EBNA-2 Transactivates a Lymphoid-Specific Enhancer in the *Bam*HI C Promoter of Epstein-Barr Virus

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Among the few Epstein-Barr virus (EBV) genes expressed during latency are the Epstein-Barr nuclear antigens (EBNAs), at least one of which contributes to the ability of the virus to transform B lymphocytes. We have analyzed a promoter located in the *Bam*HI-C fragment of EBV which is responsible for the expression of EBNA-1 in some cell lines. Deletion analysis of a 1.4-kb region 5' of the RNA start site has identified a 700-bp fragment that is required for optimal promoter activity in latently infected B lymphocytes, as shown by promoter constructs linked to the chloramphenicol acetyltransferase reporter gene. This fragment is also able to enhance activity, in an orientation-independent manner, of the simian virus 40 early promoter linked to the chloramphenicol acetyltransferase gene. The enhancer element has some constitutive activity in EBV-negative lymphoid cells, which is increased in the presence of the EBNA-2 gene product. Further deletions have shown that the EBNA-2-responsive region requires a 98-bp region that contains a degenerate octamer-binding motif. In epithelial cells there was no enhancer activity regardless of the presence of EBNA-2. These results demonstrate that *Bam*HI-C promoter activity may be dependent not on an enhancer contained in the *ori-P*, as was previously assumed, but rather on EBNA-2 transactivation of this more proximal enhancer located in the upstream region of the *Bam*HI C promoter itself.

The establishment and control of latency are principal features of infection with the Epstein-Barr virus (EBV). Latency and B-lymphocyte immortalization are biologically intertwined, in that most if not all of the identified latent EBV gene products are expressed in immortalized and transformed cells. Expression of at least a subset of the latent genes is required for the transformed state, but not all of the viral and cellular genes needed for transformation have been identified.

Maintenance of the EBV episomal genomic form, which is the basis for viral latency (29), depends on the *trans*-acting functions of a single EBV protein, EBNA-1 (35). The EBNA-1 protein is synthesized early in the viral cycle, but its appearance is preceded by the appearance of another nuclear protein, EBNA-2 (26, 37), which has been implicated in cell immortalization and transformation (7, 8, 17, 34, 41). Details of the function of this nuclear antigen are largely unknown, although it induces an increase in the expression of a B-cell activation antigen, CD-23 (48), as well as an increase in the level of RNA of the *c-fgr* proto-oncogene (22). EBNA-2 also transactivates the promoter for the latent membrane protein 1 (LMP-1), another latently expressed viral gene product (1, 49). EBNA-2 alone is unable to transform cells, but it may function to transactivate viral and cellular promoters which in turn are responsible for the expression of gene products necessary for transformation.

The messages encoding the EBNAs can arise from two different promoters located near the origin of plasmid replication (*ori-P*), with some transcripts originating from a promoter in the *Bam*HI W region (38, 42) and others originating from a promoter in *Bam*HI-C (5). Promoter usage for expression of these nuclear proteins may vary or shift in different cell lines and differentiation states (50), but it appears that *Bam*HI-W promoter activity precedes *Bam*HI-

C promoter activity in latent infection and that gene products transcribed from the *Bam*HI W promoter may be required to transactivate the *Bam*HI C promoter (51).

We focus here on an analysis of the *Bam*HI C promoter because of its central role in the expression of the EBNA-1 protein as well as the other EBNAs. We show that the EBNA-2 gene product increases expression of this promoter and that this increase is due to the activation of a tissue-specific enhancer located in the region 5' of the RNA start site.

