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Epstein–Barr virus-encoded nuclear antigen 2 activates the viral latent membrane protein promoter by modulating the activity of a negative regulatory element

(cotransfection/inducible enhancer/repressor)

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ABSTRACT **Previous studies suggest that the Epstein–Barr virus nuclear antigen EBNA2 participates in the regulation of the expression of the viral latent membrane protein (LMP). We have used reporter plasmids containing DNA fragments of the 5′ flanking region of the LMP gene in cotransfection experiments to analyze the effect of EBNA2 on the activity of the LMP promoter. The results show that the LMP promoter is controlled by positive and negative transcription elements in a DNA fragment that contains the LMP transcription initiation site and 634 base pairs of upstream sequences. The promoter is activated by EBNA2. The region between position −54 and +40 relative to the mRNA cap site contains a positive transcription element that is constitutively active in DG75 cells and independent of EBNA2. The −106 to −54 region contains a negative regulatory element that prevents adjacent positive elements from functioning in the absence of EBNA2. Regulatory sequences between −324 and −144 participate in maintaining a high level of transcription of the LMP promoter after induction with EBNA2. The regulatory elements in the −634 to −54 promoter region have the characteristics of an inducible enhancer, including orientation independence and ability to regulate a heterologous promoter.**

Epstein–Barr virus (EBV) gains entry into B lymphocytes by attaching to the CD21 molecule that is expressed on resting B cells. This elicits a complex series of events in the cell for which the details at the molecular level are still largely unknown. The interaction of EBV with B lymphocytes *in vitro* leads to the outgrowth of immortalized, EBV-carrying lymphoblastoid cell lines (LCLs). Of the >100 genes coded for by the EBV genome, only 10 are expressed in all LCL cells: the EBV nuclear antigen (EBNA) gene family (EBNA1–EBNA6), the latent membrane protein (LMP) gene, the terminal protein (TP) gene, and the genes encoding two small RNAs, EBER1 and EBER2 (reviewed in ref. 1). The products of some or all of these genes are thus likely to participate in lymphocyte immortalization.

A number of observations support the notion that EBNA2 plays a role in the early phase of B-cell transformation. The P3HR-1 substrain of EBV, which carries a deletion in the EBNA2 and EBNA5 (also referred to as EBNA-LP)-encoding region, does not induce DNA synthesis in resting B lymphocytes and also fails to transform them into LCLs (2, 3). Recent experiments by Hammerschmidt and Sugden (4) and Cohen *et al.* (5) demonstrated that the transformation-defective phenotype of P3HR-1 virus can be cured by complementation or recombination with EBNA2-carrying vectors.

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In this context it might be relevant that transfected EBNA2 could induce the expression of the activation antigen CD23 on the surface of an EBV-negative Burkitt lymphoma (BL) cell line (6). The CD23 antigen is related to the receptor for a B-cell growth factor (7) and has also been suggested to act as an autocrine growth factor for B cells after shedding from the cell surface (8).

EBNA2 is a phosphorylated polypeptide with DNA-binding properties, which is encoded by the BYRF1 reading frame of the *Bam*HI WYH region of the EBV genome (reviewed in ref. 1). The protein (the A subtype) has a rather unusual primary structure containing an almost continuous sequence of 40 proline residues, an arginine- and glycine-rich positively charged region, and a negatively charged C-terminal sequence. The overall proline content of the 487-amino acid long polypeptide is 29%. The recent demonstration of a new class of transcriptional activators with a proline-rich domain might provide some clues concerning the action of EBNA2 (9).

It is thus conceivable that EBNA2 modulates the cell phenotype by influencing the expression of cellular genes. The effect might be direct, as suggested by the induction of CD23 in EBNA2-transfected cells (6), or indirect and mediated by way of other EBV genes, whose expression is in turn controlled by EBNA2. Evidence for the latter mechanism has been provided recently. The B95-8 virus strain, but not P3HR-1, has been shown to be able to induce expression of LMP in EBV-negative BL cell lines (10). Transfection of the EBNA2 gene into P3HR-1 virus-converted B-lymphoma cell lines induced the expression of LMP and a dramatic change in growth phenotype toward a LCL-like pattern (11). Some, but not all, of these changes could be reproduced in the absence of EBNA2 by transfection of P3HR-1-converted cell lines with a recombinant vector expressing LMP (11).

