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Activation of the Adenovirus and BK Virus Late Promoters: Effects of the BK Virus Enhancer and *trans*-Acting Viral Early Proteins

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We have examined the activation of the adenovirus major late promoter (MLP) by the *cis*-acting enhancer element of the human polyomavirus BK and by the *trans*-acting simian virus 40 (SV40) T antigen and adenovirus E1A proteins. By using chloramphenicol acetyltransferase expression vectors, we found that the MLP (pLP-CAT) was *trans*-activated in human and monkey kidney cells expressing the SV40 T antigen. In addition, the MLP could be *cis*-activated by the BK virus enhancer in both human and monkey kidney cells; approximately 20 times more chloramphenicol acetyltransferase was produced from expression vectors containing a hybrid promoter (BL), in which the BK enhancer was upstream of the MLP, than from expression vectors containing the MLP alone. This same level of enhancement of the MLP by the BK enhancer was observed in cells expressing the T antigen of SV40. However, in the 293 cell line, greater enhancement of MLP activity (70-fold) was observed with the BK enhancer sequence. In contrast, MLP activity in the 293 cell line was unchanged by the SV40 enhancer. In cotransfection experiments, MLP activity, augmented by the BK enhancer, could be further stimulated with a plasmid coding for the E1A gene products. By creating deletion mutants, we determined that the high-level activation of the hybrid BL transcriptional unit by the E1A proteins requires both MLP sequences and an intact BK virus enhancer. On the other hand, activation of the BL transcriptional unit by the T antigen did not require an intact enhancer sequence. Our results suggest that the SV40 T antigen and E1A proteins *trans*-activate the BL promoter by different mechanisms. We also demonstrate in cotransfection experiments that the BK late promoter is activated 45-fold by the SV40 T antigen.

A major factor in controlling the expression of genes transcribed by RNA polymerase II is the ability to activate and repress the initiation of transcription. Many studies have demonstrated that this control is mediated by both *cis*- and *trans*-acting factors. One of the most completely studied examples of a *cis*-acting element is the enhancer first described as a repeat sequence in the regulatory region of simian virus 40 (SV40). This element can act to stimulate the transcription of adjacent genes in a position- and orientation-independent manner (reviewed in references 18 and 33). Both viral and cellular enhancer sequences have been identified (4, 7, 13, 19, 25, 27, 41, 53), and in many cases they exhibit species or host cell specificity (3, 12, 16, 37, 45, 46, 62). Specific interactions between enhancer-containing sequences and nuclear components have been observed (50), suggesting that the activity of enhancer sequences may be regulated by factors in *trans*. Additional support for this contention comes from studies by Sassone-Corsi et al. (49), suggesting that a *trans*-acting factor may be responsible for the activity of the SV40 enhancer in vitro.

As with the *cis*-acting control elements, the *trans*-acting factors have been well studied in model viral systems. For example, the early protein of SV40, T antigen, is known to stimulate transcription from the SV40 late promoter (8, 32), to repress the activity of its own synthesis from the early promoter (34, 47, 57), and to stimulate replication of the viral genome (10, 56, 59). Recent studies also suggest that the SV40 T antigen can stimulate the activity of other promoters (2). Another *trans*-acting viral factor, the adenovirus E1A protein(s), has been shown to be required for the efficient expression of early transcriptional units in adenovirus-infected cells (5, 31, 43, 64). In a number of plasmid expression studies, the E1A gene products have been shown to stimulate transcription from adenovirus early transcriptional units as well as from other promoters (15, 22, 30, 55, 60). With the SV40 early promoter, it has also been suggested that the E1A gene products can substitute or relieve the requirement for the enhancer sequence (60). In addition to the enhancement activity, products of the E1A gene have been shown to repress the activity of certain genes, e.g., the class 1 major histocompatibility antigens (51). Several recent studies also suggest that the E1A protein can repress enhancer-induced stimulation of transcription. Velcich and Ziff (61) demonstrated, in transient expression assays, that E1A reduces transcription from the SV40 early promoter and suggested that the decrease results from the ability of the E1A protein to antagonize the action of the enhancer element. Similar studies by Borrelli et al. (6) indicated that adenovirus type 2 (Ad2) E1A proteins repress the activation induced not only by the SV40 enhancer but also by the murine polyomavirus and E1A enhancers. Furthermore, repression of the immunoglobulin heavy-chain enhancer by the Ad2 E1A products has been demonstrated (28).