MATERIALS AND METHODS

Cell lines. Raji (32) is a latently infected EBV-positive Burkitt's lymphoma B-cell line, Louckes (47) is an EBV-negative Burkitt's lymphoma B-cell line, Jurkat is an EBV-negative T-cell line, and HEp-2 is an EBV-negative human epithelial line. Other lines used include the human cervical carcinoma line HeLa (which contains sequences from human papillomavirus type 18), the EBV-positive lymphoid line P3HR-1 (34), the latently infected EBV-positive lymphoid line IB4 (21), which was derived by infection of human cord blood lymphocytes, and NPC-KT, an EBV-positive epithelial line made by the fusion of an adenoidal carcinoma line with a nasopharyngeal carcinoma line (46). All cells were maintained at 37°C in a 5% CO₂ environment. Lymphoid cells were grown in RPMI medium supplemented with 10% fetal calf serum, and epithelial lines were grown in minimal essential medium with 10% fetal calf serum.

DNA transfections. Plasmid DNA was amplified in *Escherichia coli* and purified over two sequential cesium chloride gradients. For each sample, 10 µg of plasmid DNA was electroporated (27) into 10⁷ cells at 1,500 V, using the University of Wisconsin Zapper electroporation unit. Cells were then suspended in 5 ml of RPMI 1640 medium supplemented with 10% fetal calf serum and incubated for 48 h at 37°C in 5% CO₂.

Chloramphenicol acetyltransferase (CAT) assays. Extracts

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were prepared by washing cell pellets twice in phosphate-buffered saline solution, suspending them in 200 μ l of 0.25 M Tris-Cl (pH 7.5), and freeze-thawing them four times. Reactions were carried out as described previously (16) by incubating each sample with acetyl coenzyme A and [14 C] chloramphenicol at 37°C for 2 h. Acetylated reaction products were separated by thin-layer chromatography, visualized by autoradiography, and quantitated by scintillation counting. Percent acetylation is calculated as the ratio of counts per minute in the acetylated reaction products to counts per minute in the entire sample.

Recombinant plasmids. A 1.4-kb *Sau*3A restriction fragment containing the *Bam*HI C RNA start site was cloned into the *Bgl*II site of the promoterless CAT expression vector pCAT3M (24) as previously described (18). Constructs containing deletions of this fragment and constructs to evaluate possible enhancer function were also made and are described later. The pA₁₀CAT vector contains the CAT gene under the control of the simian virus 40 early promoter but lacks the simian virus 40 enhancer sequences (25). An EBNA-2-expressing construct, CMV-EBNA-2, was made by inserting a 2.1-kb *Mlu*I-*Aha*III restriction fragment containing the EBNA-2b coding region from the AG876 virus into the pGem derivative pHD101-3 (9), where EBNA-2 expression is driven by the cytomegalovirus (CMV) immediate-early promoter.

RNA analysis. An internally labeled single-stranded DNA probe complementary to plasmid pC-CAT was made by cloning a fragment from pC-CAT containing the intact promoter region plus the CAT gene into the M13 phagemid pBlueScribe (Stratagene). Single-stranded template DNA was prepared by using M13KO7 helper phage, and a 20-base primer that hybridized from 234 to 254 bases downstream of the RNA start site was annealed to it. The primer was extended and labeled with the Klenow enzyme and [32 P] dCTP. After digestion with *Sma*I, the strands were separated on a denaturing polyacrylamide-urea gel. The appropriate-size band was detected by autoradiography and electroeluted from the gel slice.

Cytoplasmic RNA from Jurkat cells was prepared 24 h after transfection (2, 13), and then 50 μ g was hybridized overnight at 30°C to 10⁵ cpm of the 32 P-labeled probe. After digestion with S1 nuclease, samples were electrophoresed on a denaturing polyacrylamide-urea gel. The protected fragment was visualized by autoradiography.