The present investigation is concerned with the mechanism by which EBNA2 activates the expression of the LMP gene. LMP is encoded by the BNLF1 reading frame and the gene is transcribed in a leftward direction from a promoter at position 169,546 in the B95-8 EBV genome (12, 13). We have transfected reporter plasmids containing the 5′ flanking sequences of the LMP gene into an EBV-negative BL cell line together with EBNA2 expression vectors. We present evidence that the LMP gene regulatory region contains a negative transcription element that prevents adjacent positive regulatory elements from functioning. Induction by EBNA2 relaxes the repression and activates the promoter.

Abbreviations: EBV, Epstein–Barr virus; EBNA, EBV nuclear antigen; LMP, latent membrane protein; LRS, LMP regulatory sequence; LCL, lymphoblastoid cell line; BL, Burkitt lymphoma; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40; TK, thymidine kinase.

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MATERIALS AND METHODS

Cell Culture, DNA Transfections, Chloramphenicol Acetyltransferase (CAT) Assays, and RNA Analysis. DG75 is an EBV genome-negative BL cell line (14). The IB4 cell line was derived by transforming human placental lymphocytes with the B95-8 EBV strain (15). The cells were maintained as suspension cultures in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum, penicillin, and streptomycin. The recombinant plasmids were transfected into DG75 cells by the DEAE-dextran technique (16) and into IB4 cells by electroporation (17). Cells were harvested 40 hr after transfection for the CAT enzyme assay. Preparation of extracts and enzyme assay were carried out as described (16). Total cytoplasmic RNA was analyzed by S1 nuclease mapping as described (16).

Plasmid Construction. All manipulations involved in plasmid constructions were carried out by standard procedures (18). The pSVECAT plasmid, which carries the reporter CAT gene under the control of the simian virus 40 (SV40) early promoter, was obtained from M. Yaniv (Institute Pasteur, Paris). The EBNA2 expression vector pEΔA6 and the mutated derivatives pEΔA7, pEΔA8, pEΔA9, pEΔA12, and pEΔA13 with defined deletions in the EBNA2 gene are constructs based on the pSV2-gpt vector and have been described (19). The deletions span from nucleotides 46,560 to 48,777 (pEΔA7), from 46,204 to 47,526 (pEΔA8), from 47,530 to 48,039 (pEΔA9), from 45,170 to 46,868 (pEΔA12), and from 45,442 to 47,010 (pEΔA13).

The pLRS(−634)CAT plasmid contains the 5′ flanking region of the LMP gene (position −634 to +40) linked to the reporter CAT gene (LRSs = LMP regulatory sequences). It was made by replacing the SV40 control region in pSVECAT with the 676-base-pair (bp) *Mst* II–*Sac* II fragment of B95-8 EBV DNA corresponding to nucleotides 169,477–170,153 using *Hin*dIII linkers (Fig. 1). The 5′ deletion series of the LMP control region was created by cleaving the purified *Mst* II–*Sac* II fragment with *Hin*fI, *Hha* I, *Alu* I, *Nla* III, *Rsa* I, and *Mlu* I, respectively, and cloning the appropriate 3′ fragments into the *Hin*dIII site of the deleted pSVECAT vector. The resulting plasmids were designated according to the position of the 5′ ends of the inserts as indicated in Fig. 1. The −634/+40 and −54/+40 fragments were also cloned into a derivative of the pSVECAT vector in which the SV40 poly(A) signal was replaced by the rabbit β-globin poly(A) signal (16). The resulting plasmids were denoted pSVECAT-β, pLRS(−634)CAT-β, and pLRS(−54)CAT-β, respectively. These constructs generally gave a higher recovery of CAT activity from the transfected cells and were used with the IB4 cell line.

pLRS(−634/−54)TKCAT and pLRS(−54/−634)TKCAT were made by replacing the EBV *Bam*HI W promoter sequences in the pTKΔW-3 plasmid (16) with the LMP gene fragment −634/−54 in either orientation. In these constructs the EBV regulatory region is inserted upstream of the herpes simplex virus thymidine kinase promoter (TK DNA sequences from −109 to +51 relative to the TK mRNA cap site; ref. 20) linked to CAT coding sequences. The vector without EBV sequences is designated pTKCAT.