In the present study, we have examined the transcriptional regulation of the adenovirus major late promoter (MLP) with an upstream sequence containing the region of 68-base-pair (bp) repeats from the human polyomavirus BK (BKV). This repeat sequence has been shown previously to have the properties of an enhancer element (48, 52). We find that the BK enhancer sequence markedly stimulates transcription from the Ad2 MLP in all cell lines tested, including those producing the *trans*-acting Ad2 E1A proteins or the SV40 T antigens. The presence of the BKV enhancer sequence resulted in a 70-fold increase in MLP activity in cells expressing E1A (293 cells). This is in sharp contrast to the enhancer-dependent repression of promoters by the E1A

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proteins as described previously (6, 61). We also demonstrate that the enhancer-stimulated activity of the MLP could be further increased by cotransfection with a plasmid coding for the E1A proteins. In both human and monkey kidney cells expressing the SV40 T antigen, the BL transcriptional unit was highly active. Furthermore, both the MLP and BL promoter could be activated in cotransfection experiments with T antigen. However, our results suggest that an intact enhancer sequence is not required for this activity. Furthermore, we demonstrate that the BKV late promoter (LP) alone, like the SV40 LP, is activated by the SV40 T antigen.

MATERIALS AND METHODS

Viruses and cells. Prototype BKV, Gardner strain, was obtained from the American Type Culture Collection (ATCC) and was grown in primary human kidney cells. Twelve days after infection, viral DNA was isolated from the cells as described previously (29). Primary human fetal liver and kidney cell cultures were grown in Delbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 10% tryptose phosphate broth. Adenovirus-transformed human kidney cell line 293 (ATCC CRL 1573), MK2 cells (ATCC CCL7), COS-1 cells (ATCC CRL 1650), and SS78 (Lilly Research Laboratories, human kidney) were grown in DMEM supplemented with 10% fetal bovine serum.

Isolation of a human kidney cell line expressing the SV40 T antigen. Primary human fetal kidney cells were transfected with a plasmid (pMK16/8–16; originally obtained from Y. Gluzman) containing an origin-defective SV40 genome (17). Morphologically transformed colonies were isolated 4 weeks after transfection and passaged. All isolated colonies were positive for SV40 T antigen by immunofluorescence assays (14) with sera from tumor-bearing hamsters (obtained from R. Frisque). Clone K816-4 was used in this study, and by immunofluorescence we estimate that this cell line produces levels of T antigen equivalent to those produced by the COS-1 cell line. K816-4 has been stably passaged for 10 months.

Plasmid constructions. The procedures for molecular cloning and purification of plasmids for transfection of mammalian cells have been described previously (23). Plasmids for the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene were constructed as follows. Plasmid pSV2-CAT was obtained from ATCC. 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-Bal1-E recombinant originally obtained from P. A. Sharp) containing the Ad2 major LP (*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. pBK-CAT was similarly derived by inserting an *Acc*I (BKV nt 4318) to *Pvu*II (BKV nt 402) restriction fragment containing the regulatory region of BKV into pSV2-CAT with the deletion described above. The plasmid pBL-CAT was constructed by inserting the same restriction fragment of BKV in the late orientation upstream of the Ad2 MLP sequence in pLP-CAT (replacing the restriction fragment *Acc*I, Ad2 nt 5758, to *Stu*I, Ad2 nt 5772).

A plasmid for the expression of the Ad2 E1A gene was constructed with a *Bal*I restriction fragment (Ad2 nt 270 to nt 2065) containing the E1A coding sequence. The E1A gene was inserted into a plasmid containing the BKV regulatory region (at the *Stu*I site at nt 5100) so that the BKV enhancer (early orientation) was upstream of the E1A cap site. This plasmid, pE1A, also contains the *Eco*RI to *Nde*I restriction fragment of pBR322 including the ampicillin resistance gene and origin of replication. A plasmid coding for the SV40 T antigen (pSV3-GPT [42]) was obtained from ATCC.

Deletions in these CAT expression plasmids were made as follows. To delete all but one copy of the SV40 enhancer in pSV2-CAT, the plasmid was digested with *Sph*I, which cleaves once in each enhancer sequence, and religated to delete the resulting 72-bp *Sph*I fragment. The resulting plasmid, pdSV-CAT, was further deleted by removal of the *Sph*I to *Nde*I restriction fragment to obtain pmdSV-CAT, lacking an intact enhancer repeat. To remove all but one copy of the BKV enhancer, pBL-CAT was digested with *Mst*II, which cleaves once in each of the three repeats, and religated to remove the resulting 118-bp fragment (pdBL-CAT). Additional deletions in the BL transcriptional unit were created by Bal 31 nuclease digestion at the *Sst*II site (Ad2 nt 5793) to remove sequences between the Ad2 MLP and BKV enhancer (described in the text).