RESULTS

Constitutive activity of the *Bam* HI C promoter in different cell types. A series of nested 5' deletions of the pC-CAT

FIG. 1. Schematic representation of deletion constructs of the *Bam*HI C promoter region. (A) Nested deletions of the parent promoter construct, a *Sau*3A fragment (EBV *Bam*HI-C coordinates 5917 to 7343) extending from -1400 to +3 relative to the RNA start site of the *Bam*HI C promoter. Deletions were made by digestion with the restriction enzymes shown and linked to the CAT gene in the promoterless vector, pCAT3M. (B) Structures of Cd6787, Cd6878, and Cd6976, which were made to localize the EBNA-2-responsive region. The pC-CAT parent promoter construct was first digested with *Kpn*I and *Sma*I and then digested with exonuclease III for various times, using the Erase-a-Base system (Promega). The sequences of transformants were verified by dideoxy sequencing (39). Constructs were named according to the EBV *Bam*HI C coordinate for the 3' end of each deletion.

parent construct (18) was made by restriction enzyme digestion (Fig. 1A). Each of these deletions was transfected by electroporation into different cell lines. The *Bam*HI C promoter is constitutively active in the EBV-negative lymphoid cell lines Louckes and Jurkat, while the promoter remains inactive in the epithelial line HEP-2 (Table 1). In the lymphoid cell lines, constitutive promoter activity drops dramatically when sequences 5' of the *Sac*I site are deleted. In the latently EBV-infected Raji cells, *Bam*HI-C promoter activity is up to 60-fold higher than in the uninfected lymphoid cells, suggesting that an EBV latent gene product is required for maximal promoter expression.

EBNA-2 induces an increase in expression of the *Bam*HI C promoter. We next examined the effect of the EBV latent gene product EBNA-2 on expression from the *Bam*HI C promoter in the EBV-negative cell line Louckes. The pC-CAT deletion constructs were cotransfected either with CMV-EBNA-2 (Fig. 2, lanes 4, 6, 8, 10, and 12), which

TABLE 1. Effects of 5' deletions on *Bam*HI-C promoter activity

Cell line	Cell type	EBV	Promoter activity				
			pC-CAT	pC-dKpn	pC-dSma	pC-dSac	pC-dMst
Louckes	B lymphoid	—	2.7 ^a (0.8–7.2)	4.3 (1.6–10)	5.3 (2.7–12.6)	1.0 (0.3–2)	1.0 (0.5–1.5)
Jurkat	T lymphoid	—	7.6 (1.0–20)	5.6 (0.9–2.3)	13.8 (1.4–21)	1.7 (1.3–2.6)	0.9 (0.7–1.2)
Raji	B lymphoid	+	175.4 (54–357)	141.0 (41–345)	117.0 (83–154)	3.1 (2.5–3.8)	0.5 (0.4–0.6)
HEp-2	Epithelial	—	0.7 (0.1–1.0)	0.2 (0.1–0.4)	0.7 (0.5–1.7)	0.1 (0.0–0.4)	0.1 (0.05–0.2)

^a Expressed as the average fold increase in CAT activity over the value for the negative control plasmid, pCAT3M. CAT activity is measured by percent acetylation. Ranges for each experiment are shown in parentheses.

FIG. 2. Effect of EBNA-2 on CAT activity driven by the *Bam*HI C promoter. pC-CAT deletion constructs were transfected into Louckes, an EBV-negative B-lymphocyte line, and assayed for CAT activity. Lanes 1, positive control plasmid, RSV-CAT; 2, negative control plasmid, pCAT3M vector alone; 3, pC-CAT; 4, pC-CAT plus CMV-EBNA-2; 5, 7, 9, and 11, pC-dKpn, pC-dSma, pC-dSac, and pC-dMst in the absence of CMV-EBNA-2; 6, 8, 10, and 12, pC-dKpn, pC-dSma, pC-dSac, and pC-dMst in the presence of CMV-EBNA-2. Numbers represent the fold increase in the percent acetylation over the value for the negative control plasmid, pCAT3M.

