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The Epstein-Barr Virus (EBV) Early Promoter DR Contains a *cis*-Acting Element Responsive to the EBV Transactivator EB1 and an Enhancer with Constitutive and Inducible Activities

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The Epstein-Barr Virus (EBV) DR promoter controlled the expression of the *PstI* repeat region IR4. This promoter was activated by the EBV *trans*-acting factor EB1, mainly at the transcriptional level, and the activation was mediated by the TATA box and two *cis*-acting regulatory regions, one proximal to the TATA box and one distal to the TATA box. The distal region had enhancer properties. In HeLa cells, it activated transcription from the herpes simplex virus type 1 thymidine kinase promoter linked to the chloramphenicol acetyltransferase gene when located in inverted orientation upstream of the thymidine kinase promoter or downstream of the chloramphenicol acetyltransferase gene coding sequence. This enhancer also activated transcription from the simian virus 40 early upstream regulatory elements. These results indicate that the DR enhancer can constitutively activate heterologous promoters in HeLa cells. However, the DR enhancer was not active in EBV genome-negative B cell lines, but it became active when these cells were infected by EBV and when the expression of the EBV early genes was induced by EB1. This suggests that an EBV early gene product induces the DR enhancer activity. The DR promoter TATA box-proximal *cis*-acting regulatory element contained EB1-responsive sequences.

In most Epstein-Barr virus (EBV)-immortalized human B lymphocytes, several transcriptionally inactive copies of the viral genome persist, from which only the few genes that define latency are expressed (for a review, see reference 15). Recently, two EBV *trans*-activating factors, called EB1 and EB2 and encoded by the *Bam*HI ZLF1 and the *Bam*HI MLF1 open reading frames, respectively, have been shown to be involved in the switch from latency to a productive infection (2–5, 12, 20, 22). In Raji cells latently infected with EBV, for example, the promoters controlling the expression of EB1 and EB2 are not detectably active. When the coding sequences for EB1 and EB2 are placed under the control of the simian virus 40 (SV40) early promoter-enhancer and transfected in Raji cells, then EB1, but not EB2, activates transcription from most of the early EBV promoters, including the DR promoter (2, 3). However, EB2 has been defined as a transcriptional activator because in Vero and HeLa cells, it activates the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene linked to the EBV EB2 noncoding upstream sequences, the herpes simplex virus (HSV) thymidine kinase (tk) promoter, the adenovirus type 5 E3 promoter, and the SV40 enhancerless early promoter (12, 22). EB2 also stimulates, in part by a posttranscriptional mechanism, the human immunodeficiency virus long terminal repeat (10). A third EBV early protein, encoded by the open reading frame *Bam*HI-RLF1, has been identified as being able to stimulate the expression of the CAT gene linked to the EA-R putative promoter (8).

The duplicated right (DR) and duplicated left (DL) promoters are duplicated with almost complete homology in some of the EBV genomes. DR and DL control regions direct the expression of abundant early mRNAs transcribed from partially homologous clusters of short tandem repeats, the *Pst*I and *Not*I repeats, respectively (Fig. 1A) (6, 11). The EBV DR and DL promoters are not detectably active in the latently infected B cells Raji, but both promoters are activated by the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate and by EB1 (2).

Here we report that the DR promoter was also activated by EB1 in HeLa cells. In order to study by which mechanisms the DR promoter is activated by the EBV *trans*-acting factor EB1, we have characterized *cis*-acting elements that are active in the presence of EB1. A *Bal* 31 deletion mutation analysis of the EBV DR promoter in HeLa cells showed that none of the 5′ deletion mutants generated were significantly active in the absence of EB1. In the presence of EB1, the *Bal* 31 deletion mutants allowed us to identify two *cis*-acting transcriptional control elements: a distal element, located 639 base pairs (bp) upstream of the CAP site (−639 to −897), and a proximal element, located 69 bp upstream of the CAP site (−69 to −220). The distal element can activate transcription from heterologous promoters relatively independently of distance and orientation when located 5′ or 3′ to the coding sequence, and therefore has properties in common with transcriptional enhancers. This enhancer is constitutively active in HeLa cells. However, it is inactive in the EBV genome-negative lymphoid B cells BJA-B, and it becomes active in these cells when they are infected by EBV and when the EBV early gene product synthesis is induced by EB1. Our results also suggest that the DR promoter-proximal *cis*-acting element contains sequences responsive to EB1.

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MATERIALS AND METHODS

Cell lines. The EBV genome-negative human B lymphoid cell lines BJA-B and Louckes were a gift from G. Lenoir. The EBV-immortalized human B-cell lines Raji-tk$^-$, P3HRI-tk$^-$, and BJA-B/B95-8 have been described elsewhere (2). Vero cells were a gift from I. Machuca.

Cloned DNA templates. The construction of DNA recombinants pSVZ1, pSVSM, and pK-CAT has been described extensively elsewhere (2). Briefly, pSVZ1 and pSVSM are pUC18 derivatives containing the open reading frames coding for the *trans*-activating factors EB1 and EB2, respectively, placed under the control of the SV40 early promoter-enhancer (see Fig. 1A). pK-CAT is a pBR322 derivative in which the EBV DR promoter (11) has been cloned in front of the bacterial CAT-coding sequences (Fig. 1A). Plasmid p5.2K contains the DR promoter and the IR4 open reading frame isolated in the M-ABA *Bgl*II K DNA fragment and cloned in the pUC18 unique *Bam*HI site (Fig. 1A). 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 (19). Plasmids pSCAT258+ and pSCAT258− were made by ligating the EBV DR promoter 258-bp *Ava*I DNA fragment in both orientations in the unique *Ava*I site of pSCAT (see Fig. 4A). Plasmid pBLCAT2, which contains the HSV-tk promoter cloned upstream of the CAT-coding sequence (see Fig. 3A), was a gift from B. Luckow. Plasmid pBLCAT2E5′ was made by ligating the DR promoter 258-bp *Ava*I DNA fragment in the unique *Xba*I site located 5′ to the HSV-tk promoter. Plasmid pBLCAT2E3′ was made by ligating the DR promoter 258-bp *Ava*I DNA fragment in the unique *Sma*I site located downstream of the CAT-coding sequence.