The globin reporter plasmids pLRS(−634)globin and pLRS(−54)globin were constructed by replacing the EBV *Bam*HI W promoter fragment in the pBΔWglobin-1 plasmid (16) with the LMP gene −634/+40 and −54/+40 fragments, respectively, using *Hin*dIII linkers. The plasmids carry the rabbit β-globin coding exons and poly(A) signal but not the β-globin promoter.

RESULTS

EBNA2 Activates the LMP Promoter. To investigate the possible role of the EBNA2 protein in regulating the transcription of the LMP promoter we performed transient transfection assays. Plasmids containing 5′ flanking sequences of the LMP gene (LRSs) linked to a reporter gene were cotransfected into DG75 lymphoid cells with EBNA2 expression vectors. Transfection with the pLRS(−634)CAT construct alone (Fig. 1) did not induce expression of CAT over background levels (Table 1). If, however, the pEΔA6 plasmid, which is capable of inducing expression of EBNA2 in DG75 cells (16), was cotransfected with the reporter plasmid, a dramatic increase in CAT activity was observed. The expression of EBNA2 in the transfected cells was verified by anticomplement immunofluorescence staining (data not shown). A similar induction of CAT expression was observed after transfection of the pLRS(−634)CAT-β plasmid into the B95-8 EBV-transformed IB4 cell line (Table 1). These cells express EBNA2 constitutively as well as EBNA1, EBNA3, EBNA4, EBNA5, EBNA6, and LMP.

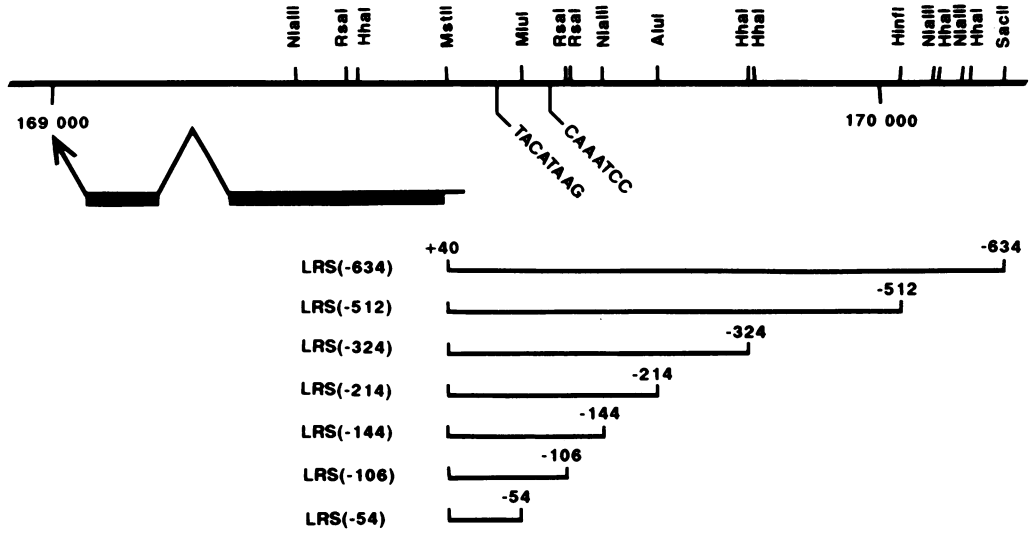

FIG. 1. Schematic map of the LMP promoter region and the LRSs in different reporter plasmids. The nucleotide sequence coordinates are from the B95-8 EBV DNA sequence of Baer *et al.* (12). The LMP transcription initiation site at position 169,516 (13) is numbered as +1 and the LRS fragments are denoted according to the relative position of their 5′ ends. Recombinant reporter plasmids containing the LRS fragments linked to the CAT or the β-globin gene were constructed as described in the text.

