 5). The time-course of appearance of BRRF1 mRNAs was similar to that of BZLF1-containing mRNAs (Fig. 2A, panel b, lanes 3, 4 and 5). Since in Raji cells there is no lytic DNA replication due to a deletion in the BALF2 gene, induction by TPA/BA of BRRF1 transcription clearly shows that it is an EBV early gene.

Since BRRF1 behaved like an early gene, i.e. it was expressed in the absence of viral DNA replication, it might probably be responsive to the viral transcription factors EB1 and/or R. We therefore transfected Raji cells with an EB1 (pCMV-EB1) or an R (pCMV-R) expression vector, and analysed by Northern blotting the mRNAs transcribed from the BRRF1 region. As shown in Fig. 2(B), the BRRF1 mRNAs were only detectably transcribed upon transfection of an EB1 expression vector (lane 2), as compared to the BRRF1 mRNA species induced in Raji cells treated with TPA/BA (lane 4). The BRRF1 gene was, however, not transcribed upon transfection of an R expression vector (Fig. 2B, lane 3), although under these conditions R induced the expression of the R-responsive early gene BMLF1 (not shown).

**Determination of the 5' end of the BRRF1 mRNA**

We next determined the 5’ end of the BRRF1 mRNA in order to locate the promoter at which it was initiated. For this, we used the RACE method. The sequence of the RACE clone with the longest 5’ end generated by this approach, which is shown in Fig. 3(A), terminated at EBV B95-8 DNA sequence position 105048 (Baer et al., 1984), 27 nucleotides longer than the BRRF1 cDNA isolated previously (Manet et al., 1989). Thus, from the cDNA cloning and the RACE data, the size of the BRRF1 mRNA should be 1080 bases without a poly(A) tail, which is very close to the size evaluated by Northern blotting, about 1.3 kb including the poly(A) tail (Manet et al., 1989).

To confirm the results obtained by the RACE method, the 5’ end of the BRRF1 mRNAs induced by TPA/BA treatment in Raji cells was also mapped by primer extension analysis using the RT probe, a 32P-labelled single-stranded oligodeoxy-nucleotide (Fig. 3B; EBV B95-8 DNA sequence 105097 (Baer et al., 1984) and by S1 nuclease protection assay using an asymmetrically 5’-32P-labelled double-stranded DNA fragment called the S1 probe (Fig. 3B; coordinates from 105120 to 104956). The primer extension products and the S1 nuclease-protected DNA fragments were run on a denaturing urea–polyacrylamide gel together with the sequence of the EBV genomic region overlapping the 5’ end of the BRRF1 mRNA (Fig. 3C, lanes 1 to 10). As shown in Fig. 3(C), no primer extension product (lanes 1 and 2) and no S1-protected DNA fragment (lanes 5 and 6) was detected with RNAs prepared from EBV-negative DG75 cells (lanes 1 and 6) or non-induced Raji cells (lanes 2 and 5). However, with RNAs...
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Fig. 3. Identification of the 5′ end of the BRRF1 mRNA transcribed in Raji cells. (A) Sequences of the 5′ end of the longest RACE cDNA and of the Na cDNA (Manet et al., 1989) are shown. (B) Radiolabelled DNA probes used for primer extension analysis (RT probe) and S1 nuclease mapping (S1 probe). (C) The primer extension products (RT, lanes 1, 2 and 3), and the S1 nuclease digestion products (S1, lanes 4, 5 and 6) were generated using RNAs extracted from the cells indicated in the upper part of the panel, and were analysed on an 8 M urea–polyacrylamide gel (lanes 1 to 6). The primer extension product (lane 11) and the S1-protected DNA fragments (lane 12) were generated using RNAs extracted from Raji cells treated with TPA/BA. A major primer extension product (lane 3, arrow RT) and two S1-nuclease-resistant DNA fragments (lane 4, arrows S1) about 72 nucleotides long were detected. In order to identify precisely the base at the 5′ end, the primer extension products (lane 11) and the S1-protected DNA fragments (lane 12) were generated using RNAs extracted from Raji cells treated with TPA/BA. They were also analysed together with the G reaction (lanes 11 and 12) of DNA sequence of the EBV genomic region overlapping the 5′ end of the BRRF1 mRNA (lanes 7 to 10). The location on the DNA sequence of the 5′ end of the Na mRNA as determined by RT and S1 nuclease digestion is shown.

Fig. 4. The BRRF1 gene promoter is responsive to the transcription factor EB1. (A) Schematic representation of nested 5′ deletion mutants in the BRRF1 promoter (pNa). (B, C) BRRF1 promoter sequences in the reporter plasmid pNa 1300CAT and in 5′-deletion mutants were transfected in EBV-negative DG75 cells (B) or in HeLa cells (C), either alone (lanes 1, 3, 5, 7), or with pCMV-EB1 (lanes 2, 4, 6, 8). Numbers over the bars indicate the -fold activation by EB1 in a representative experiment.

