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Delineation of the *cis*-Acting Element Mediating EBNA-2 Transactivation of Latent Infection Membrane Protein Expression

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To delineate the *cis*-acting element through which EBNA-2 transactivates latent membrane protein 1 (LMP1), we assayed the effect of EBNA-2 on the activity of LMP1 promoter upstream deletion mutants in the context of the LMP1 or heterologous promoters controlling chloramphenicol acetyltransferase (CAT) reporter gene expression in Epstein-Barr virus-negative Burkitt lymphoma cells. Assays of progressive 5′ deletions of the LMP1 promoter revealed low constitutive and at least eightfold EBNA-2-stimulated activity from −512 to +40 (−512/+40), −334/+40, and −234/+40 LMP1CAT plasmids. More extensive 5′-deleted −205/+40, −155/+40, and −147/+40 LMP1CAT plasmids also had low constitutive activity but were not EBNA-2 responsive. The most 5′-deleted −55/+40 LMP1CAT plasmid had moderate constitutive activity and was not EBNA-2 inducible. Either orientation of the −334/+40 LMP1 sequence conferred EBNA-2 responsiveness when positioned upstream of an enhancerless simian virus 40 or herpes simplex virus thymidine kinase (TK) promoter. EBNA-2 and the *cis*-acting LMP1 DNA were both required to increase TK promoter-initiated mRNA, indicating that the EBNA-2 effect is at the transcriptional level. Further deletion analysis of the EBNA-2-responsive *cis*-acting element defined a −234/−92 LMP1 DNA fragment which conveyed EBNA-2 responsiveness to the herpes simplex virus TK promoter. The 5′ 30 bp between −234 and −205 were essential for EBNA-2 responsiveness. Thus, these experiments define a 142-bp *cis*-acting element which is sufficient for conveying EBNA-2 responsiveness and an essential 30-bp component of that element. The role of this element in LMP1 and LMP2B expression and its possible role in LMP2A expression are discussed.

Epstein-Barr virus (EBV) infection of primary B lymphocytes results in a persistent latent infection characterized by cell growth transformation and expression from the viral genome of six nuclear proteins or EBNAs, three integral membrane proteins or LMPs, and two small RNAs or EBERs (for a review, see reference 19). The LMPs are transcribed under control of three neighboring promoters: a leftward LMP1 promoter initiating transcription at 169515 (4, 13, 15), an adjacent rightward LMP2B promoter initiating transcription at 169734 (22, 32), and a rightward LMP2A promoter initiating transcription at 166498 (23, 32), downstream of the LMP1 gene. EBV nuclear protein 2 (EBNA-2) is one of the first genes expressed after primary B-lymphocyte infection in vitro (2, 3, 29, 30). EBNA-2 transactivates LMP1 (1, 41), LMP2A and LMP2B (22, 43), and the *Bam*HI C EBNA promoter (34, 42). EBNA-2 also up-regulates expression of cellular genes, including CD23 (38–40), CD21 (9, 38), and c-*fgr* (20).

EBNA-2's transactivation of EBV LMP and cell gene expression is tightly linked to its role in cell transformation. EBNA-2 is essential for EBV-induced lymphocyte growth transformation (8, 17). LMP1 transactivation is an important pathway for EBNA-2's effects on cell growth since LMP1 has transforming effects in rodent fibroblast assays (5, 35, 36) and induces generalized activation of B-lymphoma cells (37, 38), while EBNA-2 alone has more restricted direct effects on cells (11, 20, 38, 39). Mutations in any of four separable EBNA-2 domains which remove growth transformation also remove LMP1 transactivation (7). Fifteen other EBNA-2 linker insertion or deletion mutations which do not affect or partially affect transformation do not affect or partially affect LMP1 transactivation (7).

The experiments reported here were undertaken to characterize the EBNA-2-responsive element in the LMP1 regulatory sequence. Initial experiments using a chloramphenicol acetyltransferase (CAT) reporter gene had indicated that a −512 to +40 (−512/+40) LMP1 promoter DNA fragment was more active in B-lymphoma cells infected with an EBNA-2-positive EBV strain than in cells infected with an EBNA-2-negative EBV strain (15). Subsequent transfection experiments into EBV-negative B-lymphoma cells revealed that EBNA-2 transactivates LMP1 expression from an EBV genomic LMP1 DNA fragment or CAT expression from an LMP1 −512 or −324 to +40 promoter DNA fragment positioned 5′ to a CAT reporter gene (12, 41). These latter experiments indicated that EBNA-2 is the only virus gene necessary for LMP1 transactivation and that the −512/+40 LMP1 promoter is EBNA-2 transactivatable in B-lymphoma cells. We therefore began with the −512/+40 LMP1 promoter CAT reporter construct and further defined the EBNA-2-responsive element by progressive 5′ deletion. Further 3′ deletion analysis was facilitated by the observations that an LMP1 DNA fragment conveyed EBNA-2 responsiveness to the simian virus 40 (SV40) or herpes simplex virus (HSV) beta thymidine kinase (TK) promoter.

