JOURNAL OF VIROLOGY, Feb. 1989, p. 615–623
0022-538X/89/020615-09$02.00/0

Vol. 63, No. 2

The Epstein-Barr Virus (EBV) DR Enhancer Contains Two Functionally Different Domains: Domain A Is Constitutive and Cell Specific, Domain B Is Transactivated by the EBV Early Protein R

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Received 18 July 1988/Accepted 31 October 1988

The Epstein-Barr virus (EBV) DR promoter is located upstream of the *Pst*I repeats, and besides the TATA box, it contains two *cis*-acting regulatory elements. One of them has enhancer properties. To define more precisely the functional region(s) in the DR enhancer, we generated 5′ and 3′ deletion mutants. These deletion mutants, which were transfected into various recipient cells of different origins, allowed us to identify two functionally distinct domains, A and B. Domain A was constitutively active in all cell lines tested, except in lymphoid B cells. Domain B was active in lymphoid B cells, and its activity required both EB1 (the BZLF1-encoded EBV *trans*-acting factor) and the presence of the EBV genome. This suggested that an EBV-encoded, EB1-inducible factor was activating the enhancer B domain. In effect, the B domain was *trans*-activated by R, an EBV early product encoded by the open reading frame BRLF1, and the activation by R occurred in epithelial, fibroblastic, and lymphoid cells. The R-responsive element has been reduced to 28 base pairs containing the double palindromic sequence TTGTCCCGTGGACAATGTCC. Both domains A and B act by increasing the initiation of specific RNAs.

The Epstein-Barr virus (EBV) DR promoter controls the expression of a 102-base-pair (bp) *Pst*I repeat region (the IR4 region, Fig. 1F) in the EBV genome (23). The IR4 region is homologous to another EBV repeat cluster which contains the 125-bp *Not*I repeats (the IR2 region), and the IR2 repeats are expressed under the control of the DL promoter (Fig. 1F), which is almost a perfect duplication of the DR promoter region (Fig. 1G) (1, 23). These duplicated promoters are located 100 kilobase pairs apart from each other, one in the right part (DR) and the other in the left part (DL) of the EBV genome (Fig. 1E) (15, 18, 20, 21, 32). In addition, a recent publication suggests that the DL promoter could be bidirectional (14) and could control the expression of the EA-R open reading frame (ORF) BHRF1 in a rightward direction (Fig. 1F and 1G). The DR and DL promoters are not detectably active during latency. However, after disruption of latency, they promote the synthesis of abundant mRNAs (3, 7, 10, 20, 27). The IR4 and IR2 repeats are included in ORFs, and they could potentially code for proteins with a repetitive amino acid structure. In addition, DR is conserved in the highly deleted P3HR-I defective genome, and DL is not. DR is also located in a region of intramolecular recombination as revealed by the analysis of the structure of the P3HR-I defective genome (4, 30).

Recently, several EBV *trans*-acting factors involved in the switch from latency to a productive cycle have been identified (Fig. 1B). The BZLF1 ORF encodes EB1, which seems to have a key role in the establishment of the lytic cycle (3, 5, 6, 36). EB2 is encoded by the BMLF1 and BSLF2 ORFs and activates its own promoter and several other heterologous viral promoters (25, 38). The BRLF1 ORF encodes an early protein that activates the expression of the adenovirus E3 promoter as well as the chloramphenicol acetyltransferase (CAT) gene cloned upstream of the DL promoter in the rightward direction for transcription (Fig. 1G) (14).

It has been shown that the DR promoter is activated in EBV latently infected B cells (3) and in HeLa cells (2), when EB1 is expressed. In addition, it has been shown that once activated by EB1, two *cis*-acting regulatory elements can be detected in the DR promoter; one is proximal to the CAP site, and the other is distal to the CAP site (Fig. 1G). The distal one is located 639 bp upstream from the CAP site. It is included in a 258-bp *Ava*I DNA fragment, and it has enhancer properties (Fig. 1G) (2). Briefly, in HeLa cells, it can substitute in both orientations for the simian virus 40 (SV40) enhancer, and it can activate the herpes simplex virus (HSV) thymidine kinase (tk) promoter cloned upstream of the CAT gene (tk-CAT) when located in inverted orientation 5′ and 3′ to the tk-CAT chimeric gene. However, in lymphoid B cells, the DR enhancer requires both the presence of the EBV genome and the expression of EB1 to be active. It should also be noted that the putative EA-R TATA box (14) is located about 70 bp downstream of the 5′ end of the DR enhancer (Fig. 1G).

To define more precisely the sequences involved in the enhancer functions described above, we undertook a bidirectional *Bal* 31 mutational analysis of the 258-bp DNA fragment containing the DR enhancer. The enhancer activities of the mutants were evaluated by determining their abilities to activate transcription from the SV40 enhancerless early promoter after transfection into various recipient cells of lymphoid and nonlymphoid origins. Two functionally distinct domains, A and B, were identified. Domain A was only active in nonlymphoid cells. Domain B was only active in lymphoid B cells that contain the EBV genome and when the EB1 *trans*-acting factor was expressed. However, domains A and B were inactive in all the EBV genome-negative lymphoid B cells tested. Moreover, domain B (but not domain A) became active in these cells when they were

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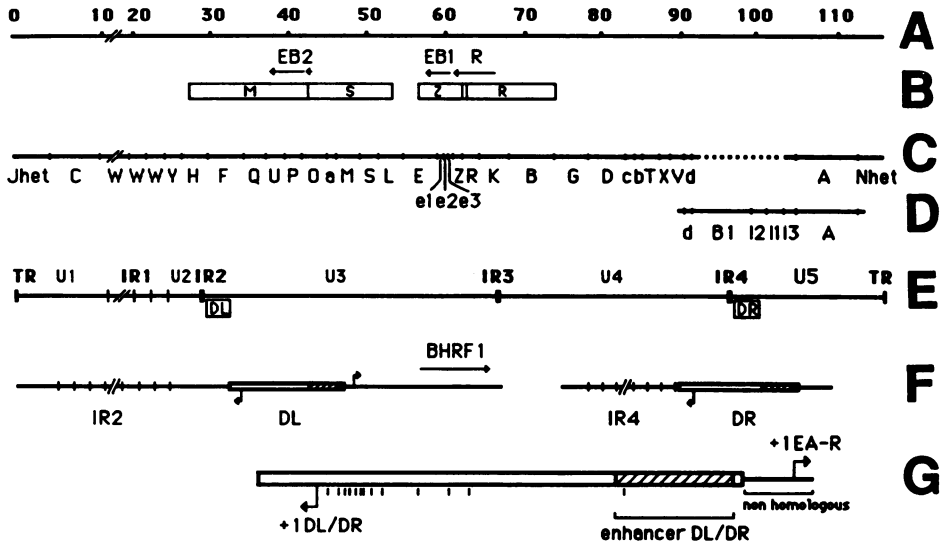

