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Negative Regulation of the Human Polyomavirus BK Enhancer Involves Cell-Specific Interaction with a Nuclear Repressor

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We have examined the cell type-specific regulation of the human BK virus (BKV) enhancer. This enhancer functions efficiently in *cis* to activate expression from the adenovirus major late promoter in the human kidney cell line, 293, and in a monkey kidney cell line, MK2, but not in the HeLa cell line. In gel retardation migration assays, specific BKV enhancer-protein complexes could be observed by using nuclear extracts prepared from each cell line. Moreover, a unique DNA-protein complex was observed by using the HeLa cell nuclear extracts. By DNase footprint analysis, four binding regions for HeLa cell nuclear proteins were defined within the BKV enhancer repeat region. Two of the protected regions encompassed nuclear factor 1 or CCAAT transcription factor binding sites. These nuclear factor 1 sites also were protected by nuclear proteins from the 293 and MK2 cell lines. The other two protected sites encompassed a region of symmetry which included a sequence similar to the simian virus 40 TC enhancer motif and to a conserved sequence present upstream or within the introns of several cellular genes. These two sites were not protected by either the 293 or MK2 nuclear proteins. Competition studies in transfected cells indicated that the reduced activity of the BKV enhancer in the HeLa cell line was due to negative regulation. Further, we have demonstrated that binding of a nuclear factor(s) to the HeLa cell-specific site is involved in the repression of enhancer activity.

The control of transcription in eucaryotic cells is a complex process involving the interaction of cellular protein factors with specific gene sequences. A number of *trans*- and *cis*-acting elements involved in the regulation of transcription initiation have been described. For example, the *cis*-acting enhancer sequence, first described as a repeat sequence in the regulatory region of simian virus 40 (SV40), can stimulate transcription from homologous and heterologous promoters, independent of position and orientation (for reviews, see references 18 and 32). The activity of enhancer sequences can be modulated by factors in *trans* as demonstrated by the ability of viral early proteins such as the adenovirus E1A proteins to repress (5, 25, 49) or stimulate (6, 21, 29) enhancer activity. In addition to viral *trans*-acting factors, a number of investigators have identified binding sites for nuclear proteins within enhancer sequences (2, 3, 15, 30, 37, 40, 41, 47), and there is evidence that the binding of *trans*-acting proteins regulates enhancer activity (1, 4, 17, 34, 35, 45, 48, 53).

For both viral and cellular enhancer sequences, host cell specificity has been observed (21, 38), and recent studies have described host cell-specific binding of nuclear proteins to enhancer sequences (2, 11, 34, 35, 51). Host cell specificity of enhancer activity could be controlled by the presence or absence of either positively or negatively acting factors. For example, Atchison and Perry (1) suggest the kappa gene is regulated in development by the presence or absence of a positively acting factor that controls kappa enhancer activity. Goodbourn et al. (19) suggest that the human beta interferon gene enhancer is controlled by a negative regulatory factor which is inactivated upon induction, while a positively acting factor has been suggested to be involved in the regulation of the mouse *H-2* and β-2-microglobulin genes (27).

In the present study, we have examined the host cell-specific regulation of the enhancer from a strain of BK virus (BKV) containing a duplicated 100-base-pair (bp) repeat. This regulatory sequence contains the 68-bp repeat previously shown to be homologous to a normal cellular sequence with enhancer activity (44). In a previous report, we determined that the BKV enhancer could activate transcription of the adenovirus type 2 (Ad2) major late promoter (MLP) in kidney cells and that this *cis* activation could be enhanced in *trans* by the E1A proteins of adenovirus (21). In this report, we demonstrate that the BKV enhancer functions with low efficiency in the HeLa cell line when its activity is compared with that in the human kidney cell line 293 and in the monkey kidney cell line MK2. We demonstrate a host cell-specific difference in the binding of nuclear proteins to the BKV enhancer and present evidence demonstrating that the BKV enhancer is under negative control in HeLa cells. Further, we show that cell type-specific binding of nuclear factors is involved in the repression of the BKV enhancer in HeLa cells.

MATERIALS AND METHODS

Cell lines, DNA transfection, and CAT assay. The adenovirus-transformed human kidney cell line 293 (ATCC CRL 1573) and the MK2 (ATCC CCL7) and the HeLa cell lines (ATCC CCL 2.1) were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum. One day prior to transfection, cells were plated at a density of 10^6 cells per 100-cm^2 culture dish. Calcium phosphate DNA precipitates (52) were prepared with various amounts of purified plasmid DNA (see text). In cotransfection experiments, a pBR322 derivative (pAT153) was used as a carrier to ensure that all transfected cultures received that same amount of total DNA. Four hours after transfection, culture medium was replaced (for 293 cells) or the cells were treated for 90 s with 10% glycerol in Dulbecco modified Eagle medium followed by medium replacement (for HeLa and MK2 cell lines). Two to three days after transfection with the chloramphenicol acetyltransferase (CAT) expression plasmids, the cells were trypsinized and

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washed and the cell lysates were analyzed for CAT activity by the method of Gorman et al. (20). The level of CAT activity from each plasmid is presented as the average of data from 2 to 8 separate experiments. Relative CAT activities varied only 15 to 20% between experiments.