Construction of 5′-deletion mutants. To generate mutants with deletions extending in a 5′-to-3′ direction from the 5′ side of the DR promoter, the plasmid pK-CAT (12 μg) was opened at a unique *Sac*I site and digested for various times with *Bal* 31 exonuclease. The extent of digestion of the DNA for each time point was determined by restriction enzyme analysis. The selected DNAs were treated with T4 DNA polymerase and redigested with *Bam*HI. The resulting DNA fragments, containing different lengths of promoter sequences linked to the CAT-coding sequences, were isolated by polyacrylamide gel electrophoresis and ligated to pUC18 cut by *Sma*I and *Bam*HI. The endpoint of the deletion for each mutant was determined by Maxam and Gilbert sequencing.

Construction of internal-deletion mutants. Sequences of the DR promoter located between the *cis*-acting element located 639 bp upstream from the CAP site and the TATA box were deleted by *Bal* 31 digestion and replaced by pBR322 sequences in order to keep the distance between the upstream element and the TATA box approximately constant and equal to about 615 bp (see Fig. 7A).

Cell culture and transfections. Lymphoblastoid cell lines latently infected by EBV (Raji-tk$^-$, P3HRI-tk$^-$, and BJA-B/B95-8 as well as the EBV genome-negative B cells BJA-B and Louckes) were grown at 37°C in RPMI 1640 (Boehringer Mannheim Biochemicals) containing 10% (vol/vol) fetal calf serum. HeLa and Vero cells were grown in Dulbecco modified Eagle medium (GIBCO Diagnostics) supplemented with 10% (vol/vol) fetal calf serum. One day before the transfection, lymphoid B cells were suspended at a density of 5×10^5 cells per ml in fresh medium. HeLa and Vero cells were seeded at a density of 10^6 per 100-mm petri dish 24 h prior to transfection, and the medium was changed 4 h before transfection. Lymphoid B cells were transfected by the DEAE-dextran method (13). HeLa and Vero cells were transfected by the calcium phosphate coprecipitation method (21). About 10^6 cells were mixed with the appropriate DNA; the DNAs were in the same topological state, as determined by agarose gel electrophoresis. Each petri dish was transfected with 15 μg of DNA containing the test promoter plasmid and equal molar quantities of SV40 promoter-containing plasmids. The overall quantity of DNA was brought to 15 μg with pUC18.

RNA extraction. At 48 h after transfection, cells were rinsed twice with cold phosphate-buffered saline and lysed by the addition of 50 mM Tris hydrochloride, pH 7.4, 150 mM NaCl, 20 mM EDTA, 2% (vol/vol) *N*-lauryl-sarcosine, and 200 μg of proteinase K per ml. The lysate was incubated for 10 min at 30°C and then vortexed thoroughly to reduce the viscosity. The clear lysate was mixed with CsCl (1 g/ml of lysate) and underlaid with 2.5 ml of 5.7 M CsCl. After 18 h at 27,000 rpm in a Beckman SW40 rotor (20°C), the RNA was recovered as a DNA-free pellet, dissolved in 200 μl of 200 mM sodium acetate (pH 5.4), and precipitated with 3 volumes of ethanol.

RNA transcription analysis. RNAs were suspended in 20 μl of 10 mM Tris hydrochloride (pH 7.4)–300 mM NaCl–0.2 mM EDTA–50% (vol/vol) formamide and mixed with an excess of ^{32}P-labeled single-stranded DNA or RNA probes. After being heated at 80°C for 5 min, probes and RNAs were allowed to hybridize for 18 h at 30°C (19). The DNA-RNA hybrids were digested by S1 nuclease (9), and the RNA-RNA hybrids were digested by RNase A and T1 (14). The radioactive protected products were analyzed on 8% (wt/vol) polyacrylamide–8.3 M urea sequencing gels.

CAT assays. CAT assays were performed essentially as described previously (7). However, instead of sonication, cell lysis was obtained by treatment with 0.05% sodium dodecyl sulfate. Half the volume of each extract was used for each CAT assay. However, when necessary, the volume of the extract or the time of reaction (or both) were modified in order to stay in the linear range of the assay. The CAT enzyme-containing extracts were diluted with extracts made from untransfected cells in order to keep the protein concentration equal in each reaction and to minimize the enzyme inactivation due to dilution.

To ascertain that the amount of CAT enzyme activities obtained after transfection of cells with CAT-containing templates were reproducible, in most experiments each transfection was duplicated; otherwise each experiment was repeated at least three times.

RESULTS

Activation of the EBV DR promoter by EB1 is mainly transcriptional. We have shown previously (2) that the EBV early promoter DR was activated by EB1 in EBV latently infected Raji cells. This was also the case in nonlymphoid cells. The EBV early promoter DR linked to the bacterial CAT gene (plasmid pK-CAT; Fig. 1A) was cotransfected in HeLa cells with plasmid pSVZ1 expressing EB1. Figure 1B shows that in HeLa cells transfected with pK-CAT (lane 1), CAT activity was undetectable. However, one could observe an activation of the DR promoter in pK-CAT by EB1 (lane 2); this activation increased linearly with the amount of