Table 1. Induction of the LMP promoter by EBNA2

Transfection vector		CAT expression, %	
A	B	DG75	IB4*
pSVECAT	—	100	100
pLRS(−634)CAT	—	<1	26
pLRS(−634)CAT	pEΔA6	12	33
pLRS(−634)CAT	pEΔA7	<1	ND
pLRS(−634)CAT	pEΔA8	8	ND
pLRS(−634)CAT	pEΔA9	4	ND
pLRS(−634)CAT	pEΔA12	<1	ND
pLRS(−634)CAT	pEΔA13	8	ND

Values are expressed as % of chloramphenicol acetylation using the result obtained with pSVECAT as 100%. The values are the average of at least two independent experiments. ND, not determined.

*IB4 cells were transfected with the pSVECAT-β and pLRS(−634)CAT-β reporter plasmids.

Equivalent results were obtained when the activation of the LMP promoter by EBNA2 was studied at the RNA level (Fig. 2). Total cytoplasmic RNA from cells cotransfected with the pEΔA6 plasmid and the pLRS(−634)globin and pLRS(−54)-globin reporter plasmids, respectively, was analyzed by S1 nuclease mapping using an end-labeled fragment of the globin gene as probe (16).

To ensure that the stimulation of CAT expression induced by the pEΔA6 vector was specific to the EBNA2 protein, further cotransfection experiments were conducted using derivatives of the vector with deletions in the EBNA2 gene. The pEΔA7 and pEΔA12 plasmids, which do not induce the expression of EBNA2 due to deletions of essential parts of the gene, failed to activate the LMP promoter (Table 1). On the other hand, cotransfection with the EBNA2-expressing pEΔA8, pEΔA9, and pEΔA13 plasmids, in which only noncoding and nonessential sequences are deleted, activated the promoter to about the same extent as with the pEΔA6 plasmid. It should be noted that segments that contain the EBNA5-encoding W2, Y1, and Y2 exons, respectively, are deleted in the pEΔA9 and pEΔA13 plasmids. Thus, at least these three exons of EBNA5 are not essential for the induction of the LMP promoter. The overlapping deletions in the EBNA2 vectors also exclude the possibility that a DNA sequence within the EBNA2 gene competes with the LRS region in the reporter plasmid for the binding of a putative negative LMP transcription factor, thereby activating the promoter.

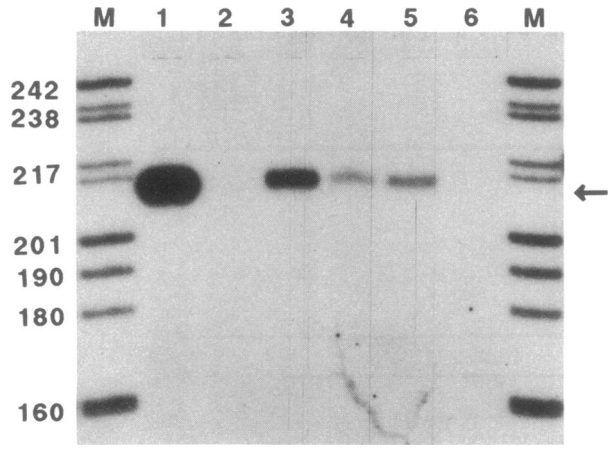

FIG. 2. Activation of the LMP promoter by EBNA2. The pSVE-globin (lane 1), pLRS(−634)globin (lanes 2 and 3), and pLRS(−54)globin (lanes 4 and 5) plasmids were transfected into DG75 cells together with pEΔA6 (lanes 3 and 5) or with an equivalent amount of pSV2-gpt (lanes 2 and 4). RNA was extracted and subjected to S1 nuclease protection analysis. The end-labeled fragment of the probe protected by globin RNA should be 210 nucleotides long. Lane 6, RNA from untransfected cells. Lanes M, size marker (in bp) ^{32}P-labeled *Msp* I digest of pBR322. The arrow indicates the position of the protected probe fragment.