Plasmid constructions. The procedures for molecular cloning and purification of plasmids for transfection of mammalian cells were as described previously (22). Plasmids for the expression of the bacterial CAT gene were constructed as follows. Plasmid pSV2-CAT was obtained from the American Type Culture Collection. Plasmid pLP-CAT was constructed by replacing the SV40 regulatory region in pSV2-CAT, *Pvu*II (SV40 nucleotide [nt] 270) to *Stu*I (SV40 nt 5171) and up to the *Acc*I site in the pBR322 sequence, with a restriction fragment isolated from pLP (a pBR322-*Bal*I-E recombinant originally obtained from P. A. Sharp) containing the Ad2 MLP (*Acc*I, Ad2 nt 5758 to *Pvu*II, nt 6071). A *Bcl*I linker (PL Biochemicals) was inserted between the resulting *Pvu*II and *Stu*I junction. pBL2-CAT was derived by inserting an *Acc*I (BKV nt 4318)-to-*Pvu*II (BKV nt 402) restriction fragment containing the enhancer region of BKV (strain BKV-P2, isolated from a preparation of prototype BKV Gardner and to be described elsewhere) in the late orientation upstream of the Ad2 LP sequence in pLP-CAT (replacing the restriction fragment, *Acc*I, Ad2 nt 5758 to *Stu*I, Ad2 nt 5772). The plasmid pUCLNb-5 is a pUC13-based plasmid containing one copy of the intact BKV enhancer sequence (*Stu*I-to-*Avr*II fragment). Competitor DNAs were prepared by ligating isolated regions of the BKV enhancer to the pBR322 derivative pAT153. The various regions were as follows: C1, *Mst*II-digested and religated BKV enhancer containing a single 100-bp repeat; C2, isolated 100-bp *Mst*II fragment; C3, *Mst*II-to-*Avr*II fragment; C4, synthetic 68-bp repeat; C5 and C6, synthetic segments of the 68-bp repeat. The base pair location of each of the competitor DNAs is indicated (see Fig. 8a).

Gel migration inhibition assay. Nuclear extracts were prepared from HeLa, 293, and MK2 cells as described by Dignam et al. (12). The probe for this assay and for the footprint analysis described below was prepared by digesting pBL2-CAT with *Avr*II or *Stu*I followed by end labeling either with Klenow fragment and [^{32}P]deoxynucleoside triphosphates or with polynucleotide kinase and [^{32}P]ATP. The labeled plasmid DNA was digested with either *Avr*II or *Stu*I, and the 316-bp fragment containing the BKV enhancer was purified from an 8% polyacrylamide gel by using methods previously described (23). Nuclear extracts were incubated with the probe under conditions similar to those described by Carthew et al. (9) at 25°C for 30 to 45 min with 10 ng (approximately 10,000 cpm) of probe in a 10-μl reaction mixture containing 200 ng of poly(dI-dC) (Pharmacia), 15% glycerol, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM dithiothreitol. Samples were subjected to electrophoresis on 4% low-ionic-strength polyacrylamide gels as described by Fried and Crothers (14). After the gels were dried, the protein-DNA complexes were visualized by autoradiography.

DNase I footprint analysis. Nuclear extracts were incubated for 30 min at 25°C with approximately 10 ng of end-labeled BKV enhancer probe in the reaction buffer described above for the gel retardation analysis. Following incubation, DNase I was added to the reaction mixture to a final concentration of 2.5 μg/ml and the digestion was allowed to proceed for 1 min at 20°C. As a control, the probe was incubated in the reaction mixture without nuclear extract and digested with DNase as described above. DNase digestion reactions were terminated by the addition of EDTA to 10 mM followed immediately by phenol-$CHCl_3$ extraction and ethanol precipitation. The precipitated samples were analyzed on 7 M urea-polyacrylamide sequencing gels as described previously (23).

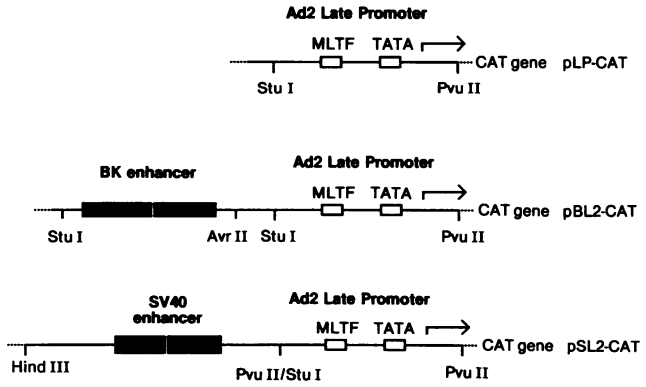

FIG. 1. Schematic representation of the promoter regions of CAT expression vectors used in this study. The core plasmid in each case was derived from pSV2-CAT (20). The SV40 regulatory region was excised and replaced with the Ad2 MLP (pLP-CAT), a hybrid transcriptional unit containing the MLP and BKV enhancer (pBL2-CAT), or a hybrid transcriptional unit containing the MLP and the SV40 enhancer sequence (pSL2-CAT) (see Materials and Methods for details of the constructions). The cap site in each regulatory region is indicated by an arrow, the TATA homology and upstream factor binding region (MLTF) of the MLP (9) are indicated, and the solid box represents the enhancer sequences.

RESULTS

Comparative activity of the BKV enhancer in HeLa, 293, and MK2 cells. To compare the activity of the BKV enhancer in the various host cells, we used a transient expression system with the CAT gene. The regulatory regions of the CAT expression plasmids used in this study are shown in Fig. 1. Each plasmid is identical except for the regulatory region driving expression of the CAT gene; pLP-CAT contains the enhancerless Ad2 MLP, pBL2-CAT contains the MLP with the BKV enhancer upstream, and pSL2-CAT contains the MLP with the SV40 enhancer upstream. pSL2-CAT was used in these studies to compare the well-studied SV40 enhancer with the BKV enhancer. The CAT expression plasmids were introduced into cells by calcium-phosphate-mediated transfection, and 48 to 72 h later, cell lysates were prepared and assayed for the level of CAT activity.

In Table 1, we show the relative levels of CAT gene activity in the HeLa, 293, and MK2 cell lines transfected with the plasmids shown in Fig. 1. The MK2 and 293 cell

TABLE 1. Effect of the BKV and SV40 enhancers on the Ad2 MLP in several cell lines

Plasmid	Relative level[a] of CAT in cell line:		
	HeLa	MK2	293
pLP-CAT	1	1	1
pSL2-CAT	26	18	1.8
pBL2-CAT	1.5	22	73

[a] The level of CAT from pLP-CAT was assigned a value of 1 in each cell line, and the levels from other plasmids were expressed relative to that value.