expresses EBNA-2b under the control of the CMV immediate-early promoter, or with the pH101-3 vector alone (lanes 3, 5, 7, 9, and 11). The presence of the EBNA-2 gene product resulted in increases, some striking, in the level of CAT expression for those constructs containing sequences 5' of the *Sac*I restriction site (lanes 4, 6, and 8). Constructs containing the two smallest deletions, pC-dSac and pC-dMst (lanes 10 and 12), were unresponsive to EBNA-2. This same pattern of transactivation was observed in Jurkat cells as well as in the EBV-infected cell line P3HR-1, in which the coding region for EBNA-2 is deleted (data not shown) (20, 34). No promoter activity was observed in the EBV-negative epithelial cell lines HEP-2 and HeLa or in the EBV-positive epithelial cell line NPC-KT, in the presence or absence of EBNA-2 (data not shown). These results indicate that the EBNA-2 gene product is needed for maximal expression from the *Bam*HI C promoter in lymphoid cells.

EBNA-2 transactivates an enhancer contained in the region 5' of the *Bam*HI C promoter. The pattern of promoter expression that emerged during the deletion studies indicated that the region 5' of the *Sac*I site might contain an enhancer. A 700-bp *Sma*I-*Mst*II fragment containing the putative enhancer was therefore cloned in both orientations (C71 and C72) downstream of the CAT gene into the pA₁₀CAT vector (Fig. 3). pC71 and pC72 were transfected by electroporation into Raji cells, in which CAT expression of the constructs containing inserts in both orientations was enhanced over the background level (Table 2).

When pC71 and pC72 were tested in the EBV-negative cell line Louckes, a low level of promoter activity was observed, which was increased when the EBNA-2b-expressing plasmid (CMV-EBNA-2) was cotransfected. Likewise, in another EBV-negative lymphoid line, Jurkat, there was an increase in CAT activity when the enhancer-containing construct was

cotransfected with CMV-EBNA-2. CAT activities for all three lymphoid cell lines are summarized in Table 2. These results clearly demonstrate that the 700-bp *Sma*-*Mst* fragment contains an enhancer element that is transactivated by EBNA-2.

Enhancer activity is tissue specific. The enhancer element is most active in lymphocytes when EBNA-2 is provided in *trans*. The enhancer-containing constructs were also tested in several epithelial cell lines. In the EBV-negative cell lines HEP-2 and HeLa, no enhancer activity was observed in the presence or absence of cotransfected EBNA-2 (Table 2). The EBV-positive epithelial line NPC-KT was also tested, and again no activity was observed (data not shown). These results indicate that maximal expression from the *Bam*HI C promoter appears to require lymphoid cell factors in addition to the EBNA-2 gene product.

RNA analysis. To confirm that the RNA start site for the *Bam*HI C promoter does not change in the presence of the EBNA-2 transactivator, S1 nuclease analysis was performed. pC-CATdKpn (5 µg) was transfected into the EBV-negative lymphoid cell line Jurkat with 5 µg of either pH101-3 (Fig. 4, lane 1) or CMV-EBNA-2 (lane 2). Cytoplasmic RNA was harvested 24 h posttransfection and hybridized to a ³²P-labeled single-stranded DNA probe. After digestion with S1 nuclease, a protected DNA fragment of 254 bp was expected, according to the previously determined RNA start site for the *Bam*HI C promoter (5, 19, 45). Our results demonstrate that in the presence of the EBNA-2 transactivator, the RNA start site remains unchanged and the level of CAT RNA is increased.

EBNA-2 responsiveness requires a 98-bp region upstream of the *Bam*HI C promoter RNA start site. Exonuclease III deletions of the pC-CAT parent promoter construct were made as shown in Fig. 1B and transfected into the EBV-

FIG. 3. Schematic representation of the plasmid construct used to test for the presence of an enhancer. A 700-bp *Sma*I-*Mst*II fragment containing the putative enhancer was cloned in both orientations (C71 and C72) into the pA₁₀CAT vector, in which the CAT gene is under the control of the simian virus 40 (SV40) early promoter.