FIG. 1. Organization of the EBV genome. (A) Size scale (in kilodaltons). (B) *Bam*HI fragments containing the coding sequences for the known EBV early gene transactivators. The arrows below the DNA fragments represent the leftward ORFs coding for the transactivators. The provisional names of the transactivators are indicated on the arrows. (C) Map of EBV B95-8 genome. Letters indicate *Bam*HI fragments; the nomenclature is according to reference 1. The dotted line represents the large deletion in the B95-8 genome compared with the M-ABA genome. (D) *Bam*HI M-ABA fragments from *Bam*HI-d to *Bam*HI-A. (E) Organization of the EBV genome. TR indicates the terminal repeats. IR1 to IR4 are the largest internal repeats. U1 to U5 are the unique sequences located between the repeats. DL and DR are the homologous duplicated promoters controlling the expression of IR2 and IR4, respectively. (F) Organization of IR2-DL and IR4-DR regions. Vertical lines are the start sites of transcription. The open boxes correspond to the promoters, and the hatched regions of the boxes correspond to the enhancer (see Results). (G) Enlarged representation of homologous DL and DR promoters. The vertical bars represent the locations of the 13 nonhomologous bp between DL and DR (23). The putative start site for the EA-R mRNAs (14) located in the nonhomologous region between DL and DR is indicated.

newly infected by EBV and when EB1 was expressed. This suggests that a product(s) encoded by the EBV genome and induced by EB1 *trans*-activates the DR enhancer B region. Indeed, an EBV early gene product encoded by the ORF BRLF1 *trans*-activated the B domain, and the R-responsive element was located in a 28-bp DNA fragment.

MATERIALS AND METHODS

Cell lines. The EBV genome-negative human lymphoid B-cell lines BJA-B, Louckes, and BL41 were gifts from G. Lenoir. The BJA-B cell line and the BL41 cell line were newly infected by the EBV strains B95-8 for BJA-B cells (cBJAB/B95) and P3HR-I for BL41 cells (cBL41/P3HR1). The EBV latently infected human B-cell lines Raji-tk$^-$, P3HRI-tk$^-$, and BJA-B/B95-8 have been described elsewhere (3). MRC5 cells are diploid human embryonic lung fibroblasts (American Type Culture Collection) and were a gift from L. Gazzolo. Vero cells were a gift from I. Machuca. MCF7 cells are immortalized human breast tumor cells and were a gift from P. Chambon. The CNE immortalized epithelial cells were a gift from H. Wolf. This cell line was established originally from an EBV genome-positive NPC tumor, and it lost the EBV genome after several passages.

Cloned DNA templates. The EB1- and EB2-expressing vectors have been described extensively elsewhere (3). Briefly, they are pUC18 derivatives containing the ORFs BZLF1 and BMLF1 plus BSLF2 that code for the EBV *trans*-activating factors EB1 and EB2, respectively, placed under the control of the SV40 early promoter-enhancer. Plasmid pSV2CAT is the SV40 early promoter-enhancer cloned in front of the bacterial CAT-coding sequences. Plasmid pSCAT was made by ligating the SV40 *Hin*dIII-*Sph*I DNA fragment containing the SV40 early promoter devoid of enhancer sequences to pUC18 cut by *Hin*dIII and *Sph*I and to a *Hin*dIII DNA fragment containing the bacterial CAT-coding sequences isolated from plasmid p1-3CAT (34). Plasmids pSCAT258+ and pSCAT258− were made by ligating the DR 258-bp *Ava*I DNA fragment in both orientations (sense and antisense relative to the SV40 T-antigen-coding sequence) in the unique *Ava*I site of pSCAT (Fig. 2). Plasmid pBLCAT2 contains the HSV tk promoter cloned upstream of the CAT-coding sequence (Fig. 3). Plasmid pBLCAT2-A5′ was made by ligating the DR enhancer A region (nucleotides −639 to −736) in the pBLCAT2 unique *Xba*I site (Fig. 3A). Plasmid pKSVR was made by ligating a *Dra*I DNA fragment isolated from a cDNA clone which contains the entire BRLF1 ORF (unpublished results) to plasmid pKSVδ, which is a modified version of the SV40 expression vector pKSV10 (Pharmacia, Inc.). The initial unique internal *Eco*RI and *Bgl*II sites were mutated, and a new *Eco*RI site was created instead of the *Bgl*II site by introducing *Eco*RI linkers (see Fig. 6A). Plasmid pG is a gift from P. Jalinot. It contains the rabbit β-globin gene with the m13mp12 polylinker cloned 5′ to the β-globin promoter (19). Plasmids pGA, pGB, and pGB0 were made by ligating the A domain, the B domain, and a subregion of the B domain (oligo B0) of the DR enhancer 5′ to the β-globin promoter (see Fig. 8).

Construction of 5′ and 3′ enhancer deletion mutants. To generate mutants with deletions extending in a 5′-to-3′ direction and in a 3′-to-5′ direction from the DR enhancer, plasmids pSCAT258+ and pSCAT258− (Fig. 2A and B) were linearized at a unique *Sac*I site located upstream of the enhancer. The linearized DNAs were digested with *Bal* 31 exonuclease for various periods of time. The extent of digestion for each time point was determined by restriction