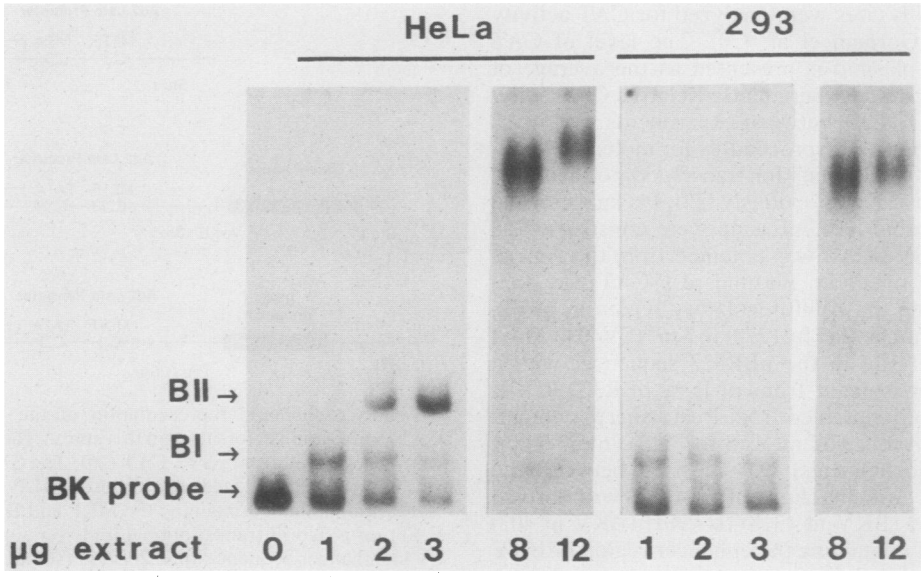

FIG. 2. Binding of HeLa and 293 cell nuclear proteins to the BKV enhancer. The probe was the *Stu*I-to-*Avr*II fragment of pBL2-CAT containing the BKV enhancer (Fig. 1). Approximately 10 ng of end-labeled probe was incubated with various amounts of a nuclear extract prepared from either HeLa or 293 cells, and the protein-bound probe was separated from the free probe on a low-ionic-strength polyacrylamide gel. BI and BII indicate the location of bound probe at low concentration of nuclear extract. The location of the free BKV probe (0 μg of extract) is indicated.

lines were used in this study because we previously demonstrated that the BKV enhancer stimulated expression from the MLP in these lines. In the E1A-producing 293 cell line, the SV40 enhancer had little effect on the level of expression from the MLP, consistent with previous reports indicating that E1A represses SV40 enhancer-stimulated transcription (5, 49). In contrast, the BKV enhancer increased expression from the MLP approximately 70-fold. Both the BKV and SV40 enhancers activated transcription from the MLP approximately 20-fold in the MK2 cell line. In HeLa cells, the SV40 enhancer stimulated transcription from the MLP (26-fold), but only a marginal level of activation (1.5-fold) was observed with the BKV enhancer. By Northern (RNA) blot analysis, we determined that differences in the level of CAT activity reflected differences in the level of CAT mRNA. In addition, we determined by Southern blot analysis that differences in plasmid copy number were not responsible for the differences in CAT activity or CAT mRNA levels (data not shown). Our data indicate that the BKV enhancer functions as an efficient *cis*-acting element in the two kidney cell lines examined but not in the HeLa cell line.

Differential binding of nuclear factors to the BKV enhancer. Because of the observed host cell specificity, experiments were performed to determine whether there were differences in the binding of nuclear factors from the HeLa, 293, and MK2 cells to the BKV enhancer. Nuclear extracts were prepared, and specific binding of protein factors to a ^{32}P-labeled fragment containing the BKV enhancer was determined in the gel migration retardation assay described in Materials and Methods. The ability of each nuclear extract to support transcription was determined in an in vitro assay by measuring runoff transcription from the MLP as described previously (23). The extracts prepared by the method described above were transcriptionally active (data not shown).

The BKV enhancer used in this study (BKV-P2) contains a 100-bp repeat which includes the 68-bp repeat found in the regulatory region of the Gardner (prototype) strain of BKV (46). As shown in Fig. 2, the BKV enhancer probe migrated as a discrete band. Addition of an increasing amount of nuclear extract resulted in the appearance of distinct bands of slower mobility. With low levels of added HeLa or 293 cell nuclear extract (1 to 3 μg), one common band (BI) was observed. This same band also was observed by using an MK2 cell nuclear extract (data not shown). At high levels of added extract from each cell line (8 and 12 μg), the probe migrated as a broad single band near the top of the gel. However, with the HeLa cell nuclear extract an additional unique band (BII) was observed when low levels of nuclear extract were used. No binding to the BKV enhancer probe could be detected when binding reactions were treated with proteinase K, suggesting that the more slowly migrating bands represent protein-DNA complexes.

To determine the specificity of the protein-DNA interactions, gel retardation assays were performed in the presence of competitor DNA fragments. For these experiments, labeled BKV enhancer probe was incubated with 3 μg of nuclear extract and increasing amounts of unlabeled enhancer fragment. An increasing amount of competitor BKV enhancer resulted in the loss of both band BI and BII. In contrast, when a 50-fold molar excess of a restriction fragment containing the SV40 enhancer sequence was used as a competitor, the HeLa cell band BII was not affected. The intensity of the common band (BI) was reduced but, in repeated experiments, never eliminated (data not shown).

Identification of the binding sites on the BKV enhancer. The gel retardation analyses demonstrated that an additional DNA-protein complex forms in the HeLa cell nuclear extract and the formation of this complex (BII) was specific. To determine the exact location of the binding region(s), we performed DNase I footprint analyses as described in Materials and Methods. The ratio of template to extract used in these experiments was that which gave differential binding in the gel shift analyses above, i.e., 10 ng of template and 3 μg of nuclear extract.