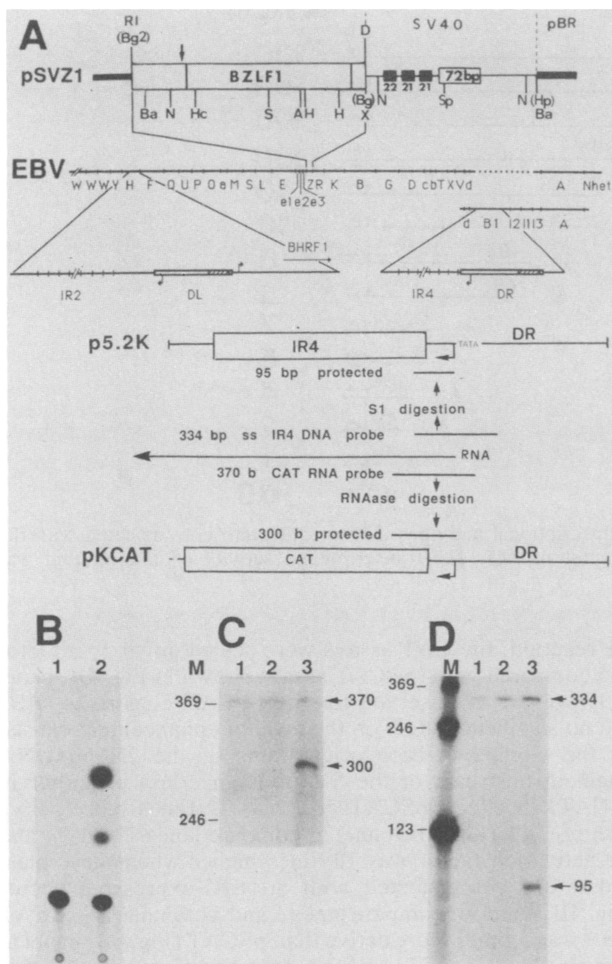

FIG. 1. EB1 activation of the DR promoter increases the amount of RNAs initiated from the DR TATA box. (A) Linear representation of the plasmids used in the transfections and of the ^{32}P-labeled probes used in RNase or nuclease S1 protection experiments. Plasmid p5.2K contains the IR4-DR transcription unit, plasmid pK-CAT contains the CAT coding sequence under the control of the EBV early promoter DR, and plasmid pSVZ1 expresses EB1 under the control of the SV40 early promoter. Restriction site abbreviations: A, *Ava*I; Ba, *Bam*HI; Bg, *Bgl*I; Bg2, *Bgl*II; D, *Dra*I; H, *Hin*dIII; Hc, *Hin*cII; Hp, *Hpa*II; K, *Kpn*I; N, *Nco*I; R, RsaI; RI, *Eco*RI; S, *Sma*I; Sp, *Sph*I. ^{32}P-labeled RNAs were synthesized in vitro by using SP6 polymerase and plasmid pSP65 (Promega Biotec) containing the *Eco*RI-*Hin*dIII fragment from mutant A11 (see Fig. 2) as indicated by the supplier. The RNA probe and the protected RNA fragment are indicated. A 5'-^{32}P-labeled single-stranded IR4 DNA probe was prepared as described previously (19) from the p5.2k 334-bp *Ava*II DNA fragment carrying the 5' end of the IR4 open reading frame. (B) CAT enzyme activity in extracts made from HeLa cells transfected with 2 μg of pK-CAT (lane 1) or 2 μg of pK-CAT and 1 μg of EB1-expressing vector (lane 2). (C) RNase protection of the 370-nucleotide CAT RNA probe by RNAs extracted from HeLa cells transfected with pUC18 (lane 1), pKCAT (lane 2), or pKCAT and pSVZ1 (lane 3). (D) S1 nuclease protection of the 334-nucleotide IR4 single-stranded DNA probe by RNAs extracted from HeLa cells transfected with pUC18 (lane 1), p5.2K (lane 2), or p5.2K and pSVZ1 (lane 3). Lane M, radiolabeled DNA fragment size markers.

EB1 expressed (not shown). The increase in CAT activity in the presence of EB1 was due to higher levels of correctly initiated CAT mRNAs (Fig. 1C). In effect, RNA extracted from HeLa cells transfected with pK-CAT alone or from HeLa cells cotransfected with pK-CAT and an EB1 expression vector were hybridized to a 370-base, ^{32}P-labeled CAT RNA made from the SP6 promoter in the antisense orientation (Fig. 1A). The hybrids were digested with RNase A and T1 as described in Material and Methods. Correctly initiated CAT mRNAs should protect a radioactive RNA fragment about 310 bases long. Such a product was detected only with RNAs extracted from cells cotransfected with pK-CAT and EB1 (Fig. 1C, lane 3). The same results were obtained with the complete DR-IR4 transcription unit (plasmid p5.2K; Fig. 1A). In effect, plasmid p5.2K was also transfected in HeLa cells either alone or with an EB1-expressing vector. The RNAs extracted from the transfected HeLa cells were hybridized with a 334-base, ^{32}P-labeled single-stranded DNA probe complementary to the IR4 RNA (Fig. 1A). After S1 nuclease digestion of the hybrids, correctly initiated IR4 mRNAs should protect a radioactive DNA fragment of about 95 bases. Such a product was only protected against S1 nuclease digestion by RNAs extracted from cells cotransfected with p5.2K and EB1 (Fig. 1D, lane 3). In conclusion, activation of the DR promoter by EB1 was mainly transcriptional, and this activation was seen at the level of RNAs transcribed from homologous and heterologous open reading frames.

EB1 activation of the DR promoter revealed two *cis*-acting regulatory regions. In order to determine whether EB1 transcriptional activation of the DR promoter was dependent on specific sequences, we searched for such sequences in the DR promoter. Recombinant plasmids containing *Bal* 31 5'-deletion mutants from the DR promoter cloned upstream of the CAT open reading frame were transfected in HeLa cells (Fig. 2). None of the mutants were detectably transcriptionally active in the absence of EB1 (not shown). In the presence of EB1, we observed that deletion of sequences located 721 bp upstream from the CAP site decreased the promoter activity by less than 10-fold. We also observed that all the mutants containing sequences located downstream of −721 were still responding to EB1. In addition, a five- to sevenfold decrease in the activity of the mutants was observed when sequences located between −221 to −69 were deleted.