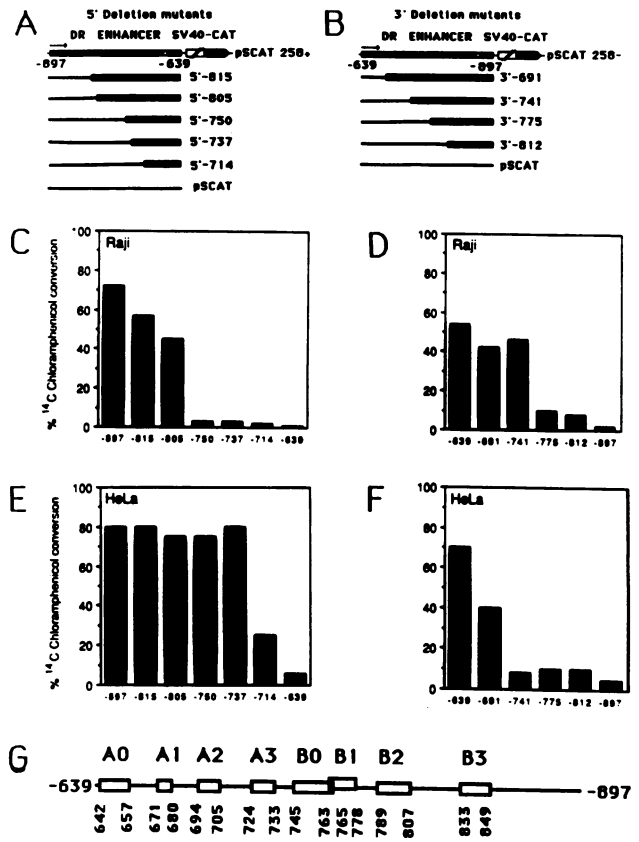

FIG. 2. The EBV DR enhancer has two functionally distinct domains, A and B. Progressive *Bal* 31 deletions were made in the DR enhancer from either the 5′ or the 3′ end as described in Materials and Methods. Numbers (−639 and −897) indicate the 3′ and the 5′ ends of the 258-bp DR enhancer fragment with respect to the CAP site of the IR4 gene. (A) Mutants generated by progressive *Bal* 31 digestion from the 5′ end. (B) Mutants generated by progressive *Bal* 31 digestion from the 3′ end. The sizes of the remaining DNA sequences in the mutants are indicated by thick lines, and the deletions are indicated by thin lines. The numbers which follow 5′- and 3′-indicate the endpoints of the deletion for each mutant. In Raji cells, the transcriptional activities of the 5′ deletion mutants (panel C) and of the 3′ deletion mutants (panel D) were assayed by cotransfection with an EB1-expressing vector. In HeLa cells, the transcriptional activities of the 5′ deletion mutants (panel E) and of the 3′ deletion mutants (panel F) were assayed by cotransfection with an EB2 expression vector. In panels C and E, plasmid pSCAT 258+ is called −897 and plasmid pSCAT is called −639. In panels D and F, plasmid pSCAT258− is called −639 and plasmid pSCAT is called −897. CAT assays were made under the following conditions: for Raji cells, 15% (vol/vol) of the extract was incubated in a 10-min reaction, and for HeLa cells, 10% (vol/vol) was incubated in a 10-min reaction. The transcriptional activity of each mutant was estimated by determining the percentage of the [^{14}C]chloramphenicol acetylated by the CAT enzyme present in cell extracts. (G) Map positions of palindromic sequences present in the DR enhancer.

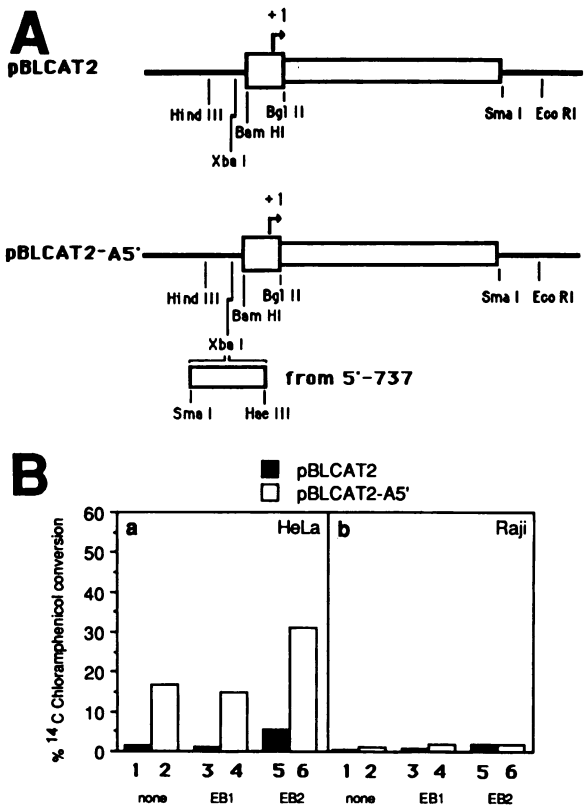

FIG. 3. Functional specificities of domain A. The DNA fragment containing the A domain of the DR enhancer was cloned upstream of the HSV tk promoter in plasmid pBLCAT2. (A) Construction of plasmid pBLCAT2-A5′. The DNA fragment extending from nucleotides −639 and −736 (*Sma*I-*Hae*III) in mutant 5′-737 (Fig. 2G) was cloned in the unique *Xba*I site in pBLCAT2. (B) CAT expression from plasmids pBLCAT2 and pBLCAT2-A5′ transfected in HeLa (panel a) and Raji (panel b) cells in the absence (lanes 1 and 2) and in the presence of EB1 (lanes 3 and 4) or EB2 (lanes 5 and 6) *trans*-acting factors.

enzyme analysis. Linear DNA molecules with an extent of digestion in the enhancer not exceeding 260 bp were treated by T4 DNA polymerase, ligated to *Sac*I linkers, and circularized. The endpoint of the deletion for each mutant selected was determined by sequencing (29).

Cell culture and transfections. CNE cells, EBV-infected B cells (Raji-tk$^-$, P3HRI-tk$^-$, BJA-B-B95-8, cBJAB/B95, and cBL41/P3HR1) and the EBV genome-negative B cells BJA-B, BL41, and Louckes were grown at 37°C in RPMI 1640 (Boehringer Mannheim Biochemicals) containing 10% (vol/vol) fetal calf serum. HeLa, Vero, MRC5, and MCF7 cells were grown in Dulbecco modified Eagle medium (GIBCO Diagnostics) supplemented with 10% (vol/vol) fetal calf serum. The plasmids used for transfection were prepared by the alkaline lysis method and purified through two CsCl gradients. One day before transfection, lymphoid B cells were suspended at a density of 5×10^5 cells per ml in fresh medium, and CNE cells were seeded at 10^6 cells per 100-mm petri dish. These cells were transfected by the DEAE-dextran procedure (26). HeLa, Vero, MRC5, and MCF7 cells were seeded at 10^6 cells per 100-mm petri dish 24 h prior to transfection, and the medium was changed 4 h before transfection. Transfections were performed by the calcium precipitate method (13). Cells were mixed with the appropriate DNA(s), and the DNAs were in the same topological state, as determined by agarose gel electrophoresis.

