Negatively cis-acting elements in the distal part of the promoter of Epstein–Barr virus trans-activator gene BZLF1

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Epstein–Barr virus (EBV) replicates in a latent or a lytic way in the infected organism, depending on the type and level of differentiation of the host cell. The switch between latency and lytic replication was previously shown, for Burkitt's lymphoma cell lines, to depend on the viral BZLF1 gene product. Protein–DNA assays were used to identify the cis-acting elements that represent the link between regulating signal transduction pathways and the viral cascade of gene expression. Specific binding of proteins to several sites of the BZLF1 promoter during latency was shown. Induction of the lytic cycle by stimulation with 12-*O*-tetradecanoylphorbol 13-acetate abolished the binding of these proteins to the distal promoter (positions −227 to −551), suggesting a functional role for the down-regulation of promoter activity during latency. Computer analysis identified a multiply repeated sequence motif, HI, in this region and exonuclease III footprints confirmed that these sites act as specific protein recognition sites. Using a set of reporter plasmids we were able to demonstrate a negative regulatory effect of the HI motif in some B lymphoid cell lines, in contrast to epithelial HeLa cells. The HI silencer elements are different from other silencer elements described so far in respect of their sequence and protein-binding pattern during the activation of BZLF1.

Introduction

Cell type-specific regulation of latency and productive lytic replication is important for the lifelong persistence of Epstein–Barr virus (EBV) after primary infection. A small proportion of lymphocytes from healthy donors contain latently replicating EBV genomes. However, the cell cycle-independent replication of viral DNA and death of the infected cell, as a consequence of a lytic cycle of virus replication, is a rare event. In epithelial tissues of the uterine cervix, parotid gland and tongue (Wolf *et al.*, 1984; Sixbey *et al.*, 1986; Greenspan *et al.*, 1985; Becker *et al.*, 1991; Young *et al.*, 1991), the virus replicates and consequently lyses the host cell. The product of the viral reading frame BZLF1 (Z'ta, EB1, ZEBRA) disrupts latency in Burkitt's lymphoma (BL) cells and leads to viral replication in distinct cell types (Countryman *et al.*, 1987; Bogedain *et al.*, 1994). Z'ta trans-activates a series of homologous and heterologous early viral promoters (Chavrier *et al.*, 1989; Chevallier-Greco *et al.*, 1986, 1989; Cox *et al.*, 1990; Kenney *et al.*, 1989*a*, *b*; Lieberman *et al.*, 1990; Rooney *et al.*, 1989) and the other immediate early genes BRLF1 (Chevallier-Greco *et al.*, 1989) and BI'LF14 (Marschall *et al.*, 1991), as well as the BZLF1 promoter itself (Urier *et al.*, 1989; Flemington & Speck, 1990*b*). Hence Z'ta has a pivotal role in the transition from latency to lytic replication. Control of transcriptional activity by positively as well as negatively acting elements has been shown (Flemington & Speck, 1990*a*; Montalvo *et al.*, 1991). In order to identify cis-acting elements that mediate the control of BZLF1 gene induction by the host, we employed DNA binding assays with protein extracts from cells that were latently infected and from cells permitting lytic replication of EBV. We identified elements that changed their pattern of protein binding during gene activation. These elements, with a common sequence motif, designated HI, were repeated five times (α, β, γ, δ and ε) within the promoter region of the BZLF1 gene. Site-directed mutagenesis and transfection assays with reporter constructs demonstrated their functional impor-

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tance for down-regulation of BZLF1 during latent infection.

Methods

Cell lines and transfection assays. For transfection assays we used the lymphoid EBV-negative BL cell line BJAB, the EBV-positive BL cell line Raji, two lymphoid cell lines (LCL), established by spontaneous outgrowth (Ru-LCL and Em-LCL) and epithelial HeLa cells. Transfection of lymphoid cells was achieved by electroporation with a Bio-Rad Gene Pulser. Cells (2×10^7) were collected by centrifugation at 1000 *g* at room temperature, washed once and resuspended in 400 µl PBS. The cells were mixed with 100 µl of DNA solution (50 µg of plasmid DNA in PBS) and incubated for 10 min at room temperature. The cells were treated with a pulse of 250 V/960 µF, incubated for another 10 min at room temperature and finally resuspended in 5 ml RPMI with 10 % fetal calf serum. After 3 to 5 days, the culture medium was tested for the presence of the reporter protein hepatitis B virus surface antigen (HBsAg; Marschall *et al.*, 1989). The epithelial cell line HeLa was transfected by calcium phosphate precipitation as described earlier (Marschall *et al.*, 1989). Promoter activities were expressed as percentage activity (the average of at least three independent experiments) in relation to a control construct which did not contain EBV inserts.