By using the HeLa cell nuclear extract, four regions were

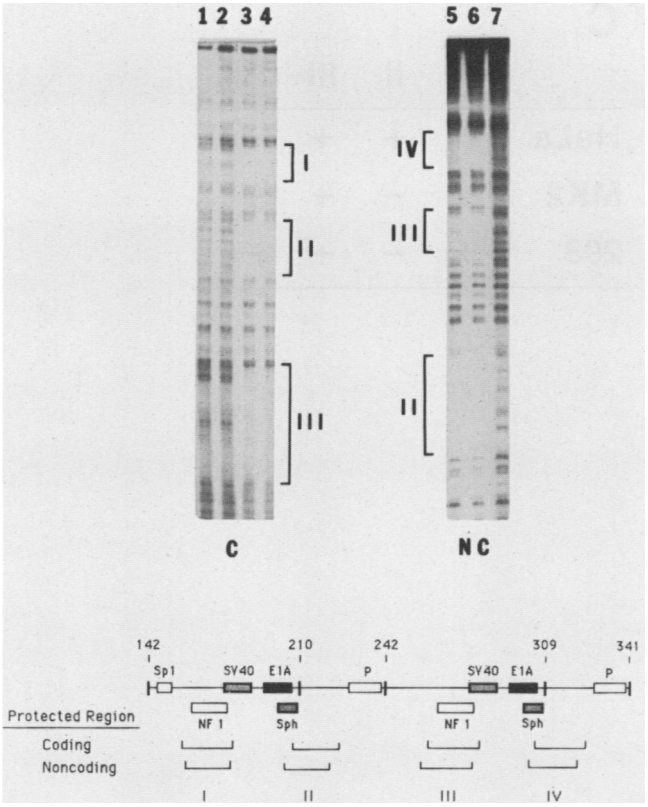

FIG. 3. DNase I footprint analysis of binding of HeLa cell nuclear proteins to the BKV enhancer. End-labeled restriction fragments containing the BKV enhancer were incubated alone (lanes 1, 2, and 7) or with a HeLa cell nuclear extract (lanes 3 to 6) and then digested with either 2.5 μg (lanes 1, 3, and 5) or 5 μg (lanes 2, 4, 6, and 7) of DNase per ml and analyzed on polyacrylamide sequencing gels. The autoradiograms show protected regions on both the coding and noncoding strands. The coding strand was defined relative to the BKV late promoter and mRNAs (46). Also shown is a schematic representation of the BKV enhancer with the regions protected by the HeLa cell nuclear extract indicated by brackets. The locations of sequences similar to previously described enhancer motifs are indicated.

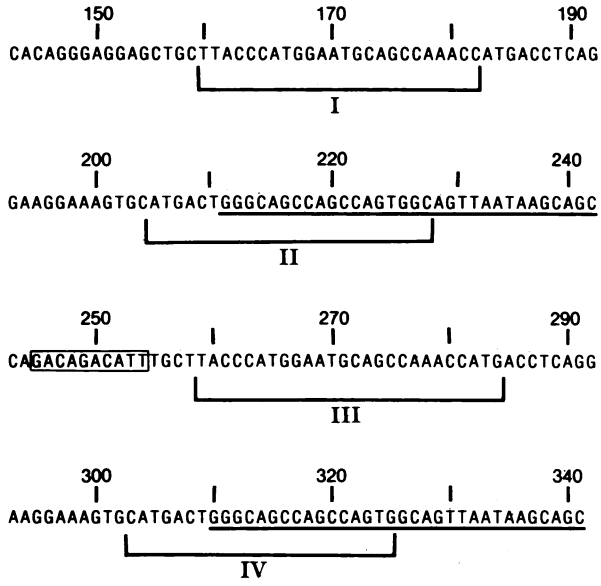

FIG. 4. Sequence of the BKV enhancer. The base pair numbering follows the convention of Seif et al. (46) and has the polarity of the late mRNAs. The 32-bp repeat sequences, which separate the 68-bp repeats, are underlined. The locations of the protected regions on the coding strand (relative to the late mRNAs) shown in Fig. 3 are indicated. The boxed region in the second 68-bp repeat indicates the nucleotides that differ from the first repeat.

protected from nuclease digestion (Fig. 3). On the coding strand, region I extended from nt 158 to nt 182, region II extended from nt 205 to nt 228, region III extended from nt 258 to nt 284, and region IV extended from nt 304 to nt 330. On the noncoding strand, the protected regions overlapped with those observed on the coding strand, with region I extending from nt 160 to nt 181, region II extending from nt 200 to nt 225, region III extending from nt 255 to nt 278, and region IV extending from nt 298 to nt 325. The BKV enhancer contains several proposed "recognition sequences" or motifs indicated schematically in Fig. 3. The sequence GGTCATGGTTTG, similar to the SV40 enhancer core sequence (50) or GT-1 motif (54) is present in each BKV enhancer repeat. The sequence GGGAGG, suggested by Rosenthal et al. (44) to be analogous to the Sp1 binding site (13), is present in the origin proximal repeat but not in the other repeat because of several nucleotide differences (boxed region in Fig. 4). In addition, the sequence AGGAAAGTGCAT, similar to the core element of adenovirus E1A enhancers (24), is present in each of the repeat elements. The sequence TGGAATGCAGCCA is present in each of the 68-bp repeats, and this sequence has been shown to be a binding site for purified nuclear factor 1 (NF1) (39). In addition, sequences homologous to the *Sph*I motif (210/309 TCAGTACGT 202/301), the SV40 enhancer P motif (229/328 AGTTAATAAG 239/338) and TC motif (215/314 ACGGGTC 209/308) are present (54). As shown in Fig. 3 and 4, the regions I and III protected by the HeLa cell nuclear extract encompass the NF1 binding sites in the 68-bp repeat and the protected regions II and IV encompass a unique sequence that includes the TC-like motif.

Under identical conditions, footprint analysis was performed with both the 293 and MK2 cell nuclear extracts. As shown in Fig. 5B, protection of region III was observed with the 293 cell extract but protection of region II (and region IV, not shown) was not observed even at high levels of nuclear extract (Fig. 5A). Protection of region I also was observed with the 293 cell extract, and the protection patterns with the MK2 cell extract were identical to those observed with the 293 cell nuclear extract. The results of the DNase I footprint analyses are summarized in panel C. Both by gel retardation and DNase footprint analyses, we have demonstrated that HeLa cells contain a nuclear factor capable of interacting with a site on the enhancer-containing sequences from the 32-bp repeat (junction of the 32- and 68-bp repeats), and this factor is not present or is nonfunctional in the MK2 and 293 cell lines.