MATERIALS AND METHODS

Cell culture. EBV-negative B-lymphoma cells of the BJAB and DG75 lines (6, 28) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum.

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Recombinant DNA. The pSG5 expression vector (Stratagene, La Jolla, Calif.) contains the SV40 early promoter, a beta-globin intron, and an SV40 polyadenylation signal. An EBV type 1 (10, 11) or type 2 (10) EBNA-2 DNA fragment (an *Rsa*I partial digest of the Ag876 EBV *Bam*HI D1 fragment) was cloned into the pSG5 vector, and these are referred to as pSG5-EBNA2A and pSG5-EBNA2B, respectively (41).

−512/+40 LMP1CAT constructs were generated by ligation of the cloned EBV B95-8 strain LMP1 DNA fragment (13, 15) into the *Bgl*II site in pCAT-3M (21). Shorter LMP1CAT constructs were derived from the −512/+40 LMP1CAT by restriction enzyme cleavage at the sites defined by the distance from the LMP1 transcriptional start site (13). After restriction endonuclease digestion, the 5′-deleted fragment was ligated into the *Xba*I site of pCAT-3M. The −55/+40 LMP1CAT, −147/+40 LMP1CAT, −155/40 LMP1CAT, −205/+40 LMP1CAT, and −234/+40 LMP1 CAT constructs were sequenced (Sequenase; United States Biochemical) by the dideoxynucleotide method (33), using primers flanking the *Bgl*II site in pCAT-3M. The 20-mer oligonucleotide primers were synthesized by an oligonucleotide synthesizer (Applied Biosystems) and butanol extracted three times before the sequencing reaction. pSV2CAT contains the SV40 early promoter and enhancer.

The TKCAT constructs were derived by excising the LMP1 upstream sequences from various LMP1 pCAT-3M plasmids by using *Hin*dIII and *Bgl*II and cloning the LMP1 fragment into the *Bam*HI site of pBLCAT2 (27). pBLCAT2 contains the HSV TK promoter upstream of CAT. Shorter LMP1 fragments were generated by restriction enzyme digestion of the purified *Hin*dIII-*Bgl*II fragment. The orientation of the LMP1 fragments in various TKCAT constructs was confirmed by dideoxynucleotide sequencing (Sequenase). The enhancerless SV40CAT (pA10CAT2) constructs (21) were similarly derived and tested.

pUC-βgal (a gift from Nadia Rosenthal) expresses β-galactosidase (β-Gal) under control of the SV40 promoter and was used as an internal control for transfection efficiency.

Plasmid DNAs were purified through two cycles of cesium chloride gradient centrifugation before transfection.

Transfections. Cells (5×10^6) in log-phase growth were suspended in 0.3 ml of RPMI 1640 medium with 10% fetal bovine serum and 40 μg of gentamicin per ml at room temperature and placed in a Gene Pulser cuvette (Bio-Rad Laboratories, Richmond, Calif.). pUC-βgal (5 μg), 15 μg of pSG5 vector or pSG5-EBNA2, and 10 μg of a CAT-containing construct were added to each cuvette. Cells were electroporated with 0.2 V at 960 μF (Gene Pulser) and then suspended in complete medium and cultured at 37°C. After 3 days, cells were harvested, counted, and then assayed. EBNA-2 expression was monitored in all experiments by immunofluorescence with an EBNA-2-specific monoclonal antibody (PE2) to ensure equivalent EBNA-2 expression in cells transfected in parallel (41). β-Gal assays were done on cell extracts to normalize for transfection efficiency before CAT analysis.

CAT assays. Cells were harvested 3 days after transfection, extracts were made by three cycles of freeze-thawing, and equivalent amounts of cell lysate (as standardized by β-Gal assay) were assayed in 30- to 45-min enzyme reactions (16). Percent acetylation represented the ratio of acetylated [^{14}C]chloramphenicol relative to total [^{14}C]chloramphenicol (nonacetylated and acetylated [^{14}C]chloramphenicol). This was calculated by scraping the nonacetylated and acetylated ^{14}C-labeled chloramphenicol from thin-layer papers and assaying ^{14}C by liquid scintillation counting. In those instances in which more than 50% of the chloramphenicol was acetylated, assays were repeated with diluted samples to ensure that the reactions were performed within the linear range of acetylation.