RNA extraction and S1 nuclease mapping. The cells transfected with plasmids carrying the β-globin gene were lysed by Nonidet P-40 as described elsewhere (19). Nuclei were pelleted, and RNAs were phenol extracted from the cytoplasmic fraction. Total cytoplasmic RNAs (10 to 40 μg) were hybridized overnight at 30°C in 50% formamide–0.3 M NaCl–0.01 M Tris hydrochloride, pH 7.4, to a 60-base

5′-^{32}P-labeled synthetic single-stranded DNA probe (see Fig. 8). The hybrids were digested for 2 h at 20°C with 5 U of S1 nuclease per 10 μg of RNA. The size of the S1-protected DNA fragment was analyzed on 6% (wt/vol) polyacrylamide–8.3 M urea gels.

CAT assays. CAT assays were performed essentially as described previously (12). However, instead of sonication, cell lysis was obtained by treatment with 0.05% sodium dodecyl sulfate. An adequate volume of each extract and the time of the reaction were chosen in order to be in the linear range of the assay. To ascertain that the CAT enzyme activities obtained after transfection of cells with CAT-containing templates were reproducible, for most experiments, each transfection was duplicated and each experiment was repeated at least two times.

RESULTS

DR enhancer is composed of two functionally distinct domains. To define more precisely the *cis*-acting elements involved in the DR enhancer activity, we progressively deleted the DR enhancer sequences either from the 5′ end or from the 3′ end. The deletions were generated from a unique *Sac*I site in pSCAT258+ for mutants deleted from the 5′ end (Fig. 2A) and in pSCAT258− for mutants deleted from the 3′ end (Fig. 2B). These two plasmids contain the DR enhancer, in either the sense (pSCAT258+) or the antisense orientation (pSCAT258−) with respect to the SV40 early coding sequences. We checked the activity of the EBV DR enhancer deletion mutants in cell lines of different origins because although it seems that EBV is only able to infect lymphoid B cells, the EBV genome was nevertheless found (37) and transcribed (35) in vivo in human epithelial cells from nasopharyngeal carcinomas. Accordingly, Raji cells were used as the EBV genome-positive B cells and HeLa cells were used as the human epithelial cells.

We have previously shown that the DR enhancer could activate transcription from the SV40 early promoter in HeLa cells (2). However, although EB2 did not increase the enhancer activity, EB2 strongly activated transcription from the SV40 early promoter linked to the DR enhancer and allowed a better visualization of the enhancer effect. In Raji tk$^-$ cells, the enhancer activity was not detectable unless induced by EB1 (2). For these reasons, in order to be able to efficiently detect every variation in the activity of the DR enhancer deletion mutants linked to the SV40 early promoter, we cotransfected the DR enhancer mutants with an EB2 expression vector in HeLa cells, and we cotransfected the DR enhancer mutants with an EB1-expressing vector in Raji cells. The results of the enhancer mutant transient expression in HeLa and Raji cells are shown in Fig. 2C to F.

In Raji cells, when deletions progressively extended in a 5′-to-3′ direction from the 5′ side of the DR enhancer (Fig. 2A, 5′ deletion mutants), there was at first a less than twofold decrease in the enhancer activity when the sequences between positions −897 and −805 were deleted (Fig. 2C). There was another decrease of more than eightfold when sequences between positions −805 and −750 were deleted. Further deletions did not detectably affect the enhancer activity. For deletions extending in a 3′-to-5′ direction (Fig. 2B), a drastic decrease in the CAT enzyme activity was observed when sequences between positions −741 and −775 were deleted, and the enhancer activity was not significantly affected by additional deletions (Fig. 2D). These results suggest that sequences located between nucleotides −741 and −897 were important for the activity of the EBV DR enhancer in Raji cells.

In HeLa cells, for deletions extended in a 5′-to-3′ direction (Fig. 2A), the DR enhancer activity was reduced by a factor of about 16-fold when sequences between −737 and −639 were deleted (Fig. 2E). This was confirmed by transfection in HeLa cells of the enhancer mutants with deletions extending in a 3′-to-5′ direction (Fig. 2B). A significant decrease in enhancer activity was observed when sequences extending between −639 and −741 were deleted (Fig. 2F). This suggested that sequences located between −639 and −737 were important for the function of the DR enhancer in HeLa cells.

From the results presented in Fig. 2, we could conclude that the EBV DR enhancer had two functional domains. Domain A, located between −639 and −737, was active in HeLa cells. We also found that region A activated transcription in other nonlymphoid cell lines (Vero, CNE, MCF7, and MRC5 [data not shown]). However, domain A was not detectably active in the EBV latently infected lymphoid B-cell line Raji (Fig. 2D) and in the other EBV genome-positive B cells tested (BJA-B-B95-8 and P3HR-1 tk$^-$ [data not shown]). Domain B, located between −741 and −897, was activated by EB1 in Raji cells. We obtained similar results after transfection of the mutants in the presence of the EB1-expressing vector in the EBV latently infected B-cell lines (P3HR1-tk$^-$ and BJA-B-B95-8 [data not shown]). The B region was not detectably active, however, in HeLa cells (Fig. 2F) and in all the other non-B-cell lines tested (Vero, CNE, and MCF7 [not shown]).

Domain A is constitutively active in HeLa cells. Domain A can activate the SV40 early promoter, but this activation was only clearly detected when an EB2-expressing vector was cotransfected. It has been previously shown that in HeLa cells, the DR enhancer stimulated transcription from the HSV tk promoter in the absence of any EBV *trans*-acting factor (2). According to the results presented above, the stimulatory effect on the HSV tk promoter should be due to domain A. To confirm this point, we cloned the putative A domain (nucleotides −639 to −736) of the DR enhancer upstream of the HSV tk promoter in plasmid pBLCAT2 (Fig. 3). We generated the plasmid pBLCAT2-A5′ (Fig. 3A), which contains the A domain cloned upstream of the HSV tk promoter. Figure 3B shows the results of CAT enzyme transient expression from this plasmid after transfection in HeLa and Raji cells compared with CAT expression from the control plasmid pBLCAT2.