The results clearly showed that in HeLa cells, there seemed to be two regions required for the activation of the DR promoter by EB1. Identical results were obtained in Vero cells (not shown). The results also show that important *cis*-acting sequences that could have enhancer properties are located distal to the TATA box.

DR promoter contains an enhancer located in the *Ava*I 258-bp DNA fragment. To test the enhancer properties of the sequences located distal to the TATA box and to determine whether these sequences contained EB1-responsive element(s), they were cloned in a 258-bp *Ava*I DNA fragment in inverted orientation either in front of a tk-CAT chimeric gene or downstream of the CAT-coding sequence (Fig. 3A). The constructs were transfected in HeLa cells, and the transient expression of the chimeric genes was analyzed at the CAT enzymatic level. The tk promoter constitutive activity in plasmid pBLCAT2 (Fig. 3B, lane 1) could be increased when the 258-bp DNA fragment was cloned upstream of the tk promoter and in inverted orientation (plasmid pBLCAT2E5'; Fig. 3B, lane 3), and the activation factor was about 10-fold (compare lanes 1 and 3). The 258-bp

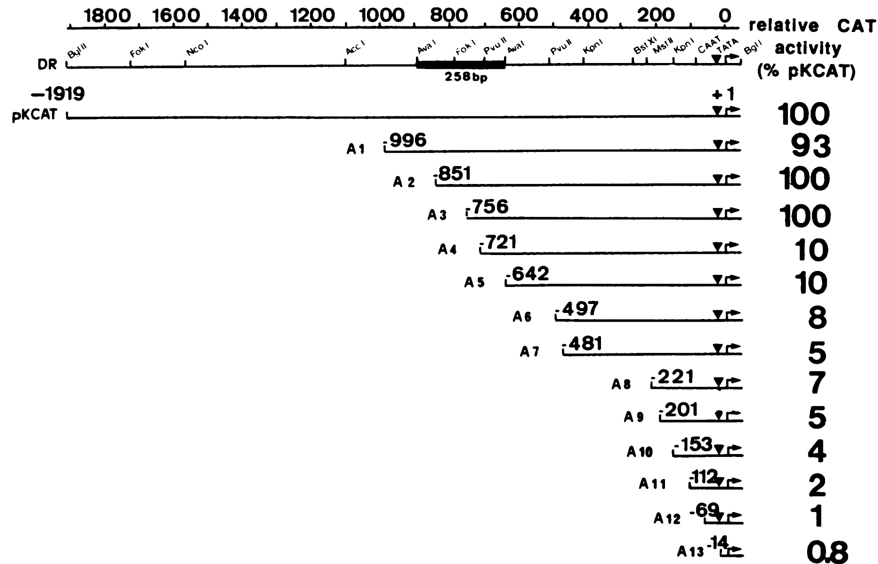

FIG. 2. Deletion mutants of the DR promoter in pK-CAT and their transcriptional activities. HeLa cells were cotransfected with the different 5′-deletion mutants in the DR promoter and EB1 expression vector pSVZ1. The transcriptional activity of each mutant was expressed as a percentage of pK-CAT activity.

DNA fragment could also activate tk-CAT transcription when cloned in inverted orientation downstream of the tk-CAT chimeric transcription unit (plasmid pBLCAT2E3′; Fig. 3B, lane 5), and the activation factor was lower (about fivefold). In conclusion, the 258-bp *Ava*I DNA fragment located 639 bp upstream of the DR promoter TATA box contains sequences with enhancer properties, since they activate a heterologous promoter when located 5′ or 3′ to the promoter and in inverted orientation with respect to the coding sequence. Moreover, these enhancer sequences were not responsive to EB1 in HeLa cells. In effect, the amounts of CAT enzyme expressed from pBLCAT2 (Fig. 3B, lane 2), pBLCAT2E5′ (lane 4), and pBLCAT2E3′ (lane 6) were unaffected by EB1.

DR enhancer increases the amount of RNAs initiated specifically from the SV40 enhancerless early promoter. It could be that the tk promoter was activated to saturation by the DR enhancer alone, and in these conditions it would have been impossible to see an additional effect of EB1 on the DR enhancer. To confirm that the DR promoter 258-bp DNA fragment had EB1-independent enhancer properties, we cloned it in both orientations upstream of the SV40 enhancerless early promoter in pSCAT and generated plasmids pSCAT258+ and pSCAT258− (Fig. 4A). These plasmids (10 μg) were transfected in HeLa cells, and their activity was compared at the CAT enzymatic level and at the CAT RNA level. The DR promoter 258-bp DNA fragment could enhance transcription from the SV40 enhancerless early promoter when cloned in the right orientation (Fig. 4B, panel a; compare lanes 1 and 2), and the activation was about 12-fold. When the 258-bp DNA fragment was placed in the inverted orientation, an increase in CAT enzyme expression could also be detected (Fig. 4B, panel a; compare lanes 1 and 3), and the activation was only about fivefold. Contrary to what was observed for the tk-CAT constructs, the DR enhancer did not activate to saturation the SV40 enhancerless early promoter. In effect, as shown in Fig. 4B, panel b, EB1 increased the overall activities of pSCAT (lane 1), pSCAT 258+ (lane 2), and pSCAT 258− (lane 3), since only 2 μg of these plasmids was transfected. To stay in the linear range of the reaction, the CAT assays were scaled down by a factor of 5 compared with the CAT assays shown in Fig. 4B, panel a. However, as observed for the tk-CAT constructs, EB1 had no significant effect on the level of enhancement caused by the cloning in both orientations of the 258-bp DNA fragment upstream of the SV40 enhancerless promoter in pSCAT. In effect, pSCAT258+ was 12 times more active than pSCAT (Fig. 4B, panel a; compare lanes 1 and 2), and the activation factor was 10 times higher when these plasmids were cotransfected with an EB1-expressing vector (Fig. 4B, panel B; compare lanes 1 and 2). Similarly, pSCAT 258− was 5 times more active than pSCAT (Fig. 4B, panel a; compare lanes 1 and 3) and the activation factor was 7 times higher when these plasmids were cotransfected with an EB1 expression vector (Fig. 4B, panel b; compare lanes 1 and 3).