Recombinant plasmids and oligonucleotides. The recombinant plasmid pEBZ contains the complete promoter region of BZLF1 (Baer *et al.*, 1984; Skare & Strominger, 1980), from the *Bam*HI site at position −551 to the *Nae*I site at position +13 (relative to the start site of transcription). The plasmid was constructed by inserting the 564 bp *Bam*HI–*Nae*I fragment into pUC18 *Bam*HI–*Hin*cII sites. The pEBZ EBV insert was subcloned further, in three fragments, for bandshift assays. The proximal 240 bp *Sph*I–*Sph*I fragment was cloned into the pUC18 *Sph*I–*Sph*I site, the central 120 bp *Sph*I–*Bal*I fragment was cloned into pUC18 *Hin*cII–*Sph*I and the distal 200 bp *Bal*I–*Bam*HI fragment into pUC18 *Bam*HI–*Hin*cII. This gave the constructs pZ240, pZ120 and pZ200 respectively (Fig. 1). The promoter region from positions −551 to −227, relative to the start site of transcription, was inserted upstream of the heterologous early enhancer–promoter of simian virus 40 (SV40), which drives the expression of HBsAg in the plasmid pSVHBs (Marschall *et al.*, 1989). The *Bam*HI–*Sph*I fragment of the promoter was treated with T4 DNA polymerase and cloned into the *Eco*RI site of pSVHBs, which had been blunt-ended with the Klenow fragment of DNA polymerase, to give pSSVHBs.

Point mutations were introduced into the BZLF1 promoter region using modifications of the PCR techniques reviewed by Higuchi (1990). The sites targeted for mutagenesis were the HI α, β and γ sequence motifs, identified in the promoter region using the GCG software package (Devereux *et al.*, 1984). The sequences of each of the five motifs are shown in Fig. 2(*a*). The mutations (indicated in bold), which effectively inactivate individual motifs from the promoter, resulted in a sequence of 5′ **TAAGCTTG** 3′ for the HI α element, 5′ **CAAGATTG** 3′ for HI β and 5′ **CAAGCTTG** 3′ for HI γ. The PCR–mutagenesis products, designated α^*, β^* and γ^*, were cut with the restriction

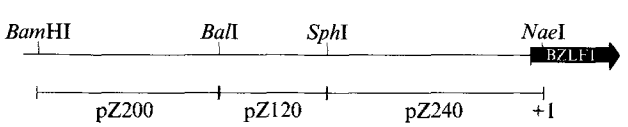

Fig. 1. The location and arrangement of the three BZLF1 promoter fragments and the restriction sites used to excise them.

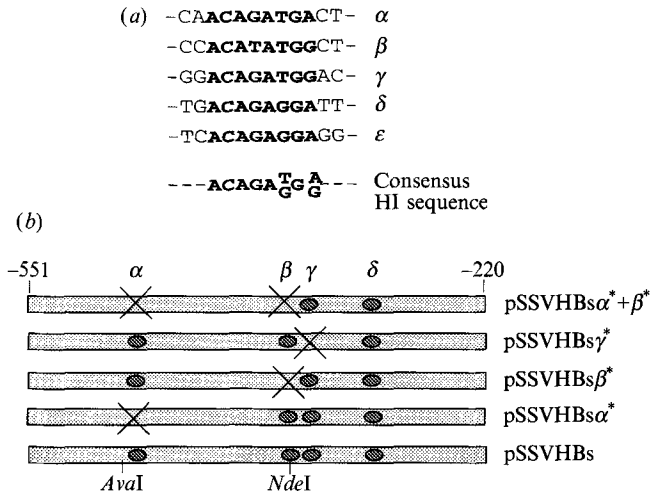

Fig. 2. (*a*) Sequence of the different HI elements and the HI consensus sequence. (*b*) Schematic representation of the BZLF1 promoter, showing the HI motifs that are functional (circles) and non-functional owing to mutation (crosses). The plasmid vectors that include the various mutations are indicated.

enzymes *Sph*I and *Ava*I (for α^*, β^* and $\alpha^*+\beta^*$) or *Sph*I and *Nde*I (for γ^*). They were then cloned into the recombinant plasmid pZΔ*Ava*I/*Nde*I at the *Sph*I and *Ava*I, or *Nde*I sites respectively, to place the original sequences of the proximal part of the promoter next to the mutated elements. The pZΔ*Ava*I/*Nde*I plasmid is identical to the pEBZ construct except that the *Ava*I and *Nde*I sites have been deleted. Finally, *Sst*I–*Sph*I fragments were purified, the *Sph*I site was blunt-ended with T4 DNA polymerase, and subcloned into the plasmid pSVHBs at *Sst*I and *Sma*I sites upstream of the SV40 enhancer–promoter. The resulting constructs, pSSVHBsα^*, pSSVHBsβ^*, pSSVHBsγ^* and pSSVHBs$\alpha^*+\beta^*$, contained one or two defective HI motifs (Fig. 2*b*). All recombinant reporter plasmids were sequenced with an Applied Biosystems automatic sequencer.