β-Gal assays. A 15-μl sample of each cell lysate was added to 3 μl of 100× magnesium buffer (100 mM $MgCl_2$, 5 M 2-mercaptoethanol), 66 μl of 4-mg/ml *o*-nitrophenyl-β-D-galactopyranoside substrate solution, and 216 μl of sodium phosphate buffer to a final volume of 300 μl. The reaction mix was incubated at 37°C for 30 min (31). The reaction was stopped by adding 0.5 ml of a 1 M Na_2CO_3 solution, and the color reaction was measured at 410 nm. β-Gal of known activity was obtained from Sigma Chemical Co. as a positive control. The cell lysate in the above reaction was substituted with 0, 1, 2, 5, or 10 μl of 1:5,000 and 1:10,000 enzyme solution (in sodium phosphate buffer).

S1 analysis. A complementary 156-base probe was synthesized from plasmid pBLCAT2 by extending a 17-mer oligonucleotide primer (+35 to +51 of the TK leader sequence) to the *Bam*HI site (−105 of the TK promoter), using Klenow fragment (Boehringer Mannheim) in the presence of [^{32}P]dCTP. The single-stranded probe was purified by 8% polyacrylamide–8 M urea denaturing gel electrophoresis. Total cellular RNA (50 μg) and 5×10^4 cpm of the gel-purified probe were hybridized in 30 μl of 1 M NaCl–0.17 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.6)–0.33 mM EDTA at 70°C. Total RNA was prepared with RNAzol B (Tel-test, Friendswood, Tex.), treated with RQ1 DNase (Promega), phenol-chloroform extracted twice, and precipitated with ethanol. After overnight hybridization, RNA and probe were digested with 400 U of S1 nuclease (Boehringer Mannheim) in 300 μl of S1 nuclease buffer (0.28 M NaCl, 50 mM sodium acetate [pH 4.5], 4.5 mM $ZnSO_4$) at 37°C for 30 min, precipitated, and separated by gel electrophoresis through an 8% polyacrylamide–8 M urea gel. The gel was dried and autoradiographed.

RESULTS

A −234/+40 LMP1 DNA fragment is EBNA-2 transactivatable, and a −205/+40 LMP1 DNA fragment is not. Progressive 5′ deletions of the EBNA-2-responsive −512/+40 LMP1 promoter fragment were cloned in front of the CAT reporter gene in pCAT-3M (21). These plasmids were tested for EBNA-2 responsiveness by cotransfection into EBV-negative B-lymphoma cells (BJAB) with an EBNA-2 expression vector or an isogenic plasmid without the EBNA-2 open reading frame. Since EBNA-2 from type 2 EBV was consistently slightly more active than EBNA-2 from type 1 EBV (40, 41), type 2 EBNA-2 was used in all subsequent assays. To control for possible differences in transfection efficiency, we did multiple experiments comparing different constructs and corrected each result for β-Gal activity expressed from a cotransfected plasmid. The −512/+40 LMP1 promoter CAT reporter plasmid and serial 5′ deletions of the LMP1 promoter down to −147 all had about the same basal activity as a promoter- and enhancerless CAT reporter plasmid (Table 1 and Fig. 1A). Further deletion of the LMP1 promoter to −55/+40 resulted in increased basal LMP1 promoter activity to about four times that of the enhancerless, promoterless pCAT-3M (Tables 1 and 2 and Fig. 1B). An increased basal activity with −55/+40 LMP1 promoter CAT plasmids had been previously noted in a study of LMP1 promoter CAT plasmids in DG75, another EBV-negative B-lymphoma cell line (12). The lower basal activity of LMP1

TABLE 1. EBNA-2 effect on LMP1 promoter-driven CAT activity in BJAB lymphoma cells

LMP1 DNA	Promoter	Median EBNA-2-induced increase[a] (range; no. of expts)	Median activity without EBNA2[b]
−512/+40	LMP1	11 (5.7–16; 3)	1.5
−334/+40	LMP1	17 (5.8–24; 3)	1.1
−276/+40	LMP1	11 (4.8–14; 3)	1.3
−234/+40	LMP1	8.4 (3.8–44; 3)	0.9
−205/+40	LMP1	1.7 (1.1–1.9; 3)	3.0
−155/+40	LMP1	0.9 (0.6–1.8; 3)	1.6
−147/+40	LMP1	1.1 (0.5–1.6; 6)	0.5
None	None[c]	1.3 (0.3–1.8; 6)	
None	SV40[d]	1.0 (0.7–1.2; 6)	