In HeLa cells, CAT enzyme was 11-fold more expressed from the plasmid pBLCAT2-A5′ than from plasmid pBLCAT2 (Fig. 3B, panel a; compare lanes 1 and 2). As has been published elsewhere (25, 38), EB2 had a stimulatory effect on the tk promoter (Fig. 3B, panel a, lane 5). However, our results show that EB2 has no effect on domain A activity when cloned upstream of the tk promoter (Fig. 3B, panel a; lane 6). In effect, although the activities of plasmids pBLCAT2 and pBLCAT2-A5′ were increased by EB2 (Fig. 3B, panel a; compare CAT activities in lanes 1 and 2 with CAT activities in lanes 5 and 6), the enhancing activity of domain A remained unchanged (compare CAT activities between lanes 1 and 2 and CAT activities between lanes 5 and 6). As expected, EB1 had no effect on the transcriptional activities of pBLCAT2 and pBLCAT2-A5′ (Fig. 3B, panel a; lanes 3 and 4). From these results, we concluded that domain A constitutively activated the HSV tk promoter in HeLa cells. As shown in Fig. 3B, panel b, domain A was almost inactive (pBLCAT2-A5′ was 1.5- to 2-fold more active than pBLCAT2, as deduced from CAT enzyme expression) in Raji cells, and this inactivity was unchanged in the presence of EB1 (lanes 3 and 4) or EB2 (lanes 5 and 6).

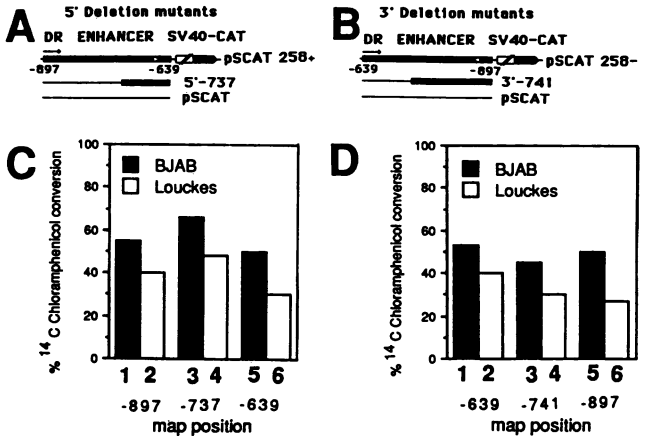

FIG. 4. Both domains A and B of the DR enhancer are inactive in the EBV genome-negative B cells B-JAB and Louckes. Deletion mutants which contain the A region (mutant 5'-737) (A) or the B region (mutant 3'-741) (B) of the DR enhancer, as well as plasmids pSCAT, pSCAT258+ and pSCAT258−, were cotransfected in BJA-B and Louckes cells with EB1- and EB2-expressing vectors. (C) CAT expression from pSCAT 258+ (−897; lanes 1 and 2), from mutant 5'-737 (−737; lanes 3 and 4) and from pSCAT (−639, lanes 5 and 6). (D) CAT expression from pSCAT258− (−639; lanes 1 and 2), from mutant 3'-741 (−741; lanes 3 and 4) and from pSCAT (−897; lanes 5 and 6). CAT assays were performed under the following conditions: 70% (vol/vol) of the extract was incubated in a 60-min reaction. Consequently, the amount of CAT enzyme expressed from pSCAT-transfected plasmid in BJAB cells corresponds to 5×10^{-3} U per 10^6 cells per min.

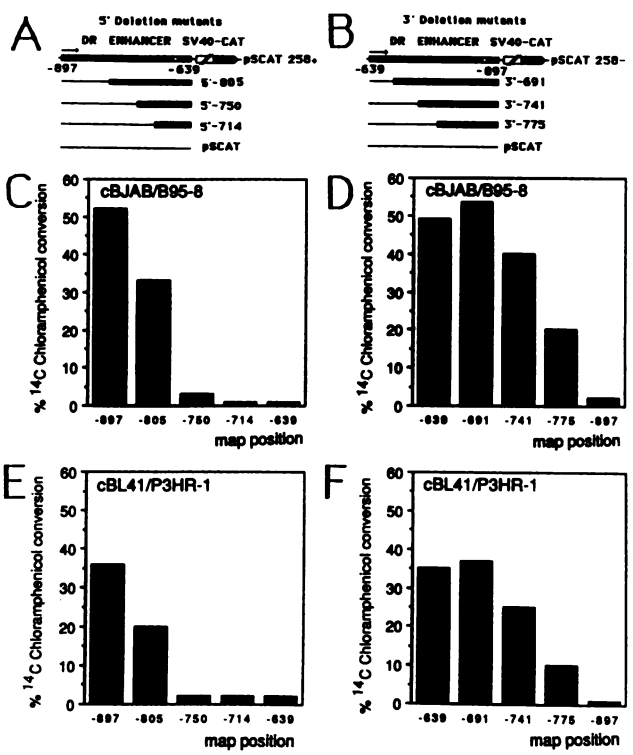

FIG. 5. Domain B of the DR enhancer became active in B cells infected by the EBV strains B95-8 and P3HR-I. cBJAB/B95-8 and cBL41/P3HR-I cell lines were obtained by infection of the EBV genome-negative cells BJA-B and BL41 with the B95-8 and P3HR-I viral strains, respectively. These new cells were cotransfected with the EB1-expressing vector and (A) with pSCAT258+ (−897), the 5' deletion mutants −805, −750, and −714, and pSCAT (−639) or (B) with pSCAT258− (−639), the 3' deletion mutants −691, −741, and −775, and pSCAT (−897). (C) CAT expression from 5' deletion mutants in cBJAB/B95-8 cells. (D) CAT expression from 3' deletion mutants in cBJAB/B95-8 cells. (E) CAT expression from 5' deletion mutants in cBL41/P3HR-I cells. (F) CAT expression from 3' deletion mutants in cBL41/P3HR-I cells. CAT assays were performed under the following conditions: 15% (vol/vol) of the extract was incubated in a 15-min reaction. Consequently, the amount of CAT enzyme expressed from pSCAT-transfected plasmid in cBJAB/B95-8 cells corresponds to 9×10^{-3} U per 10^6 cells per min.

Our results confirmed that the DR enhancer had two functionally distinct domains, A and B. Domain A had a constitutive activity in HeLa cells, and its activity was not influenced by the EBV transactivators tested. Domain A was not significantly active in Raji cells compared with HeLa cells.