Negative regulation of the BKV enhancer in the HeLa cell line. As shown above, the BKV enhancer *cis* activated expression from the MLP less than 2-fold in the HeLa cell line, compared with 20- and 70-fold in the MK2 and 293 cell lines, respectively. These data suggest that HeLa cells either lack a positively acting factor or contain a negatively acting factor that interacted with this enhancer. The detection of additional protected regions with the HeLa cell nuclear extract is more consistent with the possibility of a negatively acting factor or repressor. If, in fact, the BKV enhancer was

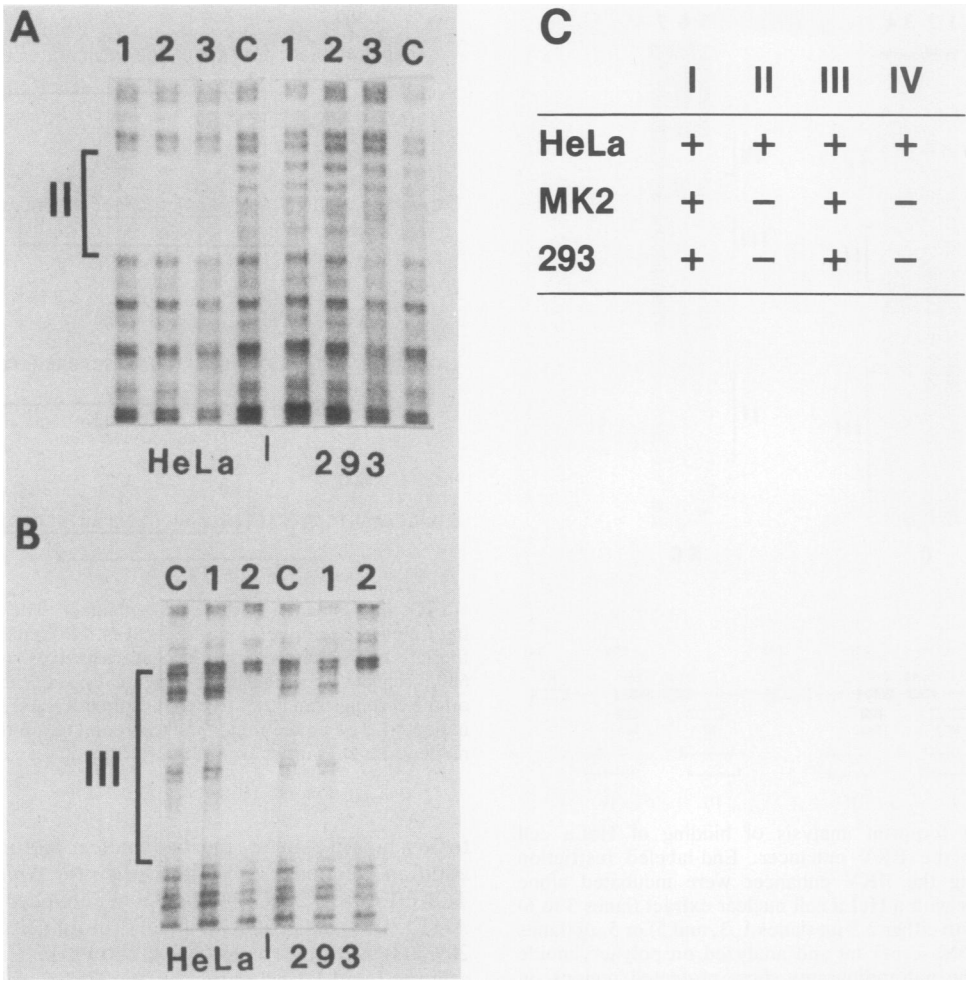

FIG. 5. Comparison of DNase footprints on the BKV enhancer with HeLa, 293, and MK2 cell nuclear extracts. (A) DNase protection pattern of region II with HeLa and 293 cell nuclear extracts. Reaction conditions were identical except for the amount of nuclear extract added: lanes 1, 2 μg; lanes 2, 4 μg; lanes 3, 8 μg; lanes C, control, or no extract added. (B) DNase protection pattern of region III with HeLa and 293 cell nuclear extracts. Lanes C, control, of no added extract; lanes 1, 2 μg; lanes 2, 4 μg. (C) Summary of regions on the BKV enhancer protected by nuclear extracts from the indicated cell lines.

being repressed in the HeLa cell line, it should be possible to titrate repressor activity.

Experiments were performed to determine whether higher input DNA concentration in the cell could overcome the apparent lack of enhancer activity. Both HeLa and MK2 cells were transfected with increasing amounts of plasmid pBL2-CAT, and 48 to 72 h later, the level of CAT activity was determined. As shown in Fig. 6A, increasing levels of input plasmid DNA resulted in an increasing level of CAT activity in the MK2 cell line. This also was observed with the 293 cell line, and the level of CAT activity was proportional to the level of plasmid DNA in the cell, as determined by Southern blot analysis (data not shown). In contrast, the level of CAT expression from pBL2-CAT in the HeLa cell line did not increase proportionally with increasing input DNA. There was very little CAT expression from pBL2-CAT at low input DNA (up to 0.25 pmol of input DNA). However, the level of CAT activity increased dramatically (15-fold) at 0.5 pmol of input DNA. Similar experiments were performed with pSL2-CAT, and in both the MK2 and HeLa cell lines, CAT activity increased proportionally with the level of input DNA (data not shown). Thus, there appeared to be a threshold of input DNA in HeLa cells above which efficient transcription from the BL transcriptional unit could occur.