TABLE 2. EBNA-2 transactivation of the *Bam*HI C enhancer

Construct	EBNA-2	Fold enhancement ^a				
		Lymphoid cells ^b			Epithelial cells	
		Raji	Louckes	Jurkat	HeLa	HEp-2
pC71	–		4.7 (2.4–8)	2.8 (1.3–4.5)	1.1 (0.8–1.6)	2.6 (1.0–2.6)
	+	5.6 (3.3–9.8)	24.7 (14.6–45)	12.6 (7.2–19)	1.9 (0.9–3.6)	2.2 (1.9–2.6)
pC72	–		11.8 (3.0–19)	2.1 (0.6–4.6)	1.1 (1.0–1.2)	1.0 (0.5–1.3)
	+	14.1 (7.9–18)	45.6 (23–68)	6.1 (4.5–7.0)	1.0 (1.0–1.2)	2.6 (1.4–3.6)

^a Expressed as the percent acetylation of the pC71- and pC72-transfected samples relative to the percent acetylation in cells transfected with the pA₁₀CAT vector alone. Ranges for each condition are shown in parentheses. Plasmid constructs are shown in Fig. 3.

^b Raji cells constitutively express EBNA-2. In Jurkat and Louckes cells, plasmid CMV-EBNA-2 is cotransfected.

negative cell line Louckes. EBNA-2 transactivation was maintained through the first two deletions (Fig. 5, lanes 2 to 5) but was eliminated altogether with the third deletion (lanes 6 and 7). These results demonstrate that this 98-bp fragment, extending from *Bam*HI-C nucleotides 6878 to 6976, is required for EBNA-2 responsiveness.

DISCUSSION

Identifying the mechanisms of regulation of EBV latent promoters is essential to an understanding of the establishment and control of latent infection as well as its contribution to lymphocyte immortalization. We have shown here that maximum expression of the *Bam*HI C promoter, one of the promoters active during latency, is lymphoid specific and requires transactivation of an enhancer by the EBV latent gene product, EBNA-2.

In established latent EBV infection, the *Bam*HI C promoter is the primary promoter used for EBNA messages in most lymphoid lines (50). However, during the early stages

of latent infection, messages encoding the EBNA's arise primarily from a promoter within the *Bam*HI W fragment, which is present in multiple copies. *Bam*HI-C promoter activity is not observable until 3 days after infection (51), implying that it requires viral or cellular factors for its activation that are not immediately available upon infection. It has been suggested by Sugden and Warren (45) that EBNA-1, which can be transcribed from *Bam*HI-W in some cell lines, activates the *Bam*HI C promoter by transactivating the enhancer in the *ori-P*. However, the promoter constructs used in those experiments did not contain the more proximal enhancer element (primarily EBNA-2 responsive) that we have now identified. The promoter constructs used in our experiments, on the other hand, did not contain the more distal EBNA-1-responsive *ori-P* enhancer. Given that the EBNA-2-responsive element that we have identified is located much closer to the RNA start site for the *Bam*HI C promoter, it seems likely that this is the dominant enhancer element used to drive expression from this promoter. However, the two enhancers may work together to regulate latent gene expression as well as replication.

At this point, it seems likely that EBNA-2 is initially transcribed from the *Bam*HI W promoter (51) and then proceeds to transactivate the *Bam*HI C promoter. Such transactivation could be mediated directly by the binding of

FIG. 4. S1 nuclease protection analysis: effect of EBNA-2 on transcription from the *Bam*HI C promoter. Shown on an autoradiogram of S1 nuclease-protected DNA analyzed on a denaturing polyacrylamide gel. Jurkat cells were transfected with pC-CATdkpn and either pHD101-3 (lane 1) or CMV-EBNA-2 (lane 2). Cytoplasmic RNA was prepared from the transfected cells and hybridized to a single-stranded DNA probe. Numbers (in nucleotides) represent DNA standard molecular size markers.