Domains A and B of the EBV DR enhancer are inactive in EBV genome-negative lymphoid B cells, but only domain B becomes active in these cells infected by EBV strains B95-8 and P3HR-1. From the results presented above, it is clear that the enhancer B domain was activated by EB1 in B cells infected by EBV and that domain A was inactive in all EBV-infected B cells tested. However, it has been shown that the DR enhancer was inactive in the EBV genome-negative BJA-B cells and that the SV40 enhancerless early promoter was only detectably active in BJA-B cells when cotransfected with EB1- and EB2-expressing vectors (2). We therefore tested the activities of domains A and B in the EBV genome-negative cell lines BJA-B and Louckes by cotransfecting the 5' (Fig. 4A) and the 3' (Fig. 4B) deletion mutants made from pSCAT258+ and pSCAT258− with EB1- and EB2-expressing vectors. Deletion of either domain B sequences (Fig. 4C) or domain A sequences (Fig. 4D) of the DR enhancer had no significant effect on the amount of CAT enzyme expressed from the deletion mutants compared with pSCAT, the control plasmid. We obtained similar results with BL41 cells (data not shown), which are also EBV genome-negative lymphoid B cells. However, domain B was active in lymphoid B cells infected by EBV when EB1 was expressed (see Fig. 2C and D). This suggested that there might be a link between the presence of the EBV genome and the activity of domain B. To test this hypothesis, we cotransfected the DR enhancer 5' (Fig. 5A) and 3' (Fig. 5B) deletion mutants and an EB1-expressing vector in new cell lines obtained after infection of BJA-B and BL41 cells with the EBV strains B95-8 and P3HR-1, respectively. As expected, both domains A and B were inactive in BJA-B and BL41 cells. However, domain B became active in cBJA-B/B95-8 cells, which are BJA-B cells newly infected by the EBV strain B95-8 (Fig. 5C and D) and in cBL41/P3HR-I cells, which are BL41 cells newly infected by the EBV strain P3HR-1 (Fig. 5E and F) when EB1 was expressed. In both cell lines, deletion in the 5' direction of the B domain sequences located between nucleotides −897 and −750 impaired the enhancer activity (Fig. 5C and E). Similarly, in both cell lines, deletion in the 3' direction of the domain B sequences located between nucleotides −691 and −897 impaired the enhancer activity (Fig. 5D and F). In no case did deletion of the A domain sequences located between nucleotides −639 and −691 have an effect on the enhancer activity (Fig. 5D and F). From these results one could conclude that some of the B domain sequences are overlapping sequences in the A domain and that these sequences are

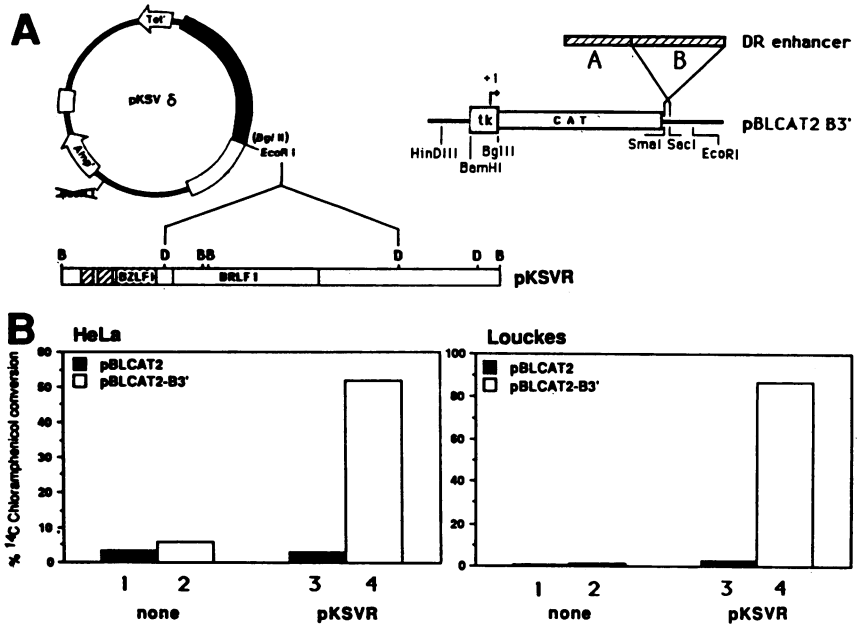

FIG. 6. The B domain of the DR enhancer is activated by the EBV R transactivator. (A) Construction of the plasmids used in the transfections. For plasmid pKSVR, a *Dra*I-*Dra*I fragment containing the entire BRLF1 ORF was excised from a cDNA clone which contains the entire *Dra*I fragment (E. Manet, unpublished results) and cloned bluntly in the T4 DNA polymerase-treated *Eco*RI site of plasmid pKSVδ (see Materials and Methods). pKSVδ contains the SV40 promoter-enhancer region (open box) and maturation signals (black box). Restriction site abbreviations: B, *Bam*HI; D, *Dra*I. For plasmid pBLCAT2-B3', the B domain of the DR enhancer was cloned in the *Sma*I-*Sac*I sites downstream of the CAT-coding sequence in plasmid pBLCAT2. (B) CAT expression in HeLa and Louckes cells from plasmid pBLCAT2 or pBLCAT2-B3' transfected in the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of plasmid pKSVR.

located between nucleotides −691 and −741. This would also explain the weak activity of the A domain in lymphoid B cells. Nevertheless, from these results one can clearly conclude that the B domain activity is linked to the presence of the EBV genome and to the expression of EB1, suggesting that an EB1-inducible EBV product was *trans*-activating domain B.

EBV early protein R, encoded by the ORF BRLF1, *trans*-activated the B domain of the DR enhancer in HeLa cells and in EBV-negative cells. The transactivator R, encoded by the BRLF1 ORF, activated the expression of the CAT gene cloned upstream of the EBV bidirectional early DL promoter in the rightward direction of transcription (14) (Fig. 1G, EA-R promoter). In the leftward direction of transcription, the DL promoter controlled the expression of a repeat cluster, the IR2 region, which contains the 125 *Not*I repeats and which is an almost perfect duplication of the DR promoter (Fig. 1F). Therefore, it could be that R *trans*-activated the B domain of the DR enhancer. To test this hypothesis, we cloned the B domain downstream of the CAT gene expressed under the control of the HSV-1 tk promoter in plasmid pBLCAT2 and generated plasmid pBLCAT2-B3' (Fig. 6A). We then investigated whether a product made from plasmid pKSVR containing the BRLF1 ORF expressed under the control of the SV40 early promoter would activate the B domain of the DR enhancer. The HSV tk promoter in plasmid pBLCAT2 had a constitutive activity in HeLa cells (Fig. 6B, lane 1), and the B domain had a twofold enhancing effect on the tk promoter activity when cloned 3' to the CAT gene in plasmid pBLCAT2 (plasmid pBLCAT2-B3') (lane 2). Cotransfection of plasmid pBLCAT2 with plasmid pKSVR (Fig. 6B, lane 3) had no detectable effect on the tk promoter activity compared with the tk promoter activity in plasmid pBLCAT2 (lane 1). However, cotransfection of plasmid pBLCAT2-B3' with plasmid pKSVR (Fig. 6B, lane 4) resulted in a 20-fold stimulation of the tk promoter activity compared with the tk promoter activity in plasmid pBLCAT2-B3' (lane 2). These results demonstrated that R, the product encoded by the BRLF1 ORF, activated the B domain of the DR enhancer in HeLa cells. This activation also occurred in the EBV genome-negative lymphoid B cells Louckes (Fig. 6C) and in Raji cells (data not shown) and was therefore not cell specific but R dependent.