[a] CAT activity from cotransfection with EBNA-2 expression vector divided by CAT activity from cotransfection with vector control DNA. The range of the EBNA-2 effect and the number of experiments are in the parentheses.
[b] CAT activity relative to pCAT-3M.
[c] pCAT-3M has no eukaryotic promoter or enhancer (21).
[d] The SV40 enhancer and promoter-driven CAT reporter gene plasmid (pSV2CAT) had 66 times the basal activity of pCAT-3M.

TABLE 2. EBNA-2 effect on LMP1 promoter-driven CAT activity in two EBV-negative lymphoma cell lines

LMP1 DNA	Cell line	Median activity with EBNA-2[a]	Median activity without EBNA-2[b]
−55/+40	BJAB	3.6	4.1
−234/+40	BJAB	16	1.4
−55/+40	DG75	5.0	6.0
−234/+40	DG75	8.6	1.5

[a] CAT activity from cotransfection with EBNA-2 expression vector relative to CAT activity from transfection of pCAT-3M.
[b] CAT activity from cotransfection with vector control relative to pCAT-3M.

promoters extending 5′ to −55 is consistent with there being a silencing element upstream of −55 (12). EBNA-2 effected a more than eightfold increase in −512/+40, −334/+40, −276/+40, and −234/+40 LMP1 promoter CAT reporter activity in BJAB cells (Table 1 and Fig. 1A, lanes 7 to 14) and had slightly less effect in DG75 cells (Table 2). The EBNA-2-stimulated activity of the −234/+40 LMP1 promoter in DG75 and especially in BJAB cells was greater than the activity of the −55/+40 LMP1 promoter (Table 2), indicating that the EBNA-2 effect was not simply attributable to a reversal of the putative negative element upstream of −55. In contrast, further 5′ deletions of the LMP1 promoter to −205/+40, −155/+40, −147/+40, or −55/+40 resulted in a loss of EBNA-2 effect (Tables 1 and 2 and Fig. 1A, lanes 1 to 6). EBNA-2 also had no effect on the low basal CAT activity of pCAT-3M or on the high CAT activity of pSV2CAT (Fig. 1A, lanes 15 to 18, and Table 1), even when the pSV2CAT-transfected samples were assayed within the linear range of CAT activity (Table 1).

A −334/+40 LMP1 DNA fragment conveys orientation-independent EBNA-2 responsiveness to an enhancerless SV40 or HSV TK promoter. A −334/+40 LMP1 DNA fragment was cloned 5′ of an enhancerless SV40 promoter-driven CAT gene (pA10CAT2 [21]) or 5′ of an HSV TK promoter-driven CAT gene (pBLCAT2 [27]) to determine whether the LMP1 DNA sequence could confer EBNA-2 responsiveness to a heterologous promoter. EBNA-2 cotransfection had no significant effect on an enhancerless SV40 promoter (Fig. 2A, lanes 5 and 6) or on the HSV TK promoter (Fig. 2B, lanes 5 and 6), with median EBNA-2-induced increases of 1.5 or 1.0, respectively (Table 3). When the −334/+40 LMP1 DNA was present upstream of the enhancerless SV40 promoter, EBNA-2 cotransfection resulted in increased CAT activity (Fig. 2A, lanes 3 and 4) with a median 3.7-fold EBNA-2-induced increase in four experiments (Table 3). A significant EBNA-2 effect was still present if the LMP1 DNA was placed upstream in the opposite orientation (+40/−334) relative to the enhancerless SV40 promoter (Fig. 2A, lanes 1 and 2), with a median 4.9-fold EBNA-2-induced increase in four experiments (Table 3). Similarly, the −334/+40 LMP1 promoter DNA fragment positioned upstream of an HSV TK promoter-driven CAT gene also showed increased CAT activity with EBNA-2 cotransfection (Fig. 2B, lanes 1 and 2), with a median ninefold EBNA-2-induced increase (Table 3). The LMP1 DNA fragment in the opposite orientation likewise conferred EBNA-2 responsiveness to the HSV TK promoter (Fig. 2B, lanes 3 and 4), with a median 5.7-fold EBNA-2-induced increase (Table 3). Thus, in either orientation, the −334/+40 LMP1 DNA fragment confers EBNA-2 responsiveness to heterologous promoters.