A

B

C

TATAAT was found at position 105022, and could therefore be the BRRF1 promoter TATA box (see Fig. 5A).

Transcription factor EB1 activates transcription initiated at the BRRF1 promoter

In order to further identify the BRRF1 promoter sequences responsive to EB1, 1300 bp of the DNA located upstream of position 105120 (Raji DNA, BamHI to PvuII site) were cloned upstream of the CAT reporter gene in plasmid pBLCAT4, to generate plasmid pNa 1300CAT (Fig. 4A), and a series of deletions extending from the 5′ end to the 3′ end of the promoter were derived from plasmid pNa 1300CAT (Fig. 4A). As BRRF1 gene expression was transactivated in EBV-infected B cells by the EBV transcription factor EB1 (Fig. 2B), we therefore asked whether EB1-responsive elements could be localized in the putative BRRF1 promoter. Thus, EBV-negative DG75 cells were transfected with the pNa 1300CAT reporter construct either alone or with the EB1 expression vector pCMV-EB1. As shown in Fig. 4(B), the weak constitutive expression of CAT protein from plasmid pNa 1300CAT (lane 1), was significantly increased in the presence of EB1 (lane 2), demonstrating in transient expression assay that DNA sequences located upstream of the BRRF1 mRNA initiation site behave like promoter sequences, and were responsive to the EB1 transcription factor. The deletion constructs were also prepared from TPA/BA-treated Raji cells, a major primer extension product (lane 3, arrow RT) and two S1-nuclease-resistant DNA fragments (lane 4, arrows S1) about 72 nucleotides long were detected. In order to identify precisely the base at the 5′ end, the primer extension products (lane 11) and the S1-protected DNA fragments (lane 12) were mixed with the G reaction (lanes 11 and 12) of a DNA sequence of the EBV genomic region overlapping the 5′ end of the BRRF1 mRNA (lanes 7 to 10). The location on the DNA sequence of the 5′ end of the Na mRNA as determined by RT and S1 nuclease digestion is shown.
transfected into DG75 cells, and activation of transcription of the reporter gene by EB1 was completely impaired when promoter sequences located between nucleotides −185 and −90 were deleted (Fig. 4C, compare lanes 7 and 8). More importantly, the basal activity of the CAT construct increased dramatically when sequences located between positions −185 to −90 were deleted (compare lane 5 to lane 7), suggesting that a negative regulatory element had been deleted.

Since the EB1 protein was present in comparable amounts in each transient transfection assay (data not shown), our results strongly suggest that the BRRF1 promoter regions located between nucleotides −185 to −90 contain EB1-binding sites essential for EB1-mediated activation of transcription in both B lymphocytes and HeLa cells.

**EB1 protein binds directly to the BRRF1 promoter**

To localize DNA sequences containing EB1-binding sites, EMSAs were performed with 5′-32P-labelled *Hae*III DNA fragments covering the Raji BRRF1 promoter sequences between positions −470 to +90 (Fig. 5A), and His-tagged EB1. As shown in Fig. 5B, lanes 1 to 10, EB1 bound efficiently to DNA fragments 124, 102, 37 and 27, with probably two binding sites formed on these DNA fragments. EB1 also bound, although more weakly, to DNA fragments 58 and 39 (lanes 4 and 5). The EB1–DNA complexes formed on DNA fragments 124, 102, 37 and 27 were specific since an excess of an unlabelled double-stranded oligonucleotide carrying the AP-1 site TGACTCA added to the binding reaction completely inhibited binding of EB1 to the 5′-32P-labelled DNA fragments (Fig. 5B, lanes 11 to 18). However, adding an excess of the unlabelled oligonucleotide carrying the mutated AP-1 site AGACTCT to the binding reaction had no effect on the formation of the EB1–DNA complexes (Fig. 5B, lanes 19 to 26). There are therefore several binding sites for the EB1 protein in the BRRF1 promoter sequences spanning nucleotides −470 to +90.

**EBV BRRF1 encodes a 34 kDa nuclear protein**

The BRRF1 mRNA contains an ORF from which a putative 310 amino acid protein could be translated. However, the product of the BRRF1 gene has not as yet been visualized. In order to characterize the Na protein, we first generated an antibody specific for Na. We constructed a chimeric gene consisting of the truncated BRRF1 ORF (∆1-86 Na) fused to the GST gene. The purified GST-∆1-86 Na fusion protein was used as the immunogen to produce a rabbit polyclonal antiserum. This polyclonal antibody (AbNa) was purified as described in Methods. In order to visualize the Na protein and evaluate the quality of the Na antibody, the coding sequence of the Na protein, the coding sequence for a tag peptide called FLAG, recognized by monoclonal antibody mAbM2, was inserted 5′ to the BRRF1 cDNA in plasmid pCMV to generate pCMV-FLAG-BRRF1. This FLAG-Na fusion protein was translated in vitro or expressed after transfection of pCMV-FLAG-BRRF1 in HeLa cells. As shown
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We also transfected the empty vector or the pCMV-FLAG-BRRF1 vector into HeLa cells to assess the subcellular localization of the Na protein. As shown in Fig. 6(C), an intense, even nuclear fluorescence was seen in HeLa cells transfected with the plasmid expressing the FLAG-Na protein.