R-responsive element localized within 28 bp of domain B. To localize the R-responsive region(s) in the DR enhancer B domain, we cotransfected HeLa cells with plasmid pKSVR (Fig. 6A) and with the DR enhancer mutants generated by progressive deletion from the 3' end of the B domain cloned upstream of the SV40 enhancerless early promoter (Fig. 7A). Deletions in the 3'-to-5' direction of sequences between bp −754 and −775 and between bp −812 and −897 impaired the induction of the B domain activity by R made from pKSVR (Fig. 7B). The region located between bp −754 and −775 contains a double palindromic sequence, TTGTC CCGTGGACAATGTCC. To test whether this sequence contained the R-responsive element, we synthesized the following double-stranded 28-bp oligonucleotide: CTGT GCCTTGTCCCGTGGACAATGTCCC, called oligoB0, and cloned it upstream of the rabbit β-globin promoter in plasmid pG.

Cloning of domain A, domain B, or oligoB0 upstream of the β-globin promoter increased the amount of specifically initiated β-globin transcripts. The A domain (plasmid pGA), the B domain (plasmid pGB), or oligoB0 (plasmid pGB0) of the DR enhancer was cloned 5' to the rabbit β-globin promoter in plasmid pG (19) (Fig. 8A). The enhancer potentials of the various DNA sequences inserted 5' to the β-globin promoter were analyzed by quantitative S1 nuclease mapping of

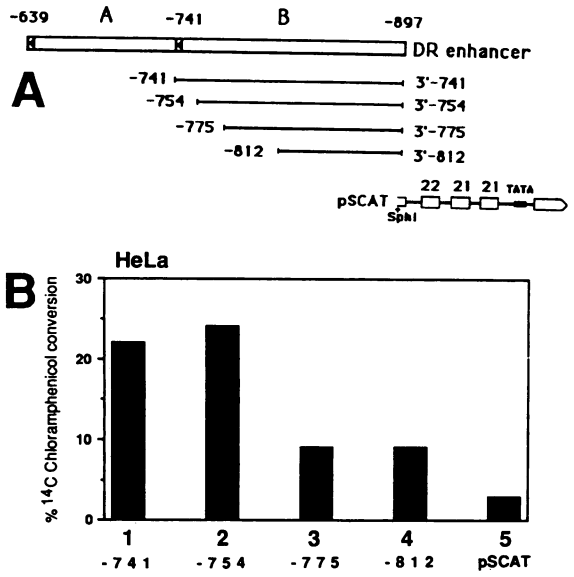

FIG. 7. Identification of the R target in the B part of the DR enhancer. (A) 3′ Deletion mutants which contain the B region (mutants 3′-741 to 3′-812) of the DR enhancer and plasmid pSCAT were transfected in HeLa cells. (B) CAT expression from the 3′ deletion mutants and pSCAT cotransfected in HeLa cells with plasmid pKSVR.

β-globin-specific transcripts. Since domain A had a constitutive activity but domain B was activated by R, the different constructions were transfected in HeLa cells either alone or with plasmid pKSVR producing R. In addition, to ascertain that a comparable amount of DNA entered the cells, 2 μg of plasmid pBLCAT2 was included in each transfection. The amount of CAT enzyme expressed was quantitated for one-fourth of the transfected cells, and a quantitative S1 nuclease mapping of specific β-globin transcripts was done on the RNAs extracted from the remainder of the cells. A comparable amount of CAT enzyme activity was found in each transfected HeLa cell extract (not shown), confirming that the tk promoter was not responsive to R (Fig. 6B) and indicating that a comparable amount of DNA entered the cells in each transfection. The results of the S1 nuclease mapping are shown in Fig. 8B. The 40-nucleotide ^{32}P-labeled DNA fragments protected from S1 nuclease digestion by specific β-globin transcripts were barely detectable in HeLa cells transfected with plasmid pG (Fig. 8B, lane 1), and the amount of transcripts was not significantly influenced by insertion of domain B (lane 3) or oligoB0 (lane 4) 5′ to the β-globin promoter. However, β-globin-specific transcripts were detected when domain A was inserted 5′ to the β-globin promoter (Fig. 8B, lane 2), which confirmed that domain A had constitutive enhancer properties. Cotransfection of plasmid pG with the R-expressing plasmid pKSVR (Fig. 8B, lane 5) resulted in a weak activation of the β-globin promoter activity. The enhancing effect of domain A seemed to be rather reduced when R was expressed (Fig. 8B, lane 6), as already observed by CAT assays (data not shown). However, R strongly stimulated the expression of specific β-globin transcripts when domain B (Fig. 8B, lane 7) or oligoB0 (lane 8) was inserted 5′ to the β-globin promoter. It should be noted that when R was expressed, insertion of domain B 5′ to the β-globin promoter resulted in a higher expression of specific β-globin transcripts compared with that observed when oligoB0 was inserted 5′ to the promoter (Fig. 8B; compare lanes 7 and 8 with lane 5), suggesting that other R-responsive sequences besides oligoB0 might be present in the B domain. These results demonstrated that an R-responsive element in the DR promoter was localized in a 28-bp double-stranded DNA fragment with enhancer properties.