EBNA-2 up-regulates CAT mRNA initiated from the TK promoter. Although the orientation independence of the LMP1 −334/+40 EBNA-2-responsive element positioned upstream of heterologous promoters is consistent with the effects of an EBNA-2-dependent regulatory element on heterologous promoter activity, the LMP1 DNA could alternatively be functioning as an EBNA-2-responsive bidirectional promoter since the LMP2B transcriptional initiation site is at −219 relative to the LMP1 transcriptional initiation site. To investigate whether the EBNA-2 effect on the LMP1 DNA fragment was through an up-regulation of TK promoter activity, we determined the transcriptional start site of

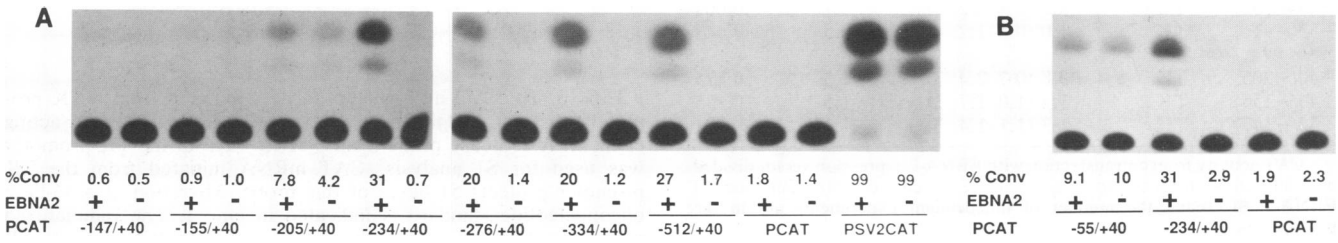

FIG. 1. EBNA-2-induced increases in LMPCAT activity. %Conv, percent chloramphenicol acetylation; +, cotransfection with the type 2 EBNA-2 expression vector, pSG5-EBNA2B; −, cotransfection with vector control DNA, pSG5. The LMP1 DNA fragments indicated by coordinates relative to the transcriptional start site were cloned 5′ to the CAT gene in pCAT-3M. pSV2CAT is the SV40 promoter- and enhancer-containing CAT plasmid and serves as a positive control.

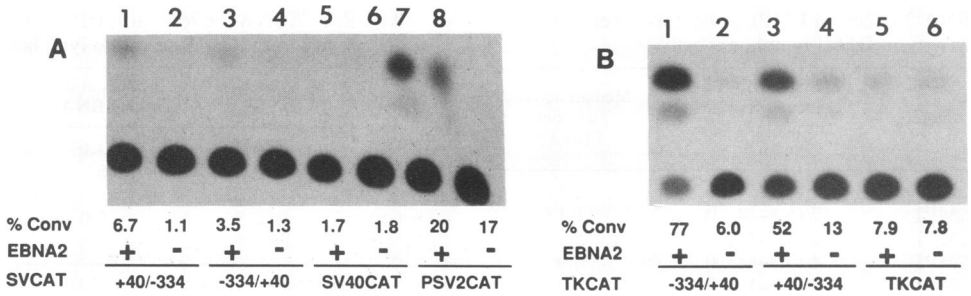

FIG. 2. EBNA-2 effects on LMP1 upstream DNA fragments positioned 5′ to the SV40 or HSV TK promoter. The −334/+40 LMP1 DNA sequence was cloned in either orientation 5′ to an enhancerless SV40 promoter in pA10CAT2 (A) or 5′ to an enhancerless TK promoter in pBLCAT2 (B). Only 1/10th of the standardized amount of cell extracts from the pSV2CAT transfection (lanes 7 and 8 in panel A) was assayed for CAT activity compared with the other SV40CAT constructs (lanes 1 to 6 in panel A).

the resultant LMP1 TKCAT mRNA by S1 analysis-using an antisense DNA probe beginning 51 bases downstream of the TK transcriptional initiation site and extending 105 bases into the TK promoter (Fig. 3). The RNA initiated at the expected site downstream of the TK promoter when there was no LMP1 DNA upstream, and EBNA-2 did not affect the initiation site or the abundance of the TK promoter-initiated RNA (Fig. 3). A 51-bp fragment was protected by both transfected cell RNAs (Fig. 3, lanes 4 and 5). LMP1 +40/−234 DNA positioned upstream of TKCAT had no effect on TKCAT mRNA abundance or initiation site when cotransfected with vector control DNA, but when cotransfected with EBNA-2 expression vector, the abundance of the TK promoter-initiated RNA increased severalfold (Fig. 3, lanes 2 and 3). Thus, EBNA-2 can transactivate the −234/+40 LMP1 DNA fragment to positively regulate heterologous promoter activity.