Activity of the LMP Promoter Is Controlled by Negative Elements. To determine in more detail which parts of the regulatory region are required for EBNA2-induced activation of transcription, a series of 5′ deletion mutants of the pLRS(−634)CAT plasmid was constructed (Fig. 1). Expression of CAT induced by the different plasmids with or without cotransfection of the EBNA2 expression vector pEΔA6 in DG75 cells is summarized in Fig. 3. When the 5′ end of the LMP regulatory region was shortened in a stepwise fashion from −634 to −106, the basal level of promoter activity in the absence of EBNA2 remained close to background levels. Further deletion of sequences to position −54 resulted in a dramatic increase of LMP promoter activity to about half the level obtained with the intact regulatory region in the presence of EBNA2. Cotransfection of pEΔA6 with the deleted plasmids produced about the same levels of CAT activity for the −512 and −324 deletions as for the undeleted construct. Further deletions to −214, −144, and −106 considerably decreased the EBNA2-induced levels of activity. The constitutively high promoter activity in the −54 deletion construct was unchanged after induction with EBNA2.

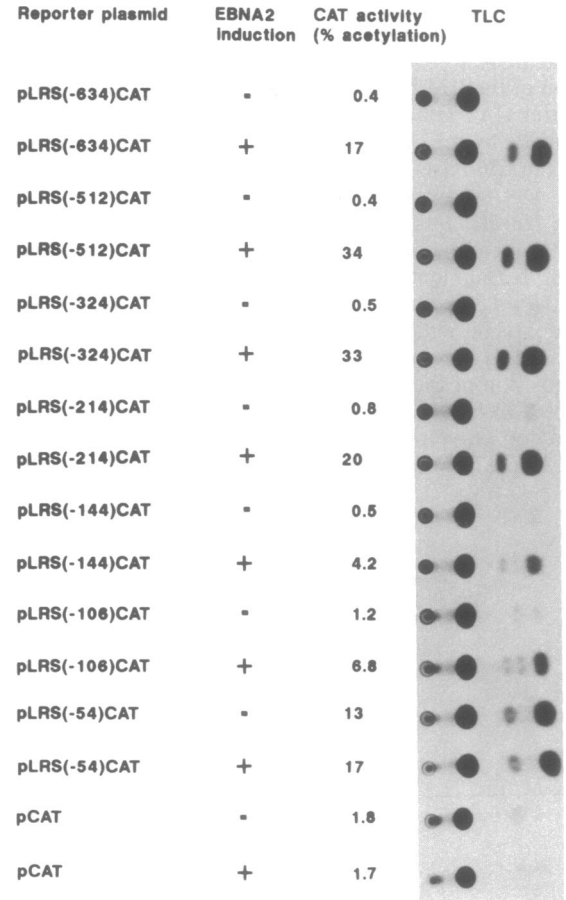

FIG. 3. Identification of positive and negative elements in the LMP regulatory region. Reporter plasmids containing the CAT gene under the control of the 5′ end-deleted LRS fragments indicated in Fig. 1 were transfected into DG75 cells together with pEΔA6 (EBNA2 induction +) or with an equivalent amount of pSV2-gpt (EBNA2 induction ▪). The cells were subsequently lysed and assayed for CAT activity as described (16). The autoradiogram of one such experiment is shown, but similar results were obtained in at least three (depending on the construct) independent experiments.

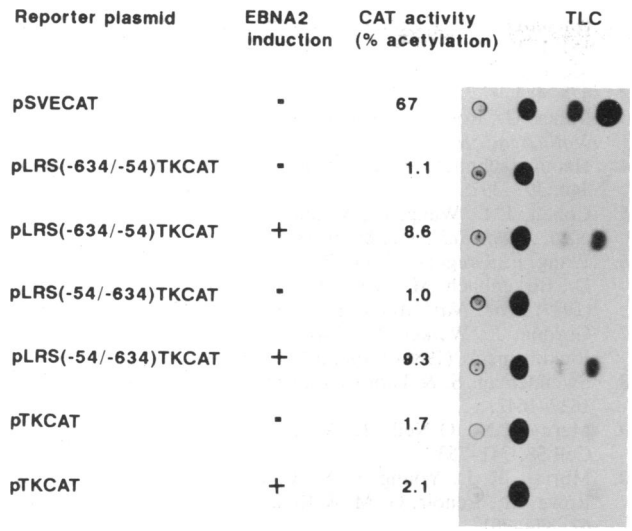

FIG. 4. LRSs can confer EBNA2 inducibility on a heterologous promoter. DG75 cells were transfected with the reporter plasmids indicated together with pEΔA6 (EBNA2 induction +) or with an equivalent amount of pSV2-gpt (EBNA2 induction •), and the expression of CAT was determined. The −634/−54 DNA fragment of the LMP regulatory region was inserted in both orientations immediately upstream of the TK promoter. Similar results were obtained in two independent experiments.