Discussion

In this report we demonstrate that a third gene, BRRF1, is located in the EBV genomic region containing the genes for transcription factors EB1 and R, which are essential for the switch from latency to productive cycle genes (Chevallier-Greco et al., 1986; Countryman et al., 1986; Rooney et al., 1989; Urier et al., 1989; Hardwick et al., 1988). Transcription of BRRF1 mRNA is initiated at a promoter called here PNa (Fig. 1A), which is EB1-responsive in B-lymphocytes and in epithelial cells, and contains several EB1-binding sites. These findings complicate seriously the relative simple model in which only two promoters, PZ and PR, are taken into account following transcription activated at the BZLF1/BRLF1 locus by various inducers (Fig. 1A).

Indeed, the BRRF1 promoter contains EB1-binding sites that cover at least 446 bp upstream of the site at which BRRF1 pre-mRNA is initiated (B95-8 nucleotide 105046). This pre-mRNA is transcribed in the opposite direction to the BRLF1/BZLF1 bicistronic pre-mRNAs initiated at promoter PR (Fig. 1A), and could interfere with their elongation. Moreover, deletion of the EB1-binding sites impairs the EB1-inducibility of the BRRF1 promoter in a transient expression assay. Since the PZ promoter extends more than 500 bp upstream of B95-8 nucleotide 103210 and promoter PNa probably extends more than 500 bp upstream of B95-8 nucleotide 105046 (Fig. 1A), it is possible that the PZ and PNa promoters share control elements which have been neglected until now. Indeed, one must keep in mind that the EBV DNA is assembled in nucleosomes in the nucleus of infected cells. Therefore, the distance between regulatory elements measured in base pairs of DNA is not valid, and apparently distant DNA elements might be close by or even associated by virtue of chromatin folding and/or protein–protein interactions.

The PNa promoter appears to contain a negative regulatory element active only in HeLa cells and located between positions −185 and −90, overlapping with EB1-responsive elements essential for the EB1-activated transcription in B cells. It is likely that this region binds a cellular repressor. However, it is not yet known whether this negative element can be transferred to an heterologous promoter and if proteins bind to it. It remains to be established if the EB1-induced transcription at promoter constructs pNa 1300CAT, pNa 470CAT and pNa 185CAT in HeLa cells (Fig. 5B), is due to EB1-induced derepression, or due to a true transcriptional activation or to a combination of the two mechanisms. It is noteworthy that in HaeIII DNA fragments 27 and 124 (Fig. 5A), there are EB1-binding sites that are also found in the promoter construct pNa.
90CAT (Fig. 5 A). However, these EB1-binding sites did not confer EB1-inducibility to the promoter sequences found in the pNa 90CAT construct (Fig. 4 B, lanes 7 and 8), suggesting that EB1 does indeed release repression of transcription at the promoter Na in HeLa cells. However, it remains to be shown if these EB1-binding sites act in synergy with the more remote PNa EB1-binding sites to counterbalance the effect of the putative repressor or are not functional per se. Site-directed mutagenesis must be used to determine if the distal EB1-biding sites act in synergy with the proximal ones in B cells, since only the deletion of the proximal sites are deleterious for the EB1-mediated activation of transcription.

In EBV-infected cells, a 34 kDa protein could be detected by Western blotting using a rabbit antibody (AbNa) raised against the GST-Na protein, but only when the viral early genes had been induced (Fig. 6 B). This, plus the fact that an in vitro-translated or a transiently transfected FLAG-Na protein of 34 kDa is seen both by an anti-FLAG monoclonal antibody and by AbNa (Fig. 6 A), demonstrate that the BRRF1 gene product is a protein with an apparent molecular mass of 34 kDa. Indirect immunofluorescence also clearly indicated that the Na protein is almost exclusively located in the nuclei of transfected HeLa cells. However, the function of this EBV nuclear protein is not yet known. A search for homologies in databases revealed that the protein Na had some identities and similarities with the cellular nuclear protein Na. This, together with the fact that this EBV protein is almost exclusively located in the nuclei of transfected HeLa cells, suggests that the Na protein is an early gene product. However, the regulation of early/late gene expression or in the ORFllyt-dependent EBV DNA replication. This is what we are currently investigating.

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