The above results are consistent with the presence of a negatively acting element that can be titrated out with increasing DNA input. To obtain more direct proof, competition experiments were performed (Fig. 6B). Both HeLa and MK2 cells were transfected with 0.25 pmol of pBL2-CAT along with increasing amounts of a plasmid containing the *Avr*II-to-*Stu*I fragment of BK virus which encompasses the BKV enhancer sequence. With an increasing amount of intact BKV enhancer as competitor, the level of CAT activity from pBL2-CAT in the MK2 cell line increased slightly at a two- to fourfold molar excess, decreasing to low levels with higher levels of competitor. In contrast, the relative level of CAT activity from pBL2-CAT in the HeLa cell line increased to high levels, between 20- and 25-fold with a 2- to 4-fold molar excess of competitor. When competition studies were performed at levels above 0.5 pmol of input pBL2-CAT, i.e., above the threshold shown in Fig. 6A, increasing levels of competitor had little effect on CAT levels (data not shown). These data strongly suggest that

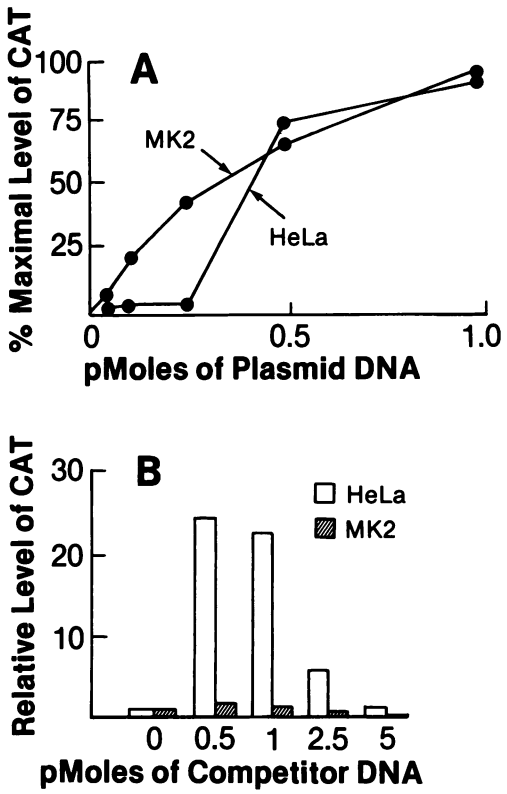

FIG. 6. Plasmid titration and competition assays of transfected pBL2-CAT in HeLa and MK2 cells. Cells were transfected with pBL2-CAT alone (A) or with a competitor plasmid pUCLNb-5 containing the BKV enhancer (B) and assayed for CAT activity 72 h later. (A) Dose responses for the activity of plasmid pBL2-CAT in the HeLa and MK2 cell lines. The percent of maximal level of CAT was determined from the actual level of CAT obtained at a saturating level of input plasmid DNA, determined in this experiment to be approximately 1 pmol/10^6 cells (5.5-kbp plasmid) for both cell lines by using the transfection method described in the Materials and Methods. (B) Activation of pBL2-CAT in HeLa cells by increasing competitor plasmid containing the BKV enhancer. pBL2-CAT (0.25 pmol) was used for each assay, with the indicated level of competitor plasmid pUCLNb-5. The relative level of CAT with no competitor was assigned a value of 1 for both cell lines.

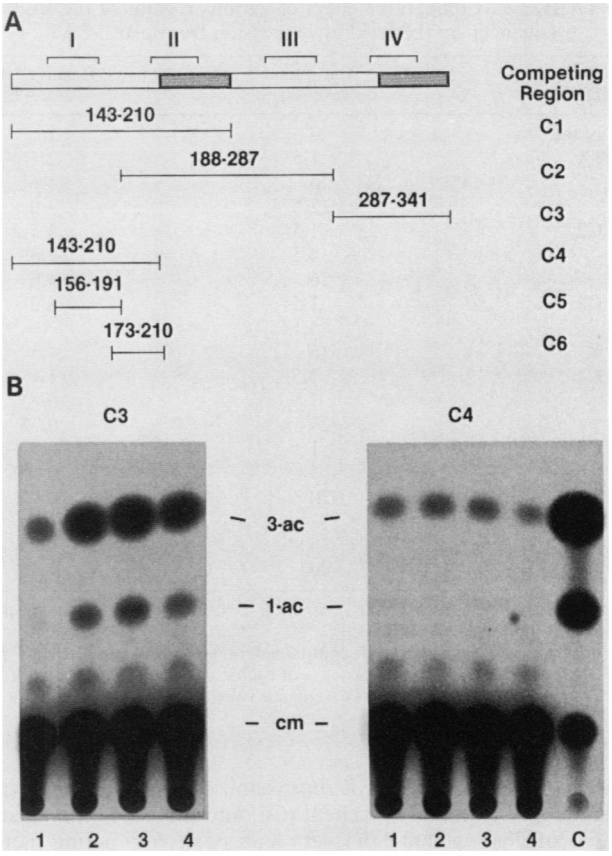

FIG. 7. Competition of pBL2-CAT with various regions from the BKV enhancer in HeLa cells. (A) Location of various regions of the BKV enhancer used in the competition studies. The numbers above each region are the beginning and ending base pair locations. (B) Cells (10^6/dish) were transfected with approximately 0.25 pmol of pBL2-CAT alone or with increasing levels of plasmid DNAs containing either region C3 or C4. After 48 h, a cell extract was prepared and assayed for CAT activity as described in Materials and Methods. Unreacted [^{14}C]chloramphenicol (cm) and its acetylated forms, chloramphenicol 1-acetate (1-ac) and chloramphenicol 3-acetate (3-ac), were separated by thin-layer chromatography and detected by autoradiography. Lanes 1, No competitor: lanes 2, 0.35 pmol of competitor; lanes 3, 1.1 pmol of competitor; lanes 4, 4.1 pmol of competitor. C, Control purified CAT (PL Biochemicals).