TABLE 3. EBNA-2-induced increases in enhancerless SV40 or TK promoter-driven CAT expression

LMP1 DNA	Promoter	Median EBNA-2-induced increase[a] (range; no. of expts)	Median activity without EBNA2[b]	*P* value[c]
None	SV40	1.5 (0.4–2.3; 4)		
−334/+40	SV40	3.7 (2.1–5.0; 4)	1.0	0.057
+40/−334	SV40	4.9 (3.4–14; 4)	0.7	0.014
None	TK	1.0 (0.9–2.0; 4)		
−334/+40	TK	9.0 (1.6–20; 4)	0.8	0.029
+40/−334	TK	5.7 (1.9–9.1; 4)	0.6	0.029
+40/−234	TK	6.9 (4.4–14; 4)	1.1	0.014
−55/−234	TK	5.3 (3.9–15; 5)	0.7	0.004
−92/−234	TK	3.7 (2.4–8.2; 5)	1.0	0.004
−234/−92	TK	3.5 (2.1–6.2; 5)	1.7	0.004
−55/−91	TK	1.0 (0.8–1.2; 4)	0.5	0.443
+40/−205	TK	0.8 (0.7–2.4; 3)	1.3	0.135
+40/−155	TK	1.4 (1.0–1.7; 3)	1.5	0.135
+40/−147	TK	1.3 (1.1–1.4; 3)	1.1	>0.571

[a] CAT activity from cotransfection with EBNA-2 expression vector divided by CAT activity from cotransfection with vector control DNA. The range of EBNA-2 effect and the number of independent experiments are in the parentheses.

[b] Ratio of CAT activity to enhancerless parental SV40CAT or TKCAT, which was approximately 2 or 15% of pSV2CAT, respectively.

[c] Mann-Whitney U tests comparing the EBNA-2 effect on the indicated LMP1 DNA fragment positioned upstream of SV40CAT or TKCAT with the effect on the enhancerless SV40CAT or TKCAT parent.

The EBNA-2-responsive element is between −92 and −234 and at least partly between −205 and −234. To further define the EBNA-2-responsive element, we cloned 5′ or 3′ deletions of the −234/+40 LMP1 promoter sequence in the opposite orientation upstream of the HSV TK promoter and assayed them for EBNA-2 responsiveness. The +40/−234, −55/−234, and −92/−234 TKCAT constructs were consistently EBNA-2 responsive in at least four independent experiments (Fig. 4A, lanes 1 to 6), with a median 6.9-, 5.3-, and 3.7-fold EBNA-2-induced increase, respectively (Table 3). The −234/−92 LMP1 TKCAT plasmid gave a similar

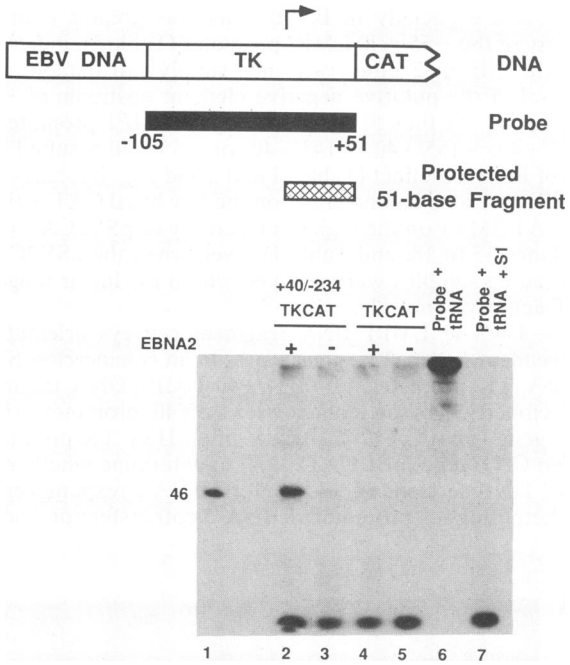

FIG. 3. EBNA-2 up-regulates mRNA initiated from a TK promoter construct, and the up-regulation requires the LMP1 *cis*-acting element. A 156-base probe from +51 to −105 of the TK promoter was used for S1 analysis. CAT mRNA initiated from the TK promoter protects 51 bases of this probe. Migration of a 46-base oligonucleotide standard is indicated in lane 1. The radiolabeled probe was hybridized overnight with 50 μg of total RNA from BJAB cells transfected as indicated and then digested with S1 nuclease (lanes 2 to 5). Lanes 6 and 7 show the probe after hybridization overnight with 50 μg of control tRNA (Sigma) without (lane 6) or with (lane 7) subsequent S1 nuclease digestion.