The results of the deletion analysis suggest that sequences between −324 and −144 participate in maintaining a high level of transcription from the LMP promoter after induction with EBNA2. Deletion of sequences from −106 to −54 results in a truncated fragment that is an active transcription element in the absence of the inducer. Thus, the −106 to −54 region appears to contain a negative regulatory element, whereas the −54 to +40 region contains a constitutively active transcription element.

LMP Regulatory Element Can Act as an Inducible Enhancer on a Heterologous Promoter. To determine whether the −634 to −54 part of the LMP regulatory region contains sequences that can regulate transcription independently of the constitutively active promoter element, this DNA fragment was placed upstream of the TK promoter in a reporter plasmid. As shown in Fig. 4, expression of CAT was similar or slightly lower in DG75 cells transfected with the pLRS(−634/−54)TKCAT construct than in cells transfected with the TK promoter-containing vector (pTKCAT) without the LRSs. Induction by cotransfection with the pEΔA6 plasmid resulted in a significant increase in CAT expression. Very similar results were obtained with the pLRS(−54/−634)TKCAT plasmid, which carries the LMP gene fragment in the opposite orientation, positioning the LMP negative element at a considerable distance from the TK promoter (Fig. 4). One interpretation of these results is that the distal (−634 to −54) part of the LMP regulatory region contains, in addition to the negative regulatory element, a positive element with some characteristics associated with viral enhancer elements, including orientation independence, ability to regulate a heterologous promoter, and ability to function at a distance from a promoter.

DISCUSSION

Murray *et al.* (10) have found that LMP is regularly expressed in originally EBV-negative BL lines converted with the transforming B95-8 virus but is absent from parallel sublines converted with the EBNA2 defective P3HR-1 viral substrain. By transfection of plasmids capable of expressing EBNA2 into P3HR-1 virus-converted BL lines, Abbot *et al.* (11) demonstrated that expression of EBNA2 is consistently associated with activation of LMP expression (11). However, it was not possible from these studies to infer whether the induction of LMP expression occurred at the level of initiation of transcription or at some other level of regulation. Very recently, Ghosh and Kieff (21) showed that the LMP regulatory region contains at least two positive cis-acting transcription elements. The data suggest that LMP promoter activity is regulated by EBNA2 or EBNA5 trans-activation. Using a cotransfection assay, we have now demonstrated that the 5′ flanking region of the LMP gene contains positive and negative regulatory elements and that EBNA2, directly or indirectly, can activate transcription of the LMP promoter. The LMP regulatory region contains inducible enhancer elements capable of acting on a heterologous promoter. The simplest interpretation of our results is that EBNA2 acts by interfering with the function of a transcriptional repressor. The possibility that LMP expression may be negatively regulated was previously suggested by studies on somatic cell hybrids showing that the EBV-negative Ramos BL line contains negative trans-acting factors that can inhibit LMP expression in Raji cells (22).

Evidence for the importance of cis-acting negative regulatory sequences in eukaryotic transcriptional control has accumulated recently, and different models for the mechanism of transcriptional repression involving sequence-specific DNA-binding proteins have been proposed (reviewed in ref. 23–25). It is certainly premature at this stage of our investigation to speculate on the detailed mechanism by which the expression of the LMP gene might be regulated. However, certain similarities between the regulatory region of the LMP gene and that of the well-characterized human β-interferon gene might be worth considering. Virus or double-stranded RNA induction of the expression of the β-interferon gene requires a 40-bp DNA sequence in the 5′ flanking region of the gene (the interferon gene regulatory element or IRE) (26–30). The IRE sequence contains two positive transcription elements that are prevented from functioning in the uninduced cell by an adjacent or overlapping negative regulatory element. Like LRS, the IRE is an inducible enhancer capable of acting on a heterologous promoter. The prevalent idea is that the β-interferon gene is switched off by a repressor and induction involves the inactivation or replacement of the repressor and binding of transcription factors to both positive regulatory domains (29).