The HI δ sequence motif was used as a probe in several experiments. The motif was incorporated into the central region of a double-stranded oligonucleotide, oligo-Z120HI. This oligonucleotide was created by annealing two complementary single-stranded sequences, 5′ GATCCACTAGAGTCCATGACAGAGGATTTGAATCTGG-ACTCG 3′ and 5′ GATCCGAGTCCAGATTCAAATCCTCTGTC-ATGGACTCTAGTG 3′, both of which contain 5′-terminal *Bam*HI sites. They were purified by PAGE, denatured for 10 min at 95 °C and annealed in 10 mM-Tris–HCl pH 8·0, 1 mM-EDTA and 150 Mm-NaCl at room temperature. The resulting double-stranded oligonucleotide has 3′-protruding *Bam*HI ends. It was purified by gel electrophoresis and end-labelled with [γ-^{32}P]ATP.

Labelling of DNA fragments and oligonucleotides. For band shift assays, the subcloned DNA fragments were excised from the recombinant plasmids with the restriction enzymes *Bam*HI and *Hin*dIII, purified by agarose gel electrophoresis and end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (5′ end-labelling kit, Boehringer Mannheim). For footprint assays the recombinant plasmids were linearized at one restriction site, dephosphorylated with calf intestinal phosphatase and end-labelled with [γ-^{32}P]ATP. The T4 polynucleotide kinase was then inactivated by heat and the insert was excised with a second restriction enzyme, to yield an asymmetrically 5′-end-labelled fragment, which was then purified by agarose gel electrophoresis.

Nuclear extracts. These were prepared by a modification of the procedure described by Dignam *et al.* (1983). Cells (10^8) were harvested by centrifugation (600 *g* for 10 min at 4 °C), washed twice with PBS, resuspended in 5 packed-cell volumes of buffer I (10 mM-HEPES, 1 mM-$MgCl_2$, 10 mM-KCl, 0·5 mM-DTT, 0·5 mM-PMSF pH 7·9) and incubated on ice for 5 min. Thereafter the nuclei were prepared by a few strokes with an all-glass Dounce homogenizer, monitored by microscopy. The nuclei were washed three times in buffer I (100 *g* for 10 min at 4 °C), the pellet was resuspended in 300 µl buffer II (20 mM-HEPES, 25% glycerol, 420 mM-NaCl, 1·5 mM-$MgCl_2$, 0·5 mM-PMSF, 0·5 mM-DTT) and the proteins eluted for 30 min on ice. The supernatant was clarified by centrifugation (25000 *g* for 30 min at 4 °C) and aliquots were stored at −80 °C. Further purification and fractionation of the protein extracts was achieved by chromatography. DNA-binding proteins were enriched on a heparin–agarose column (Sigma) and fractionated further on an ion-exchange column (S Sepharose HP, Pharmacia).

DNA–protein binding assays. Frozen nuclear protein extracts were diluted with binding buffer without NaCl (50 mM-HEPES pH 7·9, 1 mM-EDTA, 1·5 mM-$MgCl_2$, 5 mM-DTT, 10% glycerol) to the optimum NaCl concentration of 200 mM. In band shift assays, 2 to 5 µg of nuclear proteins were preincubated for 30 min, in binding buffer containing 150 mM-NaCl to a volume of 24 µl, with 2·0 µg of dAdT to reduce non-specific binding of proteins and with increasing concentrations of specific competitor DNA. Next, 1 ng (approximately 8 fmol) of ^{32}P-labelled probe was added and incubated for 15 min. After electrophoresis in a 4% polyacrylamide gel, the DNA was visualized by autoradiography. For footprint assays 2×10^4 c.p.m. (Cerenkov counts) of asymmetrically ^{32}P-end-labelled DNA was incubated with increasing concentrations of protein, to a volume of 38 µl, using the same conditions as for gel retardation. Then 2 µl of 0·1 M-$MgCl_2$ and 175 units of exonuclease III (Boehringer Mannheim) were added and incubated for 10 min at 37 °C so that the DNA fragments were digested unidirectionally, starting at their 3′ ends. Proteins bound to DNA caused the enzyme to stop, yielding DNA fragments with distinct sizes relative to the 5′ label (Hennighausen & Lubon, 1987). The reaction was stopped with 150 µl of 0·5% Sarcosyl, 10 mM-EDTA, 0·4 M-NaCl, 2·5 µg/µl glycogen, extracted once with phenol–chloroform–isoamyl alcohol, precipitated with ethanol and separated by electrophoresis in a 6% sequencing gel in 0·5 × TBE.