Transfection of cells and CAT assay. 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 were prepared (66) with 10 μg of plasmid DNA and no carrier DNA. In cotransfection experiments, 10 μg of each plasmid was used. Four hours after transfection, the culture medium was replaced. Two to three days after transfection, the cells were trypsinized and washed, and the cell lysates were analyzed for CAT activity by the method of Gorman et al. (20) or used for the RNA and DNA analyses described below. The level of CAT activity by each plasmid is presented as the average of data from three to eight separate experiments. Relative CAT activities varied by only 10 to 15% between experiments.

RNA and DNA extraction and analysis. RNA was isolated from transfected cells by two procedures. For Northern blot analysis, RNA was isolated by the LiCl precipitation procedure of Cathala et al. (9). For cytoplasmic RNA dot hybridization, the formaldehyde method of White and Bancroft (65) was used. Northern analysis was performed as described previously (36) with a ^{32}P-labeled probe consisting of the *Hin*dIII to *Bam*HI restriction fragment of pSV2-CAT (CAT coding sequence plus SV40 splice and polyadenylation sequences). The isolation of total cellular DNA and Hirt supernatant DNA, as well as the procedures for DNA dot blot and Southern analysis, have been described previously (24).

RESULTS

Expression of CAT plasmids in human and monkey kidney cells. We used a transient expression system with the CAT gene to study the effect of *trans*-acting factors on several regulatory regions; the Ad2 MLP, the BKV LP, and a hybrid transcriptional unit composed of the MLP and regulatory region (including the enhancer) of BKV. Shown in Fig. 1 are the CAT expression plasmids used in this study. Each plasmid is basically the same except for the regulatory region driving expression of the CAT gene; pLP-CAT contains the enhancerless Ad2 MLP, pBK-CAT the BKV regulatory region in the orientation of the late promoter, pBL-CAT the hybrid transcriptional unit described above, and pSV2-CAT the SV40 regulatory region in the orientation of the early promoter. pSV2-CAT was used in these studies for compar-

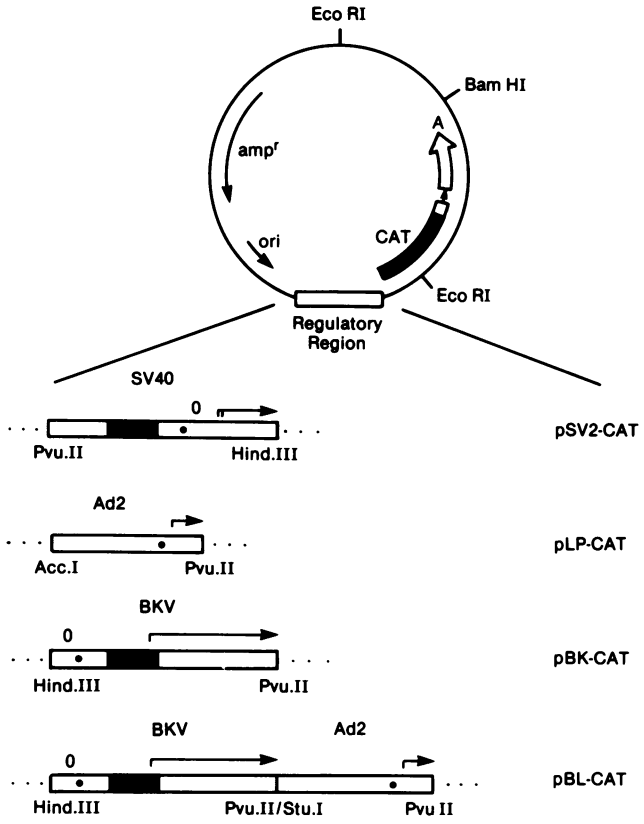

FIG. 1. Schematic representation of the recombinant CAT expression plasmids used in this study. The core plasmid in all cases was derived from pSV2-CAT (20), in which expression of the CAT gene is under control of the SV40 early promoter. The SV40 regulatory region was excised and replaced with the Ad2 MLP (pLP-CAT), the BKV LP (pBK-CAT), or a hybrid transcriptional unit (pBL-CAT) containing both adenovirus and BKV regulatory sequences (see Materials and Methods for details of the constructions). The potential transcriptional start sites are indicated by arrows. Dots indicate the locations of TATA homologies, and the solid box represents the enhancer sequence.