HeLa cells contain a repressor of the BKV enhancer. In the experiment shown in Fig. 6B, the level of CAT activity was still higher than the control at a 10- to 20-fold excess of competitor (5- and 2-fold, respectively) but lower than that observed at a 2- to 4-fold molar excess of competitor. This drop in CAT activity at high molar excess of competitor may be due to competition for positive transcription factors. On the basis of DNA input titration curves, 2.5 and 5 pmol of input plasmid DNA (for a 5.5-kbp plasmid) are over the saturating concentration for both cell lines. In addition, the fragment of BKV used as the competitor contained sequences encompassing the early promoter of the virus and these sequences could compete for positive factors necessary for efficient MLP activity. The slight increase in the level of CAT in the MK2 cell line with increasing competitor suggests that low levels of the repressor may be present in this cell line as well.

Competition studies with different regions of the BKV enhancer repeats. Above, we have demonstrated that the activity of the BKV enhancer is repressed in HeLa cells, and the enhancer contains a HeLa cell-specific binding site. From these data, it is tempting to speculate that the additional protected region in the enhancer represents the binding site for the repressor protein. To test this hypothesis, competition experiments were performed by using, as competitors, plasmid DNAs containing various regions of the enhancer repeats (Fig. 7A). The effect of two of these competitor DNAs on the level of CAT expression from pBL2-CAT is shown in Fig. 7B. With increasing concentration of C3, which contains the HeLa cell-specific binding site, we observed a six-to eightfold stimulation in the level of CAT from pBL2-CAT. In contrast, no increase in CAT expression was observed with competitor C4, representing the intact 68-bp repeat. In fact, a slight decrease in CAT expression was observed at the highest concentration tested. These results are summarized, along with the data from experiments with the other competitors, in Table 2. Only competitors containing the HeLa cell-specific binding site were capable of stimulating expression from pBL2-CAT.

TABLE 2. Competitive effect of various regions of the BKV enhancer on the level of expression from pBL2-CAT

Competitor DNA[a]	Amt (molar excess)	Relative level of CAT[b]
None	0	1
C1	1.5	2.5
	5	4.5
	12.5	3.0
C2	1.5	3.5
	4.5	5.3
	16	4.0
C3	1.5	6.2
	4.5	7.9
	16	4.5
C4	2.5	1.1
	7.5	0.85
	25	0.35
C5	1	0.9
	5	0.75
	20	0.45
C6	1	1.3
	5	0.95
	20	1.1

[a] The location of each of the competitor DNAs on the BKV enhancer is shown in Fig. 7A.

[b] Cells were transfected with approximately 0.25 pmol of pBL2-CAT per 10^6 cells alone or with a molar excess of each competitor. The level of CAT activity with no competitor was assigned a value of 1 in each experiment.

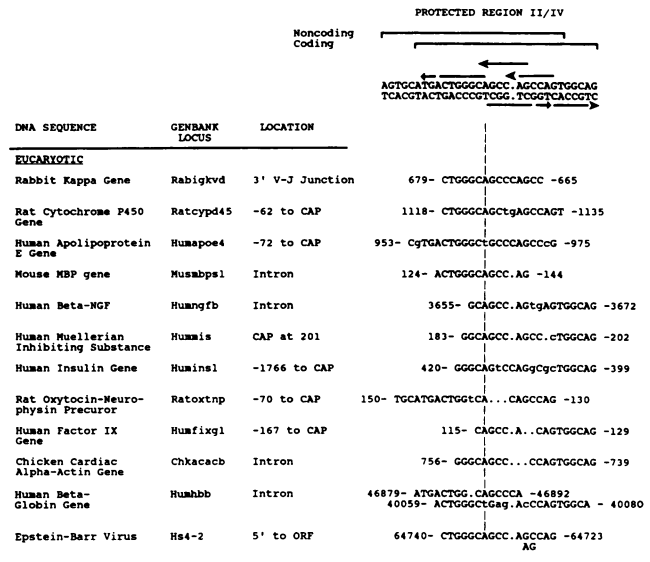

FIG. 8. Identification of eucaryotic gene sequences that share homology with the HeLa cell-specific binding site in the BKV enhancer. The sequences listed are from a homology search of the GenBank data base by using the sequence analysis software package of the University of Wisconsin Genetics Computer Group (Wordsearch/Segments). The GenBank locus for each entry is given, and the nucleotide numbers are those assigned in the locus. The centers of symmetry in the HeLa cell-specific binding site II/IV are indicated by arrows.

However, we observed a repression of CAT activity with high levels of the 68-bp repeat (C4) and the NF1 binding site (C5), suggesting that NF1 acts as a positively acting factor for the BKV enhancer. We were unable to achieve the 20- to 25-fold increase in CAT observed when the intact BKV enhancer was used as a competitor (Fig. 6B). This may indicate that optimal binding of the repressive factor requires the context of both repeat sequences. Nevertheless, only competitors containing binding site II/IV were able to relieve repression of pBL2-CAT, and the results are consistent with these regions being the binding site(s) for a HeLa cell-specific repressor.

DISCUSSION

An understanding of the individual molecular events involved in the initiation of transcription is central to our understanding of tissue-specific and temporal gene regulation. Positively acting factors are required for the efficient initiation of transcription in eucaryotes. In addition, the negative regulation of eucaryotic genes has been suggested on the basis of the observations that inhibition of protein synthesis can activate the transcription of certain genes and that tissue-specific expression can be abolished in cell fusion experiments (10, 19). The recent results of Colantuoni et al. (10) and Goodbourn et al. (19) have indicated that negative regulation can be mediated through an enhancer sequence. In this report, we have studied the host cell specificity of the human polyomavirus BKV enhancer sequence in an effort to determine the reason for differences in its activity in several host cell lines. We find that the BKV enhancer, a homolog of a cellular enhancer sequence, is under negative regulation in HeLa cells and that an additional protein factor from these cells binds to a specific site in the enhancer repeat. Further, we present evidence from competition studies that the negative regulation of the enhancer observed in this cell line involves binding of a cell-specific repressor.