Moreover, as shown in Fig. 4B, panel b, and as already published (12, 22), the SV40 early promoter in pSCAT could be activated by EB2 (lane 4), and this activation was also seen on pSCAT258+ (lane 5) and pSCAT258− (lane 6). However, again the DR enhancer activity was not affected by EB2, since pSCAT258+ and pSCAT258− were 13 and 7 times more active, respectively, than pSCAT. The effect of the DR enhancer was detected at the level of specific CAT RNAs (Fig. 4C). RNAs were extracted from HeLa cells transfected with pSCAT or pSCAT258+ either alone or with the EB1 or EB2 expression vector and were hybridized with a 450-base ^{32}P-labeled RNA probe (Fig. 4A). Properly initiated CAT RNAs should protect a 310-base radioactive RNA fragment against RNase digestion. No specific CAT RNAs could be detected in HeLa cells transfected with pSCAT (Fig. 4C, lane 1) or pSCAT258+ (lane 4), although CAT enzyme activity was detected in HeLa cells transfected with pSCAT and pSCAT258+ (Fig. 4B, panel a, lanes 1 and 2). No increase in the amount of specific CAT transcripts expressed from pSCAT cotransfected with EB1 could be detected (Fig. 4C, lane 2), but specific CAT RNAs expressed from pSCAT258+ cotransfected with EB1 were detected (lane 5). EB2 increased the amount of CAT RNAs expressed from pSCAT (Fig. 4C, lane 3), and the specific CAT RNAs were even more highly expressed from pSCAT258+ cotrans-

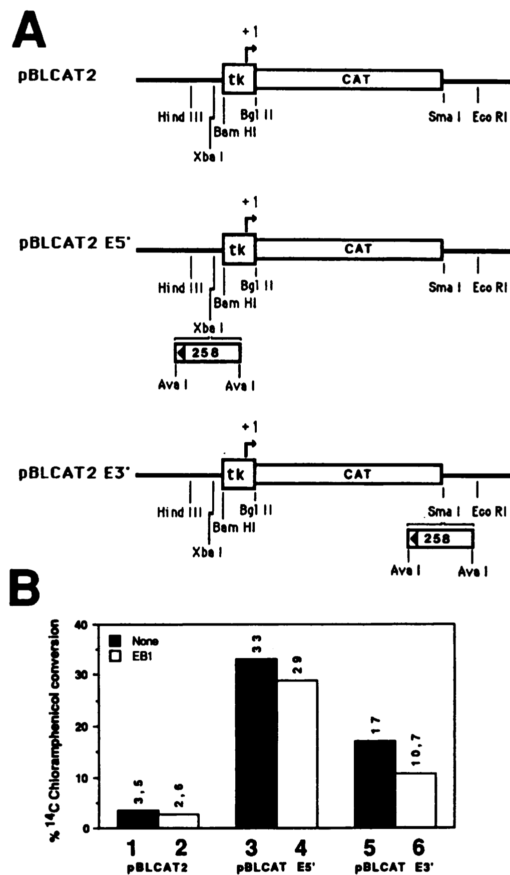

FIG. 3. The DR promoter 258-bp *Ava*I DNA fragment has constitutive enhancer properties in HeLa cells. (A) The 258-bp *Ava*I DNA fragment was cloned in inverted orientation 5′ (plasmid pBLCAT2 E5′) or 3′ (plasmid pBLCAT2 E5′) of the herpes simplex virus tk promoter in plasmid pBLCAT2. (B) Plasmids pBLCAT2 (lanes 1 and 2), pBLCAT2 E5′ (lanes 3 and 4), and pBLCAT2 E3′ (lanes 5 and 6) were transfected in HeLa cells either alone (lanes 1, 3, and 5) or with an EB1-expressing vector (lanes 2, 4, and 6). The transcriptional activities of the chimeric genes are expressed as percentages of [^{14}C]chloramphenicol acetylation.