It seems clear that EBNA2 can act as an inducer of the expression of the LMP gene, and the present study provides data supporting the notion that induction occurs at the level of transcription initiation. However, it is also clear that EBNA2 is not an essential factor for LMP expression in all EBV-transformed cells. The LMP gene was expressed in about half of the nasopharyngeal carcinoma tumor biopsies tested in spite of EBNA2–EBNA6 being down-regulated (31). It is also expressed in the P3HR-1 cell line, which harbors a virus with a deleted EBNA2 gene, although the virus itself does not induce the expression of LMP in BL cells (10). Obviously the LMP promoter can be activated by means of mechanisms not involving EBNA2 but cellular factors only. An attractive hypothesis would be that the transcriptional activity of the LMP regulatory region is determined by a balance between the activity of negative and positive cellular factors. The role of EBNA2 would then be to tip the balance either by increasing the activity of the positive factors or by decreasing the activity of the negative factors. These factors might be present in a different state either quantitatively or qualitatively (or both) depending on the cell type, in some cases eliminating the need for EBNA2 in LRS activation. A similar model has been proposed to explain the cell type specificity of the polyoma virus enhancer (32). The activity of the α-domain of the enhancer was determined by the balance

between the activities of two factors, PEA2, a labile repressor, and PEA1, an inducible positive factor that resembles the transcription factor AP1. Changes in repressor activity contributed to the increase in α-domain activity after differentiation of F9 cells.

As noted above, a similar balance between the activities of negative and positive factors seems to be involved in the regulation of β-interferon transcription (29). Furthermore, β-interferon mRNA is detected as early as 1.5 hr after the addition of inducer, and induction does not require protein synthesis (33), suggesting that the β-interferon promoter is activated by posttranslational modification of a preexisting factor in response to the induction signal. The finding that LMP expression is induced in the EBNA2-defective, LMP-negative Daudi BL cell line within 3 hr after superinfection with P3HR-1 virus or UV-inactivated B95-8 virus (34) suggests that a similar mechanism may operate in the activation of the LMP promoter.

The EBV persists in the small, heavy B-cell fraction in healthy seropositive individuals (35). It has been postulated that these cells may express EBNA1, but not EBNA2–EBNA6 or LMP, in analogy with the phenotypically somewhat similar BL cell (36). Studies on EBV gene expression in virus-carrying cells of different phenotypes, EBV convertants of EBV-negative BL lines, and somatic cell hybrids between EBV-carrying and EBV-negative cells have indicated that the genes for the seven growth transformation-associated EBV-encoded proteins (EBNA1–EBNA6 and LMP) relate to their phenotype-dependent host cell control in three different ways. EBNA1 is expressed in all EBV genome-carrying cells so far studied, irrespective of phenotype. EBNA2 (and probably EBNA3–EBNA6 that may be coregulated with EBNA2) is only expressed in B cells of an immunoblastic (LCL-like) phenotype. LMP can be expressed independently of the EBNA2–EBNA6 complex in cells of certain phenotypes (e.g., in P3HR-1 and NPC cells as already mentioned), whereas in others, like the DG75 BL line, it requires EBNA2 for expression. The following tentative scenario can be formulated on the basis of these facts. Primary EBV infection of normal resting B lymphocytes is followed by blast transformation and the expression of all seven proteins. EBNA1 and EBNA2 are expressed prior to the induction of the cellular S phase. At this stage transcription of the EBNA1 and EBNA2 genes is presumably initiated at the *Bam*HI W promoter, which is B-cell specific and does not require viral gene products for its activity (16). A cascade of events is initiated where EBNA1 activates *ori*P and the *Bam*HI C promoter, and EBNA2 serves as an inducer of LMP expression. The precise time kinetics for the appearance of EBNA3–EBNA6 has not been established.

If the BL cell can be taken as the neoplastic counterpart of the normal resting B-cell responsible for the latent persistence of the virus, and if DG75 is taken to represent a typical BL cell, it would follow that such cells down-regulate EBNA2 and LMP through at least two different cellular regulatory factors. Reactivation of such cells—e.g., by mitogens or antigens—would induce the expression of EBNA2. This, in turn, would derepress LMP synthesis by inhibiting the effect of a negative cellular transcriptional factor of the type detected in the present study.

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