Results

Proteins bind specifically during latent replication to the distal promoter of BZLF1

Control of BZLF1 gene transcription is one possible way to control viral replication. It has been shown earlier that negative (Montalvo *et al.*, 1991) as well as positive (Flemington & Speck, 1990*a*, *b*) regulatory elements control the activity of this gene. We compared binding of protein in the regulatory region of BZLF1 in latently infected cells and cells permissive for lytic replication. To visualize specific binding of regulatory factors, the region from −551 to +13, relative to the start of the transcription, was subcloned in three fragments which were designated Z200 (distal region), Z120 (central region) and Z240 (most proximal region), according to their length in bp, (Fig. 1). Fig. 3 shows the formation of several protein–DNA complexes after incubation of these promoter fragments with crude nuclear extracts. EBV-negative BJAB cells and EBV-positive, latently-infected Raji cells were compared to 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA)-stimulated Raji cells, which are a model for cell types supporting the lytic replication of EBV. In latently infected Raji cells a specific complex is visible with fragments Z120 and Z200 (lanes 5 and 12). With extracts of EBV-negative BJAB cells only a very faint specific complex is visible with both fragments (lanes 3 and 10). Competition of protein binding with pUC18 DNA *Alu*I fragments did not weaken the binding of these proteins to the labelled DNA. When binding was competed specifically with the unlabelled homologous fragment, the indicated specific bands disappeared (lanes 4 and 11). The specific complexes with fragments Z120 and Z200 did not form with protein extracts from TPA-treated cells (lanes 7 and 14). The most proximal fragment Z240 also showed specific protein–DNA complexes but these complexes were unaltered after TPA treatment (lanes 15 to 20). With all promoter fragments, additional non-specific protein–DNA complexes were visible that were insensitive to TPA treatment (compare lane 4 with lane 6 and lane 11 with lane 13). These results demonstrate specific binding of cellular factors during latent, but not during lytic, replication in the distal

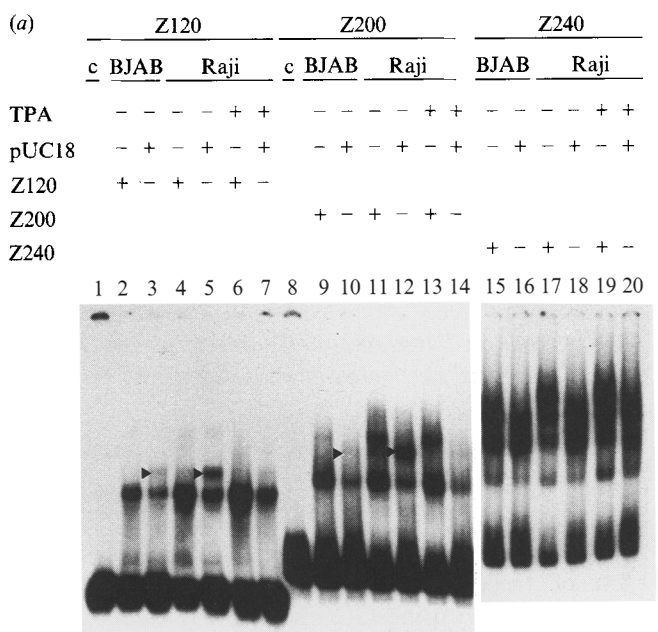

Fig. 3. Band shift experiment with the distal region of the BZLF1 promoter and nuclear extracts from EBV-negative BJAB cells and EBV-positive Raji cells. Specific protein–DNA complexes (lanes 5 and 12) are indicated by arrowheads. When extracts from TPA-stimulated cells were used these complexes disappeared (lanes 7 and 14). Use of nuclear extracts from unstimulated or TPA-stimulated Raji cells, as well as specific (Z120 and Z200) or non-specific (pUC18) competition, is indicated above the respective lanes. The pattern of protein complexes was not altered after TPA stimulation with fragment Z240 (lanes 15 to 20).