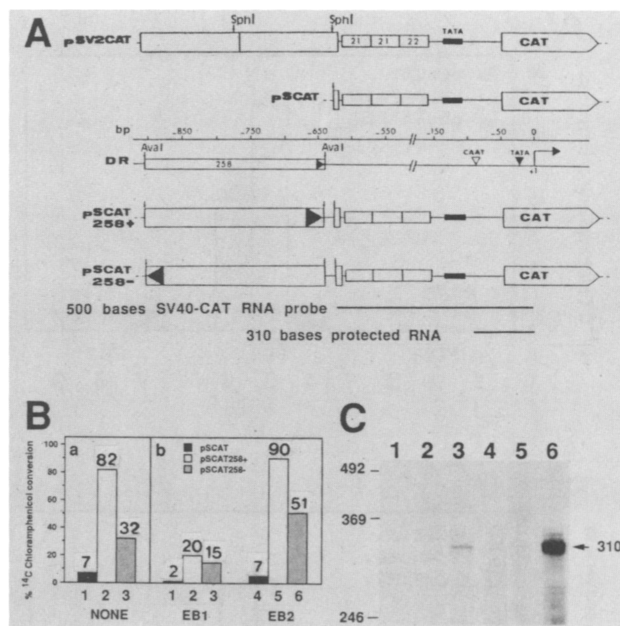

FIG. 4. The DR enhancer increases the expression of specific transcripts from the SV40 enhancerless early promoter. (A) The DR enhancer located in the 258-bp *Ava*I DNA fragment was cloned in the sense orientation (plasmid pSCAT 258+), or in the antisense orientation (plasmid pSCAT 258−), upstream of the SV40 enhancerless early promoter in pSCAT. (B) Panel a. Ten micrograms of plasmids pSCAT (lane 1), pSCAT 258+ (lane 2), and pSCAT 258− (lane 3) was transfected in HeLa cells. CAT assays were performed in 50 μl of HeLa cell extracts during 150 min. Panel b. Two micrograms of plasmids pSCAT (lanes 1 and 4), pSCAT 258+ (lanes 2 and 5), and pSCAT 258− (lanes 3 and 6) was transfected in HeLa cells with 1 μg of EB1-expressing vector (lanes 1, 2, and 3) or 1 μg of EB2-expressing vector (lanes 4, 5, and 6). These transfections were done together with those presented in panel a, but in order to stay in the linear range of the assay, the CAT assays had to be performed in 25 μl of extract during 60 min. (C) RNase protection of the 500-nucleotide SV40-CAT ^{32}P-labeled RNA by RNAs extracted from HeLa cells transfected with pSCAT (lane 1), pSCAT and EB1 (lane 2), pSCAT and EB2 (lane 3), pSCAT258+ (lane 4), pSCAT258+ and EB1 (lane 5), or pSCAT258+ and EB2 (lane 6). The RNA probe was transcribed by SP6 polymerase from plasmid pSP65 (Promega Biotec) containing the *Eco*RI-*Bam*HI DNA fragment isolated from pSCAT and cloned downstream from the SP6 promoter in the antisense orientation. Size markers and the protected probe are indicated.

fected with EB2 (lane 6). However, quantification of the radioactivity present in the protected RNA bands in lanes 3 and 6 indicated that in the presence of EB2, the DR enhancer increased the amount of specific CAT RNAs expressed from the SV40 early promoter by about 10-fold. Altogether, the results presented above strongly suggest that sequences with constitutive enhancer properties are located in the DR promoter 258-bp *Ava*I DNA fragment, and the enhancing effect is seen at the level of CAT enzyme activity and at the level of correctly initiated CAT RNAs. These enhancer sequences were not significantly responsive to EB1 and EB2 in HeLa cells.

DR enhancer was induced by EB1 in the EBV-infected B cells Raji. Since the DR promoter in pK-CAT is inactive in the EBV latently infected Raji cells (2), we then investigated whether the DR enhancer was also inactive in EBV-infected B cells. We first transfected the tk-CAT chimeric genes (Fig. 3A) in the EBV latently infected B cells Raji-tk$^-$. The tk promoter was poorly active in Raji-tk$^-$ cells (Fig. 5A, lane 1). Moreover, addition of the DR enhancer upstream (Fig. 5A, lane 2) or downstream (lane 3) of the tk promoter did not increase CAT expression significantly. Since EB1 activated the DR promoter in pK-CAT in Raji cells, we investigated whether EB1 could activate the DR enhancer in these cells. As expected, the DR enhancer activity was induced by EB1; this was true whether the enhancer was placed upstream (Fig. 5A, lane 5) or downstream (lane 6) of the tk promoter. Since in Raji cells EB1 induces the expression of most of the EBV early genes (2), including the BMLF1-encoded *trans*-acting factor EB2 (M. Buisson, unpublished results), we checked whether EB2 would be the EB1-inducible factor that activates the DR enhancer in Raji cells. As shown in Fig. 5A, EB2 activated equally the tk promoter in plasmids pBLCAT2 (lane 7), pBLCAT2E5′ (lane 8), and pBLCAT 2E3′ (lane 9) (12, 22), and had no effect on the DR enhancer activity. We obtained identical results with the SV40-CAT constructs (Fig. 5B). The SV40 enhancerless early promoter in pSCAT was not significantly active in Raji-tk$^-$ cells (Fig. 5B, lane 1), and CAT activity was not significantly increased when the DR enhancer was cloned either in the sense (lane

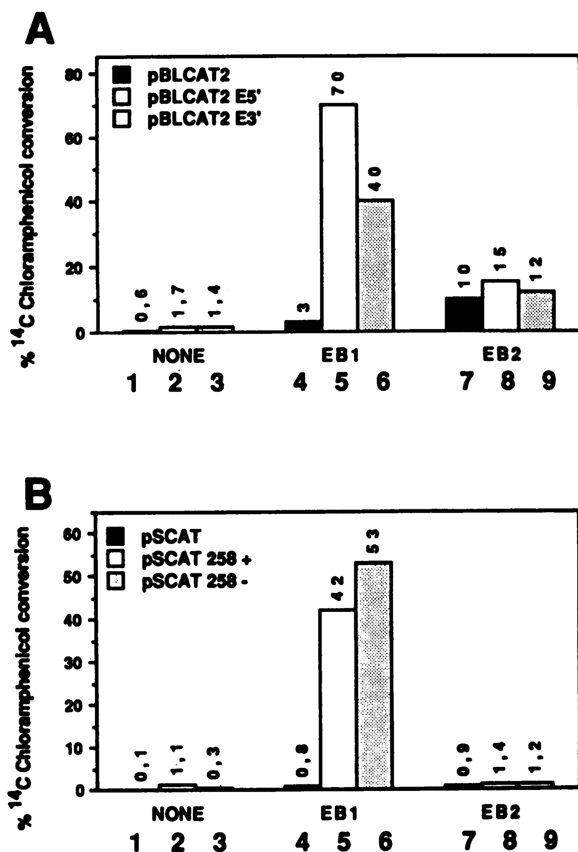

FIG. 5. DR enhancer activity induced by EB1 in Raji cells. (A) Raji cells were transfected with pBLCAT2 (lanes 1, 4, and 7), pBLCAT2 E5' (lanes 2, 5, and 8), or pBLCAT2 E3' (lanes 3, 6, and 9) without EB1- and EB2-expressing vectors (lanes 1, 2, and 3), with an EB1-expressing vector (lanes 4, 5, and 6), or with an EB2-expressing vector (lanes 7, 8, and 9). (B) Raji cells were transfected with pSCAT (lanes 1, 4, and 7), pSCAT 258+ (lanes 2, 5, and 8), or pSCAT 258− (lanes 3, 6, and 9) without EB1- and EB2-expressing vectors (lanes 1, 2, and 3), with an EB1-expressing vector (lanes 4, 5, and 6), or with an EB2-expressing vector (lanes 7, 8, and 9). The transcriptional activities of the various chimeric promoters were expressed as percentages of [^{14}C]chloramphenicol acetylation.