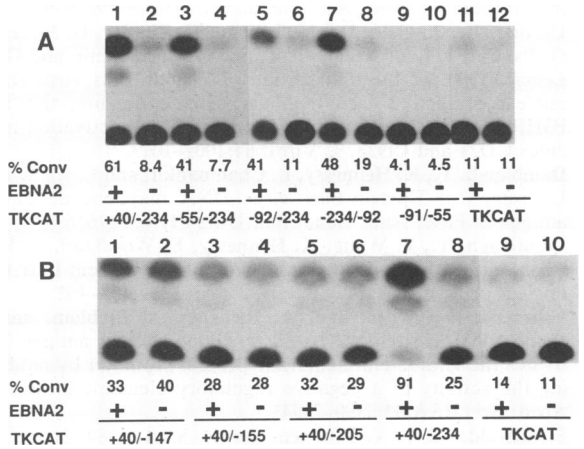

FIG. 4. EBNA-2 effects on the activity of the HSV TK promoter with LMP1 upstream DNA fragments. (A) Activity of 3′ deletions of the −234/+40 LMP1 *cis*-acting element cloned upstream of the HSV TK promoter in pBLCAT2. The −91/−55 TKCAT construct contained the −91 to −55 LMP1 DNA fragment in the opposite orientation relative to the TK promoter. (B) Activity of 5′ deletions of the LMP1 *cis*-acting element cloned in the opposite orientation upstream of the HSV TK promoter in pBLCAT2. In this experiment, only one-half of the standardized amount of cell extracts from the TKCAT transfection (lanes 9 and 10) was assayed for CAT activity compared with the other LMP1 TKCAT constructs (lanes 1 to 8).

3.5-fold median EBNA-2 response (Fig. 4A, lanes 7 and 8, and Table 3). The slightly lower EBNA-2 responsiveness of the −92/−234 TKCAT as compared with the −55/−234 TKCAT (3.7-fold versus 5.3-fold) was not readily attributable to a putative second EBNA-2-responsive element between −55 and −91 since a −55/−91 TKCAT construct was not EBNA-2 responsive (Fig. 4A, lanes 9 and 10, and Table 3).

To confirm the 5′ limits of the EBNA-2-responsive element, we cloned progressive 5′ deletions of the −234/+40 LMP1 promoter sequence in the opposite orientation upstream of the HSV TK promoter and assayed them for EBNA-2 responsiveness. As described above, the +40/−234 LMP1 TKCAT construct was EBNA-2 responsive (Fig. 4B, lanes 7 and 8, and Table 3), but EBNA-2 had no effect on the +40/−205, +40/−155, and +40/−147 TKCAT constructs (Fig. 4B, lanes 1 to 6), with a median 0.8-, 1.4-, and 1.3-fold EBNA-2-induced increase, respectively (Table 3). The loss of EBNA-2 responsiveness with deletion of the 30 bp between −234 and −205 is consistent with the effect of this deletion on the EBNA-2 responsiveness of the LMP1 promoter (Table 1). Thus, at least part of the essential EBNA-2-responsive element is between −234 and −205.

DISCUSSION

Previous experiments had indicated that EBNA-2 plays a central role in up-regulating LMP expression. LMP1 promoter DNA fragments which extended to −512 or beyond were previously shown to have substantially greater activity in EBV-infected lymphoblasts or Burkitt tumor cells than in non-EBV-infected Burkitt tumor cells (15, 41). Also, the LMP1 (1, 15, 41) and LMP2A and LMP2B promoters (22, 43) were noted to be more active in B-lymphoma cells infected with EBNA-2-positive EBV strains than in cells infected with an EBNA-2-negative EBV. Furthermore, EBNA-2 had been shown to transactivate LMP1, LMP2A, and LMP2B promoter CAT reporter plasmids in EBV-negative B-lymphoma cells (12, 41, 43).