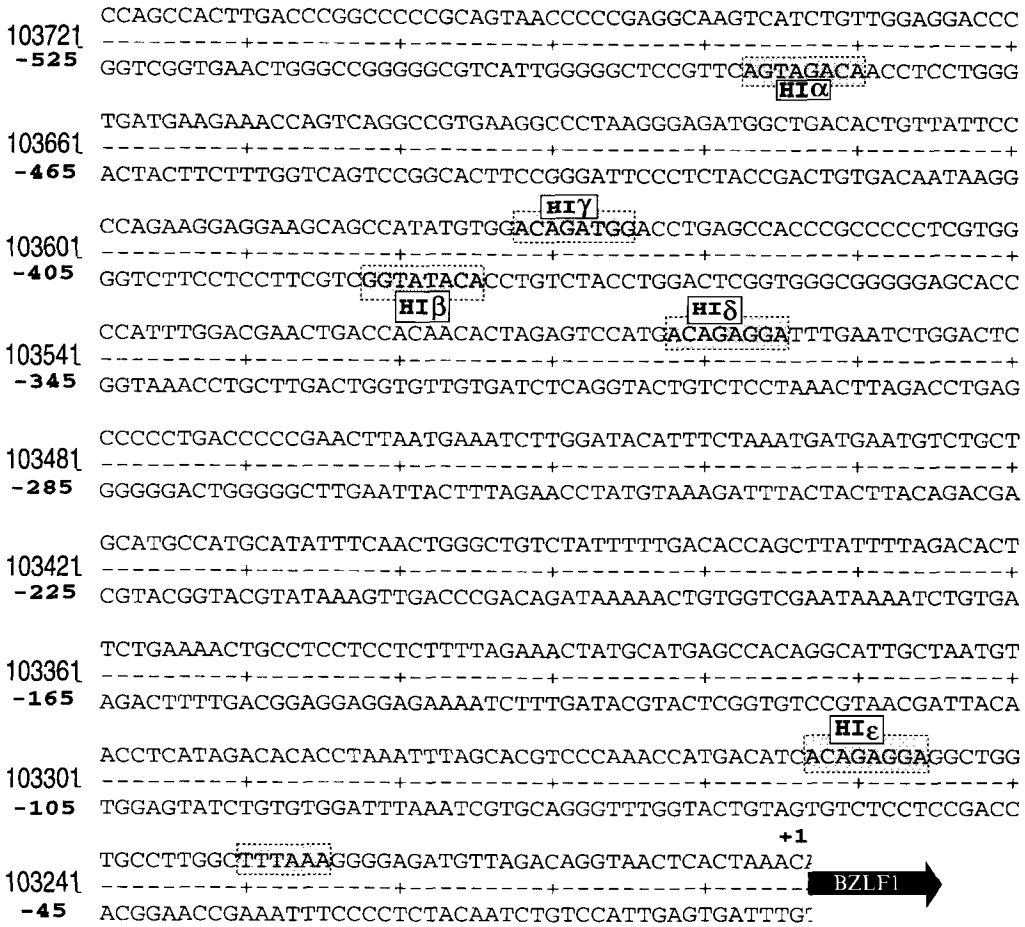

Fig. 4. Sequence of the upstream regulatory region of the BZLF1 reading frame. The HI elements that have been identified by computer analysis and confirmed by protein–DNA binding assays are depicted as boxes.

promoter region of BZLF1, indicating a functional role for the down-regulation of this promoter during latency.

The site that mediates specific binding of protein during latency is multiply repeated in the promoter of BZLF1

Band shift competition experiments with the three subfragments showed that all three would compete for specific complex formation. This was in contrast to competition with pUC18 DNA (data not shown), suggesting a binding site for the protein is present on each of these fragments. Sequence analysis of the promoter region using GCG software (Devereux *et al.*, 1984) detected a multiply repeated sequence. This motif, designated the HI element, with the consensus sequence 5′ ACAGA(T/G)G(A/G) 3′ was found three times in region Z200, once in region Z240 and once in Z120 (Fig. 4 and Fig. 2*a*). Footprint experiments were performed to test whether these sites were occupied by proteins in latently infected cells. In agreement with another report (Montalvo *et al.*, 1991), no clear footprinting was possible in this region using crude nuclear extracts. Exonuclease III footprints were therefore employed. These are much more sensitive than DNase I assays (Vogel *et al.*, 1989) because each bound protein molecule theoretically yields a positive signal. The results of the footprint assays are depicted in Fig. 5, in relation to the location of the HI sequences identified by computer analysis. Using increasing concentrations of protein, binding was detected at various distances from the 3′ end of the DNA. On each of the fragments used in band shift assays, proteins bound to one HI sequence. These sites specifically bound proteins from unstimulated lymphocytes and have been described, in part, previously (Flemington & Speck, 1990*a*, *b*). Indeed, the HI motif did function as a specific binding site, and in latently infected Raji cells three (α, δ and ε) of the five elements were bound by protein.

To confirm that the HI elements were involved in the formation of TPA-sensitive protein–DNA complexes, protein fractions from unstimulated and TPA-stimulated Raji cells were tested for the ability to bind to the HI sequence motif using the probe oligo-Z120HI. Crude nuclear extracts were divided into two fractions by