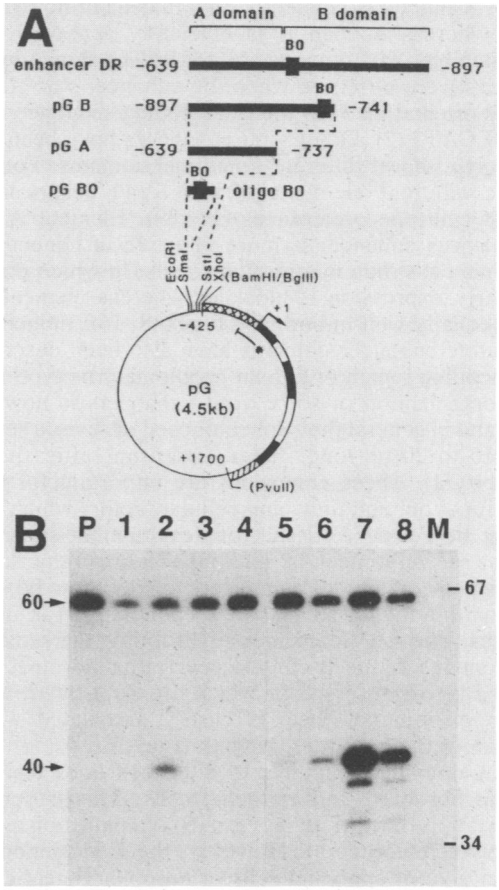

FIG. 8. Activation by R of the B0 sequence as measured by the amount of specific β-globin transcripts. (A) Construction of the plasmids pGB, pGA, and pGB0. The original plasmid pG contains the rabbit β-globin gene (19). The black and open boxes depict the exon and intron sequences, and the crossed and hatched boxes correspond to the upstream and downstream noncoding sequences, respectively. The DNA probe used for S1 nuclease mapping of the globin transcripts is indicated. It is a 5′-^{32}P-labeled 60-nucleotide-long single-stranded oligonucleotide from which a 40-nucleotide DNA fragment should be protected against S1 nuclease digestion by specific β-globin transcripts. The B and A domains of the DR enhancer as well as the B0 oligonucleotide were cloned in the polylinker of plasmid pG 425 bp upstream of the β-globin cap site. (B) Analysis of β-globin transcripts by S1 nuclease assay. Plasmids pG (lanes 1 and 5), pG A (lanes 2 and 6), pG B (lanes 3 and 7), and pG B0 (lanes 4 and 8) were transfected in HeLa cells either alone (lanes 1 to 4) or with pKSVR (lanes 5 to 8). pBLCAT2 (2 μg) was added to each transfection as an internal control. The RNAs were extracted from 75% of the cells and analyzed by the S1 nuclease assay. P, Full-length globin probe. M, Molecular weight markers. Arrows on the left side of the panel indicate the entire probe fragments (60 nucleotides) and probe fragments protected by specific transcripts initiated at the β-globin CAP site (40 nucleotides). Sizes of the markers (67 and 34 bases) are indicated on the right.

DISCUSSION

Analysis of deletion mutants in the DR enhancer allowed us to identify two functionally distinct domains, A and B.

Domain A functions efficiently only in cells of nonlymphoid origin, whereas domain B is efficiently activated by the EB1-inducible, EBV-encoded factor R. The same functional properties were observed when the enhancer was cloned in inverted orientation 3′ to the CAT-coding sequence in plasmid pBLCAT2 (2). Other viral enhancers have been shown to be composed of different functional domains. For example, the different elements of the SV40 enhancer show different cell-type preferences (17, 39). Element A of the polyomavirus enhancer is more effective in nonembryonic carcinoma cells than in embryonic cells, in which polyomavirus early expression is blocked, whereas element B enhances equally well in both cell types (8, 16). Inducible and constitutive enhancer domains have also been described in the noncoding region of human papillomavirus type 18 (11).

The organization of some viral enhancers is now established, and in general they are composed of several sequence motifs 10 to 20 bp long, called enhansons after the SV40 enhancer (31). These enhansons are important for the enhancer function, and they bind cellular factors which may or may not be cell-type, tissue, or development specific (for reviews, see references 28 and 33). The resulting loose or restricted specificity of enhancers could then be due to functional binding of subsets of specific factors in different cell types. The EBV enhancer might follow the same rules, since domains A and B contain several palindromes (A0 to A3 and B0 to B3), some of which are duplicated, and the deletion of some of them is clearly associated with the alteration of the enhancer strength (Fig. 2).

The A domain is inactive in all B-cell lines tested and active in fibroblast and epithelial cells. This is surprising since EBV is known to have a B lymphotropism (for a review, see reference 9). However, the EBV genome was found in vivo in epithelial cells of nasopharyngeal carcinomas (37), where it is able to replicate (24, 35). In addition, the A domain (but not the B domain) is active in the myeloid lineage K562 cells (not shown). The A domain activity is therefore not restricted to nonhematopoietic cells. A computer search revealed that palindromes A2 (GGTCA-X_2-TGACC) and A3 (GGTCATGACC) had some sequence homology with the estrogen receptor-binding site GGTCA-X_3-TGACC (22). To investigate whether the DR enhancer responded to estrogen, we transfected the human breast tumor cell line MCF7, which contains receptors for estrogen, with the DR deletion mutant 5′-737, which contains both A2 and A3 palindromes. Preliminary results suggest that estrogen did not activate transcription from mutant 5′-737 (not shown). The palindromic sequences present in domain A probably represent candidates for binding sites for transcription factors. Experiments are in progress to determine whether and how each of the palindromes present in the DR enhancer A domain contribute to the enhancer strength and to identify the factors binding to these palindromes.

The BRLF1 ORF codes for a factor, R, which is the enhancer B domain EB1-inducible transactivator. One target for R is localized in a 28-bp oligonucleotide and seems to be the palindromic sequence TGTCC-X-GGACA. However, there are probably other elements which contribute to the activity of the B domain and may be responsive to R, but these elements are not yet identified. We also do not know whether R interacts directly with the R-responsive element; this is under investigation. What we know is that other EBV promoters are activated by R, and these promoters do not have the palindrome described above. This suggests that R might activate transcription by different mechanisms.

In this study we have shown that the noncoding region located upstream of the IR2 and IR4 genes contains an enhancer with inducible and constitutive domains. This enhancer is probably part of a bidirectional promoter, as has been suggested by Hardwick et al. (14).

ACKNOWLEDGMENTS

This work was financially supported by the Federation Nationale des Centres de Lutte contre le Cancer, by the Institut National de la Santé et de la Recherche Médicale (contrat 871015), and by the Association pour la Recherche sur le Cancer.

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