FIG. 5. Localization of the EBNA-2-responsive region. Exonuclease III deletions of pC-CAT were transfected into Louckes cells, in the presence and absence of cotransfected CMV-EBNA-2, and assayed for CAT activity. Numbers represent the fold increase in percent acetylation relative to the value for the pCAT3M negative control.

FIG. 6. Regions of homology between the LMP and C promoters. (A) 12-of-16-bp match; (B) 9-of-10-bp match, containing a degenerate octamer-binding sequence, located within the 98-bp EBNA-2-responsive region. Numbers reflect the distance from the RNA start site for either promoter. (C) Consensus binding sequence for the Oct-1 and Oct-2 transcription factors (11, 12, 31).

the EBNA-2 protein to a sequence in the upstream region, although EBNA-2 has never been demonstrated to bind to a specific DNA sequence. Another possibility is that EBNA-2 and other viral gene products may contribute to an alteration in the differentiation state of the infected cell (7), resulting in the expression of cellular factors that may in turn activate the *Bam*HI C promoter. This explanation seems more feasible given the evidence that EBNA-2 induces the expression of vimentin (4) and the B-cell activation antigens CD-23 and CD-21 (6, 48, 49), as well as inducing an increase in the level of RNA of the *c-fgr* proto-oncogene (22).

EBNA-2 also transactivates the promoter for LMP, another EBV latent gene product (1, 49). Ghosh and Kieff (15) have identified an upstream region in the LMP promoter which, when deleted, results in a decrease in LMP promoter activity. Sharing homology with sequences in this region of the LMP promoter are two short sequences within the *Bam*HI C promoter (Fig. 6). The first homologous sequence (-559 CCCCCAGATGCCCCC -543) (Fig. 6A) appears not to be needed for EBNA-2 responsiveness of the *Bam*HI C promoter. However, deletion of 98 bp (Fig. 5, lanes 6 and 7) containing the second homologous sequence (-435 ACATGCACCC -425) (Fig. 6B) results in a complete inability of EBNA-2 to transactivate the promoter. This 98 bp is therefore required, but may not be sufficient, for EBNA-2 responsiveness.

The -435 to -425 sequence (relative to the RNA start site for the *Bam*HI C promoter) within this 98 bp contains a five-of-eight-base match with the consensus binding site for the Oct-1 and Oct-2 transcription factors (Fig. 6C) (11, 12, 31). This sequence could possibly act as a degenerate octamer-binding motif. These motifs can mediate activation of certain cellular and viral promoters and enhancers by a ubiquitous cellular octamer-binding protein (3, 40, 44). Since EBNA-2 can transactivate the *Bam*HI C promoter only in lymphoid cells, one possible mechanism of action for EBNA-2 could be an interaction with a lymphoid-specific octamer-binding protein (43), which may in turn activate the *Bam*HI C promoter through binding to this degenerate octamer motif. This scenario could be analogous to the mechanism by which VP16 activates the immediate-early promoters of herpes simplex virus (14, 23, 30).

Although the *Bam*HI C promoter appears to be the primary promoter used to activate EBNA expression in latently infected B-cell lines, at this point it is not known whether the same promoter is used to drive EBNA expression in epithelial cells. EBV infection of epithelial cells is of particular

interest, since infection of nasopharyngeal epithelial cells is strongly associated with the development of nasopharyngeal carcinoma (10, 28, 33). Our data suggest that it is unlikely that the *Bam*HI C promoter is the primary promoter used to drive EBNA expression in epithelial cells, since we were unable to detect constitutive activity of the promoter in epithelial cells, nor could we detect transactivation by EBNA-2 in this cell type. It is therefore likely that EBNA expression is differentially regulated in different cell types. Moreover, since the *Bam*HI W promoter, which can be used alternatively for EBNA-1 expression, is also inactive in epithelial cells (19, 36), there is a possibility that EBNA-1 mRNA arises from yet a third promoter or start site in epithelial cells.

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