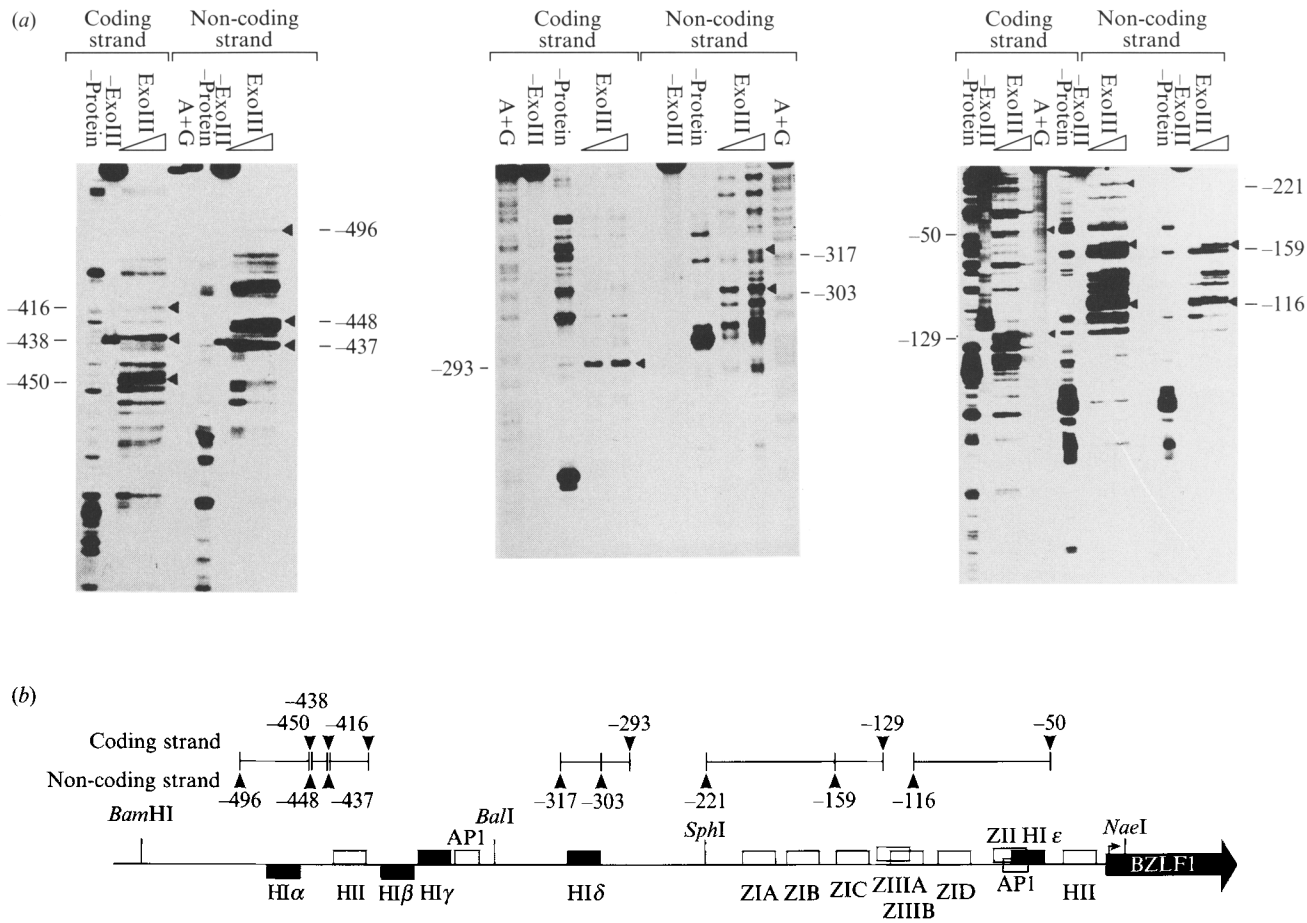

Fig. 5. Exonuclease III footprint assay (*a*) with the BZLF1 promoter region from positions −551 to +13 and nuclear extracts from latently infected EBV-positive Raji cells. Boundaries of protein–DNA complexes are indicated by arrowheads and the nucleotide positions relative to the transcriptional start site are given beside the lanes. Also included are a control without protein but with exonuclease (−protein), a control without exonuclease (−ExoIII) and a sequencing lane (A+G). The lanes marked ExoIII are exonuclease digests with increasing amounts of protein. The extreme right-hand assay is a duplicate of its neighbour, using a shorter exposure time. (*b*) A diagram of the upstream regulatory region of BZLF1. The regions between exonuclease III stops, which are bound by proteins, are depicted as bars. ZI, ZII and ZIII are regulatory elements described previously (Flemington & Speck, 1990*a*, *b*).

heparin–agarose and ion exchange column chromatography. Fig. 6 shows that in latently infected cells only (lanes 1 and 2) a protein in fraction I bound to the HI motif, but did not bind after TPA treatment (lane 3), nor to the larger fragment Z120 (lane 6). In fraction II however, there were proteins that bound to the larger Z120 fragment after TPA stimulation (lanes 7 and 8) but did not bind to the HI motif (lanes 4 and 5).