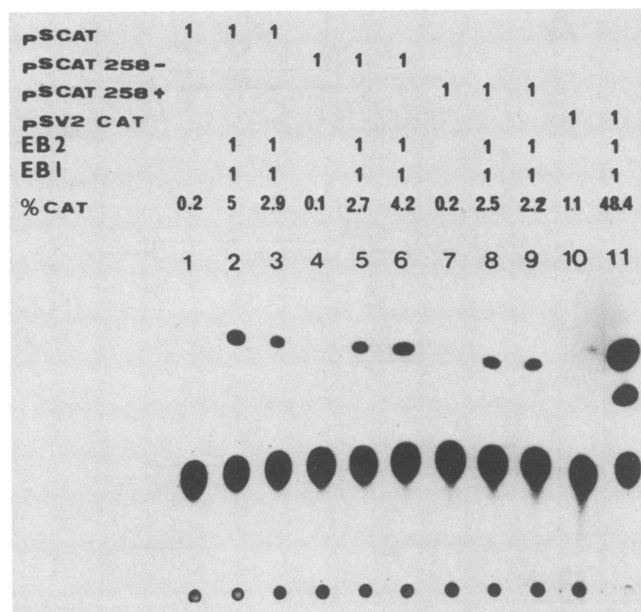

FIG. 6. DR enhancer was not active in BJA-B cells. BJA-B cells were cotransfected with the various DNA templates indicated in the upper part of the figure. The DR enhancer activity in the transfected cells was estimated by determining the percentage of [^{14}C]chloramphenicol acetylated by the CAT enzyme in cell extracts (% CAT).

2) or in the antisense (lane 3) orientation upstream of the SV40 early promoter. However, EB1 strongly induced the activity of the DR enhancer placed in the sense orientation (Fig. 5B, lane 5) or in the antisense orientation (lane 6) upstream of the SV40 early promoter compared with activation of CAT expression from pSCAT (lane 4). EB2 equally increased CAT expression from plasmids pSCAT (Fig. 5B, lane 7), pSCAT258+ (lane 8), and pSCAT258− (lane 9) and had no effect on the activity of the DR enhancer. The same functional specificities were observed in two other EBV genome-positive B cells (P3HRI-tk$^-$ and BJA-B/B95-8) and when the DR enhancer was placed in front of its homologous promoter (not shown). These results strongly suggest that the DR enhancer is induced by EB1 in EBV-infected B cells and that EB2 is not the EBV product involved in this activation. The results also suggest that the functional specificities of the DR enhancer in EBV-infected B cells were due to the enhancer itself rather than to the promoter sequences.

DR enhancer is not active in the EBV genome-negative lymphoid B cells BJA-B. From the results presented above, it was difficult to conclude that EB1 induction of the DR enhancer was B-cell specific. In effect, the activity of EB1 on the DR enhancer was assayed only in human B cells which were latently infected by EBV, and it has been shown that EB1 can induce the expression of numerous early genes in these cells (2). We therefore tested for EB1 induction of the DR enhancer in BJA-B cells, which have been used for reference as a noninfected human lymphoid B-cell line. pSCAT (Fig. 6, lane 1), pSCAT258− (lane 4), pSCAT258+ (lane 7), and pSV2CAT (lane 10) were not detectably active in BJA-B cells. EB1 alone could not detectably activate CAT expression from pSCAT, pSV2CAT, pSCAT258+, and pSCAT258− (not shown). However, we previously showed (2) that the effect of EB1 on DR promoter activity can be increased in BJA-B cells by cotransfection with an EB2-expressing vector. This effect is probably due to the facts that the SV40 promoter activity was very low in BJA-B cells (Fig. 6, lane 10) and that EB1 and EB2 were expressed under the control of the SV40 early promoter-enhancer sequences from plasmids pSVZ1 and pSVSM, respectively. However, the SV40 promoter is strongly activated by EB2 (22). Therefore, even if poorly expressed from pSVSM, EB2 will activate its own synthesis in BJA-B cells and will also activate expression of EB1 from plasmid pSVZ1. As expected, cotransfection of pSCAT (Fig. 6, lanes 2 and 3), pSCAT258− (lanes 5 and 6), and pSCAT258+ (lanes 8 and 9) with EB1- and EB2-expressing vectors resulted in significant increases in the amounts of CAT enzyme expressed from these chimeric genes. However, the DR enhancer placed upstream of the SV40 enhancerless early promoter in inverted orientation (pSCAT258−; Fig. 6, lanes 5 and 6) and in direct orientation (pSCAT258+; lanes 8 and 9) had no effect on the amount of CAT enzyme expressed compared with pSCAT (lanes 2 and 3). It should be noted that the SV40 enhancer effect could be observed in those cells in the presence of EB1 and EB2. Indeed, pSV2CAT (Fig. 6, lane 11) was about 10 times more active than its enhancerless

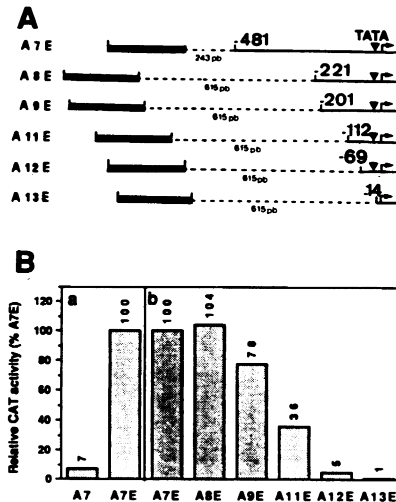

FIG. 7. DR promoter TATA box-proximal element induced by EB1 in HeLa cells. (A) The various DNA templates indicated were cotransfected in HeLa cells with an EB1-expressing vector. (B) Panel a. Transcriptional activity of mutants A7 and A7E. Panel b. Transcriptional activities of the different deletion mutants.

counterpart pSCAT (Fig. 6, lanes 2 and 3) when these plasmids were cotransfected with EB1- and EB2-expressing vectors in BJA-B cells.