The experiments described here significantly contribute to the delineation of the *cis*-acting factors which regulate LMP gene expression by precisely defining one end of the LMP1-LMP2 bidirectional promoter's EBNA-2-responsive element to be within 30 bases of −234, by localizing the other end of the responsive element to upstream of −92, and by showing that the LMP1-LMP2B element can convey EBNA-2 responsiveness to heterologous promoters. These observations strongly support the hypothesis that EBNA-2 transactivates LMP1 and LMP2B transcription through a *cis*-acting regulatory element(s) in the −234 to −92 DNA fragment. The orientation independence demonstrated here for the −234 to −92 *cis*-acting element is compatible with it being an EBNA-2 transactivatable upstream or enhancerlike positive regulatory element. Three similar studies of the EBNA-2-responsive elements upstream of the *Bam* C EBNA promoter (34, 42) or the CD23 promoter (40) have also recently demonstrated orientation independence of those EBNA-2-responsive elements in their effects on heterologous promoters. The EBNA-2-responsive element in the *Bam* C promoter is between −245 and −429 relative to the *Bam* C EBNA start site, and at least one component of that element is between −340 and −429 (42). The *Bam* C element is active when positioned downstream of CAT in reporter plasmids, consistent with an enhancerlike element (34).

The LMP1 promoter EBNA-2-responsive element is likely to be complex. The 5′ 30 bases of the −234 to −92 DNA fragment are one essential component required for EBNA-2 transactivation of the LMP1 promoter or of the heterologous HSV TK promoter. There is no obvious nuclear factor cognate sequence in the 5′ 30 bases (14). The 5′ 30 bases are not sufficient for conveying EBNA-2 responsiveness since an oligonucleotide which extends from −234 to −178 was not sufficient to convey EBNA-2 responsiveness to the HSV beta TK promoter (data not shown). Further, the −234 to −92 fragment has a potential Oct-1 site at −147 which is important for LMP1 promoter activity in EBV-transformed lymphoblastoid cell lines (15). In addition to the Oct-1 site, there is a 5-of-6-nucleotide match for an Sp1-binding site (18) at −135 and a 7-of-11-base match for an NF-κB-like factor consensus sequence at −117 (24). However, the putative Oct-1- and NF-κB-binding sites are not sufficient for conferring EBNA-2 responsiveness, as exemplified by the results obtained with the +40/−155 and +40/−205 LMP1 TKCAT plasmids. The necessity for the 5′ 30 bases of the −234/−92 responsive element, the lack of sufficiency for the −234/−178 oligonucleotide, and the multiplicity of potential binding factor recognition sites are most consistent with a model for EBNA-2 recognition of the LMP1 responsive element through more than one DNA site-specific interaction. Experiments to verify this model by reassembling the requisite components of the −92/−234 polynucleotide are currently in progress.

Studies of EBNA-2 mutants and their effects on LMP1 transactivation or cell growth transformation are consistent with the hypothesis that gene transactivation is the principal essential role of EBNA-2 in mediating B-lymphocyte transformation (7). EBNA-2 transactivates several cell genes in addition to the LMPs and the *Bam* C EBNA promoter (9, 20, 38, 39). The LMP and CD23 *cis*-acting EBNA-2-responsive elements are in fact likely to differ, at least in part. EBNA-2 genes from type 1 and type 2 EBV isolates have substantially diverged (10). The type 1 EBNA-2 is a better transactivator

of CD23 in stable or transient transfection assays (38, 40). Isogenic EBVs with type 1 EBNA-2 induce consistently higher levels of CD23 than EBVs with type 2 EBNA-2 (8). However, cells transformed with EBVs with type 1 or 2 EBNA-2 have similar LMP1 levels, and type 2 EBNA-2 induces similar or higher levels of LMP1 promoter activity in transient transfection assays (8, 41). Thus, there must be CD23- or LMP1-specific components to the type 1 or type 2 EBNA-2 and to the respective *cis*-acting elements.

The LMP1 *cis*-acting element described here is also immediately upstream of the LMP2B transcriptional start site (at −219 relative to the LMP1 transcriptional start site) and may also affect LMP2A expression. The LMP2A transcriptional start site is 3.0 kb downstream of the LMP1 start site, and LMP2A is EBNA-2 responsive (43). In acute EBV infection, LMP1 and LMP2A do not accumulate until after EBNA-2 expression (2). LMP1 and LMP2 may need to be jointly regulated so that they can functionally interact. In contrast to the well-established role of LMP1 in rodent fibroblast transformation (5, 35, 36) or in B-lymphocyte activation (37, 38), the exact role(s) of LMP2A and 2B in transformation is largely unknown. LMP2A associates with LMP1 in the transformed B-lymphocyte plasma membrane and may modulate LMP1's constitutive activating effects through its interaction with tyrosine kinase (25, 26). The location of the EBNA-2-transactivatable *cis*-acting element defined in this study further strengthens the link between these integral membrane proteins.

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