HI elements act as negative regulatory silencer elements

As the pattern of protein binding points towards a silencing mechanism during latency, we constructed reporter plasmids with site-specific mutations in the HI motif to investigate their regulatory influence. The results are shown in Table 1. When the intact region from positions −551 to −227 was tested upstream of the heterologous enhancer–promoter in the EBV-negative BJAB cell line, a 60% decrease in promoter activity was observed. In none of the other tested cell lines was a comparable influence detected. Each of the four mutations abolished the negative regulatory effect, suggesting a contribution of each of the destroyed elements to the silencing function in this cell line. Interestingly, mutations β^*, γ^* and $\alpha^*+\beta^*$ not only abolished the negative regulation, but increased the promoter activity from 40 to 144% (relative to the control) in BJAB cells. Similar results were observed for the Raji cell line, where mutation of the HI elements β, γ and α plus β reproducibly increased the promoter activity to about 160%. Raji cells showed no repression of activity with the intact distal region; a mutation in the α HI element had no influence and activity remained at 100% of the control. With the two LCLs however, strong activation occurred with mutation β^* and γ^*, of up to 217% in Ru-LCL and up to 302% in Em-LCL. The α^* mutation

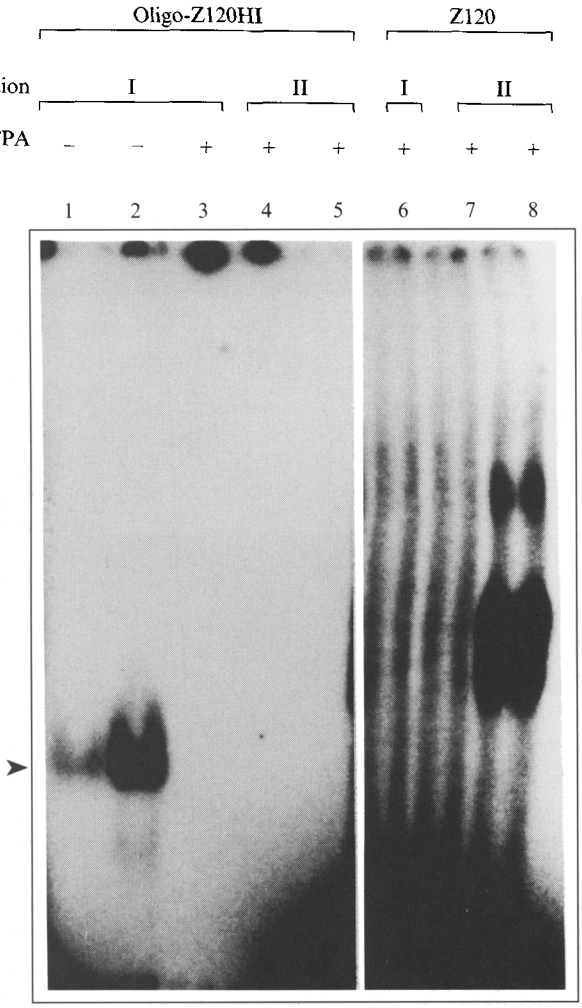

Fig. 6. Band shift experiment showing latency-specific binding of protein to the HI sequence motif, using two purified protein fractions (I and II). The arrow indicates the specific complex of the HI motif oligo-Z120HI with protein in latently infected cells (lanes 1 and 2), which did not bind after TPA treatment (lane 3). Lanes 4 to 8 are controls described in the text.

resulted in the strongest enhancement of promoter activity (up to 280% in Ru-LCL and 760% in Em-LCL) and there was no negative effect of the unmutated distal region. These results demonstrate that the HI elements are negative regulatory silencing elements under appropriate conditions, depending on the indivdiual cell line and also confirm the presence of positive regulatory elements located in the promoter region between positions −227 and −551, described earlier by Montalvo *et al.* (1991). In HeLa cell line, neither an effect of the intact distal promoter nor of the different HI mutations could be measured.

Discussion

We have identified a sequence motif, HI, that is repeated five times in the promoter region of BZLF1 (Fig. 4). Band shift assays with specific oligonucleotides and exonuclease III footprints showed that there are cellular proteins in latently EBV-infected lymphoid cells that bind to the HI motif. In the distal region of the promoter (Z120 and Z200) these sites showed loss of bound protein following TPA treatment, indicating a role in repression of the BZLF1 promoter during latency. Cloning of the distal promoter, from positions −551 to −227, in front of the heterologous SV40 enhancer–promoter in a reporter plasmid and *in vitro* mutagenesis of individual HI elements showed that the HI elements confer a negative regulatory influence on the downstream promoter.

Flemington & Speck (1990*a*) have shown that the proximal region of the promoter, but not the distal region, contains regulatory elements that directly respond to TPA in EBV-negative cell lines and that are directly involved in promoter activation. However, they could not identify the HI silencer elements which downregulate stimulatory elements nearby. This HI mechanism is cell line-dependent and functions only very poorly in EBV-negative lymphoid BJAB cells and in EBV-positive Raji cells, and not at all in epithelial HeLa cells. After TPA treatment the HI elements lose their negative regulatory effect. We assayed HI function by inactivation of these elements without TPA stimulation of the cells and thereby demonstrated HI activity during

Table 1. *Regulatory activity of the promoter region of the BZLF1 reading frame in different cell lines*