DR promoter-proximal element contains EB1-responsive sequences. From the *Bal* 31 deletion mutagenesis, it seemed that an EB1-responsive element was localized proximal to the DR promoter TATA box. In order to clearly identify this other regulatory element, we placed the 258-bp *Ava*I DNA fragment containing the DR enhancer in front of the mutants, which had less than 700 bp of promoter sequence, and kept this 258-bp DNA fragment at approximately the same distance from the CAP site as the wild-type promoter (Fig. 7A). The different mutants were then transfected in HeLa cells. In the presence of EB1, the 258-bp DNA fragment activated about 14 times more CAT expression from mutant A7E as it did from mutant A7. In addition, the mutants A8E to A12E allowed us to unambiguously identify a second *cis*-acting element located between positions −69 and −201 upstream of the CAP site (Fig. 2B). Since the DR enhancer did not respond to EB1 in HeLa cells, we concluded that there was an EB1-responsive element located upstream to the DR promoter TATA box.

DISCUSSION

The EBV early promoter DR in the plasmid pK-CAT is not detectably active in EBV latently infected B cells. In these cells, it is activated after transfection of a plasmid expressing EB1 (2). In HeLa cells, the DR promoter in plasmid pK-CAT is also not detectably active, but it can be activated after transfection of a plasmid expressing EB1.

From our *Bal* 31 mutagenesis experiments, it seemed that two regions in the DR promoter are required for the activation by EB1. One of them has properties of transcriptional enhancers. It activated transcription of heterologous promoters relatively independently of orientation and distance. This enhancer was entirely located in a 258-bp *Ava*I DNA fragment, as confirmed by progressive deletions made from either the 5′ or the 3′ end of this DNA fragment and by analysis of the enhancer effect of larger DNA fragments containing the 258-bp fragment (not shown). Our results also showed that the activities of both the HSV tk promoter and the SV40 early promoter are increased in HeLa cells by the DR enhancer and that the level of enhancement of the activities of both promoters by the DR enhancer is not significantly affected by EB1. This suggests that the DR enhancer does not contain EB1-responsive sequences.

Surprisingly, although the DR enhancer had a rather strong constitutive activity when located upstream of the HSV tk or the SV40 enhancerless early promoter, it did not detectably activate its homologous TATA box-proximal element (not shown). This can be explained by the fact that both the tk and the SV40 early promoters have a constitutive activity that can be increased by heterologous enhancers. On the contrary, the DR promoter TATA box-proximal element has no significant constitutive activity and is inducible by EB1. Therefore, the effect of the DR enhancer on its homologous TATA box-proximal element was seen only when this element was activated by EB1. This was also true when the SV40 enhancer replaced the DR enhancer (not shown). These observations also suggest that apart from the EB1-inducible sequences, there seem to be no other important *cis*-acting elements localized between the DR enhancer and the DR TATA box.

In the EBV latently infected B cells Raji-tk$^-$, P3HR-I-tk$^-$, and BJA-B/B95-8, the DR enhancer activity was induced by EB1. However, the DR enhancer was not active in EBV genome-negative BJA-B cells. This suggests that the DR enhancer is repressed in EBV genome-negative B cells or that some enhancer factors are absent or not active in these cells. In both cases, infection of the B cells by EBV was required together with the EB1-induced expression of the EBV early genes to render the enhancer active. Altogether, the results suggest that a not-yet-identified EBV-encoded product(s) other than EB1 is required for the activity of the DR enhancer in B cells. However, it is already clear that these EBV-encoded products are not EB2 or those synthesized during latency. Nor are they late gene products, since the activation by EB1 is seen in Raji cells which do not express late gene products. This is the second enhancer to be identified in the EBV genome. An enhancer has been found previously in the EBV *cis*-acting element ORI-P, which allows the replication and maintenance of recombinant plasmids in cells that express EBNA I. One component of ORI-P, the 30-bp family of repeats, acts as a transcriptional enhancer that is activated in *trans* by direct binding of the EBNA-I protein (16–18).

The other regulatory region of the DR promoter was responsive to EB1. This region was clearly located between −69 and −201 upstream from the CAP site. This region did not seem to show any cell specificity, since its activation by EB1 occurred in all cell types tested and in the absence of the enhancer (not shown). Nothing is known about the precise location of the EB1-responsive element in this region or whether EB1 binds directly to this element. Experiments are in progress to answer these questions.

In conclusion, we found that the DR promoter contains two important regulatory regions. One region, located proximal to the TATA box, contains EB1-responsive sequences. The other region, located distal to the TATA box, has enhancer activity. It is constitutive in HeLa cells, and in Raji cells it could be activated by an EB1-inducible early EBV gene product. These findings suggest the existence of functionally distinct subregions in this enhancer. This was indeed the case. The DR enhancer had two domains. One domain had constitutive activity in HeLa cells, was not influenced

by EB1, and was inactive in Raji and BJA-B cells. The other domain was inducible by R (8), an EBV EB1-induced early gene product, and the induction occurred in all cell lines tested, including EBV-negative B-cell lines (1).

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