The HeLa cell-specific binding site identified in the BKV enhancer has not previously been identified as an enhancer-regulatory motif. As shown in Fig. 8, this site contains regions of symmetry. With one gap, the binding site contains one center of palindromic symmetry around A 215/314 and another around G 224/323. In addition, the sequence CAGC CAG is repeated twice. A search of the GenBank data base identified several sequences with various degrees of homology to the BKV binding site. Of interest, the homologous sequence in the rat oxytocin-neurophysin gene is within the promoter region and is either an identical or conserved sequence among species (28). In addition, the homologous sequence upstream of the rat cytochrome gene cap site is in the region identified to be required for transcription (16). In relation to the BKV repressor site, we do not know the functional significance of the homologous sequences shown in Fig. 8. However, all of the sequences are located in regions where regulatory elements would be expected, suggesting that the BKV binding site is a conserved sequence with potential regulatory function in other genes.

Nowock et al. (39) demonstrated that purified NF1 could bind to the BKV enhancer. Using nuclear extracts from three different cell lines, we have demonstrated that the NF1 sites were protected and this interaction occurred at the lowest concentration of extract used. Recently, NF1 and the CCAAT binding transcription factor (CTF) were reported to be the same protein (31), and CTF is required for the transcription of certain genes. In our competition studies, the single copy of the 68-bp repeat containing the NF1/CTF-binding site or the NF1 site itself (C5) did not act as a competitor to relieve the repressor activity but actually reduced the level of expression from pBL2-CAT (Table 2). These data suggest that the NF1 site may be acting as a competitor for a positively acting factor and that NF1 (CTF) could be involved in the positive regulation of the BKV

enhancer. However, the presence of the 68-bp repeat alone is not sufficient for enhancer activity (21). Possibly, interactions between sites on the duplicated enhancer repeat or with BKV promoter elements are required for optimal activity. The presence of sites for both positively (I and III) and negatively (II and IV) acting elements in the BKV enhancer is similar to that observed by Goodbourn et al. (19), who demonstrated that the β-interferon enhancer consisted of constitutive transcription elements and a negative regulatory sequence. As with the interferon gene enhancer, our data support the contention that enhancer activity requires interactions with factors present in all cells but can be regulated by specific repressors that block activity in certain cells, rendering those cells nonpermissive for enhancer function. In further support of this concept, Imler et al. (26) have proposed that the lack of heavy-chain enhancer activity in nonlymphoid cells is due to the presence of negative regulatory elements that prevent activation by ubiquitous stimulatory factors.

In previous studies by Rosenthal et al. (44), the BKV repeat region of the Dun strain was shown to stimulate the activity of the SV40 early promoter in a number of host cells, including HeLa. The Dun strain of BK virus contains a repeat region which includes the 68-bp sequence but not the 32-bp sequence shown in Fig. 4. Thus, this strain of the virus does not contain the HeLa cell-specific binding site. The ability of the enhancer region from BKV Dun to stimulate promoter activity in HeLa cells is consistent with our results demonstrating that the repression of the BKV-P2 enhancer requires a binding site which includes sequences in the 32-bp repeat.

As shown in Fig. 3, the BKV enhancer contains sequence elements similar to motifs previously shown to be important for the activity of other enhancers. For example, the SV40 core (GT motif) and Sph motifs have been shown to bind nuclear factors and to be required for SV40 enhancer activity in HeLa cells (54). However, the homologous motifs in the BKV enhancer do not interact with nuclear factors in our assays, suggesting that these motifs are not important for BKV enhancer function in HeLa cells. In the competition studies (Table 2), the BKV enhancer fragments containing the NF1 binding site (C4 and C5) reduced the level of expression from pBL2-CAT at high molar excess. However, the C6 competitor, which contained the sequences similar to the E1A enhancer motif and the SV40 core and Sph motifs, did not reduce the level of expression from pBL2-CAT. These data suggest that the nonprotected motifs in the 68-bp repeat are not involved in the function of the BKV enhancer, and they emphasize the fact that the identification of sequences similar to known regulatory motifs in one enhancer does not guarantee that they will function in another regulatory element.

In a previous study, we demonstrated that the E1A proteins of adenovirus could stimulate the activity of the BKV enhancer in the MK2 cell line as it does in the 293 cell line (21). However, we recently have found that E1A further represses the activity of the BKV enhancer in the HeLa cell line (unpublished observation). The repression of other enhancers by E1A has been observed in the HeLa cell line (5, 49). Although we do not know whether there is a relationship between the repression of BKV-P2 enhancer activity in the HeLa cell line and the further repression of its activity by E1A, it is conceivable that E1A stimulates the interaction of the HeLa cell repressor with the BKV enhancer. In fact, E1A has been shown to increase either the amount or the activity of transcription factors (33). We demonstrated previously that the 68-bp repeats alone were not sufficient for the E1A responsiveness of the BKV enhancer. By examining deletion mutants, we demonstrated that deletion of sequences that would have included the 32-bp repeat in the prototype BKV enhancer eliminated the E1A responsiveness (21). Thus, the E1A-responsive region of the enhancer and the putative repressor binding site may overlap. Further studies will be needed to determine whether, in fact, E1A interacts with or modulates the repressor activity in the HeLa cell line.

It is not clear how the binding of a nuclear protein to the HeLa cell-specific site might repress the activity of the BKV enhancer, and until we understand how the enhancer activates expression we can only speculate as to mechanism of its repression. Possibly, as in procaryotes (43), binding of the repressor molecule prevents polymerase entry or alters the local DNA structure, thereby preventing the interaction with or formation of transcriptionally active complexes. Because of the close positioning of the repressor site and the potential positive motifs (e.g., NF1 sites), binding at the repressor site could affect protein-protein interactions at the positively acting sites. The mechanism of BKV enhancer repression probably is different from that of the silencer sequence identified in yeast (7, 8) and mammalian (36, 42) genes. These sequences act at a distance and are not thought to work by direct steric interference between regulatory elements. Regardless of the mechanism of repression, the identification of repressor sites within an enhancer sequence suggests that the overall activity of genes in nature can be modulated by a balance between positive and negative interactions at the enhancer. This balance may be critical in determining tissue- and developmentally regulated expression.

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