	Construct* HBsAg expression (%)†					
Cell line	pSVHBs	pSSVHBs	pSSVHBs-α*	pSSVHBs-β*	pSSVHBs-γ*	pSSVHBs-α*β*
BJAB	100	40 (10)‡	100 (19)	144 (26)	123 (44)	122 (17)
Raji	100	116 (7)	102 (3)	160 (10)	154 (44)	152 (35)
LCL-Em	100	164 (57)	760 (28)	211 (26)	302 (73)	650 (6)
LCL-Ru	100	119 (2)	280 (16)	197 (24)	217 (10)	233 (42)
HeLa	100	92 (3)	88 (6)	98 (6)	88 (4)	96 (6)

* The plasmids designated pSS have mutated versions of the promoter (Fig. 1).
† Percentage activity in relation to the positive control plasmid pSVHBs.
‡ Figure in parentheses is the S.D.; at least three assays were performed for each result.

latent infection. Furthermore, these experiments show that the activity of positive regulatory elements is controlled by the HI silencer during latency in some cell lines. Taking into account the results of Flemington & Speck (1990*a*), the target elements that are suppressed by the HI motifs are not directly stimulated by TPA. TPA sensitivity concerns silencer inactivation but not induction of the positive elements, which seem to be regulated in a TPA-independent way. In the EBV-negative BJAB cell line, the presence of the intact distal promoter, from position −227 to −551, decreased activity of the SV40 enhancer–promoter to about 40%. In all other cell lines tested, whether lymphoid or epithelial, no silencing function of the intact distal upstream region was observed. A likely explanation for this difference is the weak activity of positive control elements in BJAB cells, which results in a stronger suppression by the silencer elements. The model of a negative and a positive regulatory module also explains the results obtained in Raji cells. In these cells we could demonstrate binding of protein to the HI sequences (Fig. 3), but inactivation of these sites had only a slight effect on the activity of a downstream promoter (Table 1) because the positive regulatory target elements were inactive. Experiments to identify the trans-acting cellular proteins that interact with these positive and negative regulatory cis-acting elements are in progress.

The HI elements α, β and γ are different from some of the negative regulatory elements located in the proximal region of the promoter from −227 to +13 described by Flemington & Speck (1990*a*). They also differ from a 48 bp silencer described by Montalvo *et al.* (1991). Fig. 7 shows the HI elements β and γ in relation to the 48 bp silencer. The inverted repeat sequences on the right side of the 48 bp silencer are identical to the HI β and γ but there are two important properties of HI that distinguishes the two silencers. Firstly, mutation of the most distal HI element, α, blocks the observed silencer activity on the tested EBV promoter fragment in BJAB cells, Ru-LCL and Em-LCL, hence a negative regulatory effect is clearly demonstrated. This element is located at a considerable distance from the 48 bp silencer. Secondly, the HI mechanism does not function in HeLa cells, as described for the 48 bp silencer. Since our reporter constructs, irrespective of any HI mutations, show very high levels of activity in the HeLa cell line, we believe that this results from inactive HI silencer modules and active enhancer elements. Observations of different patterns of protein binding in this cell line are in accordance with this hypothesis (F. Schwarzmann, N. Prang, B. Reichelt, B. Rinkes, S. Haist, M. Marschall & H. Wolf, unpublished results). Therefore there is evidence that, in epithelial cells, lytic replication of EBV may be significantly influenced by non-functional HI elements releasing suppression of the enhancing modules.

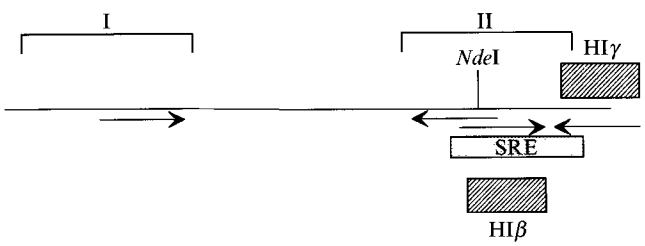

Fig. 7. Schematic drawing of the distal promoter of BZLF1. The area containing the 48 bp silencer element described previously (Montalvo *et al.*, 1991) partially overlaps with the HI elements, but they are not functionally identical. Regions I and II are protein-bound elements of the silencer region described earlier (Montalvo *et al.*, 1991) as are the inverted repeats, indicated by arrows, which are important for activity. SRE denotes a serum response element overlapping region II, the right inverted repeat pair and one HI element.

In conclusion, we have identified a new type of silencing sequence motif, HI, in the promoter of the BZLF1 gene, which is involved in the down-regulation of this lytic trans-activator in latently infected lymphoid cells. The proximity of the HI motif to other protein binding regions, identified by footprinting, and to binding motifs for other regulatory cellular factors like YY1 (Seto *et al.*, 1991), SRE (Gualberto *et al.*, 1992; Treisman, 1986) and AP1 (Rauscher *et al.*, 1988) suggests that the HI binding factor works through protein–protein interactions or by steric hindrance exerted on binding sites in the proximity.

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