ative purposes because of the well-studied nature of the SV40 regulatory region. We examined the relative activity of each of these regulatory regions in kidney cells expressing the SV40 T antigen or the E1A gene products, as well as in kidney cells not known to express either of these *trans*-acting factors. As described in Materials and Methods, the various 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 Fig. 2A and B, we show the level of CAT gene activity in the 293 cell line, a human kidney line that constitutively expresses the E1A protein of adenovirus type 5 (Ad5) (21). The level of CAT produced in cells transfected with pLP-CAT, containing the MLP alone, was relatively low but easily detectable (Fig. 2A, lane 3). The level was approximately fivefold less than that observed with pSV2-CAT (lane 1). The level of CAT activity in 293 cells transfected with pBK-CAT was significantly lower than that from cells transfected with either pSV2-CAT or pLP-CAT, suggesting that the BKV late promoter is only weakly active in the E1A-positive cell line (lane 2). However, as will be shown below, the level of CAT mRNA from pBK-CAT, unlike that from the other plasmids, did not correlate with the level of CAT activity; the level of CAT mRNA from pBK-CAT was actually much higher, twice that of pLP-CAT. In 293 cells transfected with pBL-CAT, we observed a high level of CAT gene activity, approximately 70-fold higher than from the enhancerless pLP-CAT. This suggested that the presence of the BKV enhancer markedly stimulated transcription from the MLP even in the presence of the E1A proteins. This observation is in sharp contrast to the results reported for other enhancers (see Introduction). In addition, we examined the level of CAT gene activity in 293 cells transfected with a plasmid (pSL-CAT) containing, as a regulatory region, the MLP with an upstream SV40 enhancer sequence. The regulatory region in this plasmid is similar to that in the plasmid pSVBA34 (6), in which enhancer-induced stimulation of transcription was shown to be repressed by E1A products. In basic agreement with previous reports (2, 6, 61), we determined that the SV40 enhancer had no effect on the activity of the MLP in the 293 cell line; the levels of CAT from pLP-CAT and pSL-CAT were approximately the same (data not shown).

In Fig. 2C and D we compare the level of CAT activity from the expression plasmids in the K816-4 cell line, which expresses another *trans*-acting factor, the SV40 T antigen. Like the adenovirus-transformed 293 cell line, K816-4 was derived from human fetal kidney cells. In these T-antigen-producing cells, the BL transcriptional unit (pBL-CAT) was more active than the enhancerless MLP (pLP-CAT), but the relative difference was not as great as that observed in the 293 cell line. The level of CAT activity from the BKV LP (pBK-CAT) appeared to be higher in the K816-4 cell line than in the 293 cell line, suggesting that the BKV LP was stimulated by the SV40 T antigen. This observation was confirmed in studies described below.

The relative activities of the CAT expression plasmids were examined in three additional kidney cell lines: COS-1, an SV40 T-antigen-producing monkey kidney line, and two kidney cell lines, MK2 (monkey) and SS78 (human), that did not produce either the SV40 T antigen or adenovirus E1A proteins (Table 1). The level of CAT in cells transfected with pSV2-CAT was chosen as the reference for each cell line. In the two cell lines not expressing *trans*-acting factors (MK2 and SS78), we observed a marked increase in the level of MLP transcription in the presence of the BKV enhancer (pBL-CAT), approximately 20-fold. Likewise, the relative difference in the level of CAT from pLP-CAT and pBL-CAT in the T-antigen-positive K816-4 cell line was approximately 20-fold. This is in contrast to the 70-fold difference in activity between pLP-CAT and pBL-CAT in the 293 cell line. These data indicate that in all cells tested, the presence of the BKV enhancer stimulated the level of transcription from the MLP and furthermore that the activity of the MLP was not only stimulated by the upstream BKV sequence but that this enhancer-stimulated transcription was further augmented in the presence of the E1A gene products. Very little CAT activity could be detected in either MK2 or SS78 cells transfected with pBK-CAT, whereas substantially higher activity than was obtained with the other expression plasmid could be detected in the T-antigen-producing cell lines. In fact, the level of CAT mRNA from pBK-CAT and pSV2-CAT in the COS-1 and K816-4 cell lines was approximately the same (see below). These data also suggest that the BKV LP is *trans*-activated in the presence of the SV40 T antigen.

As indicated above, we examined the activity of the MLP in the presence of the SV40 enhancer in the 293 cell line and

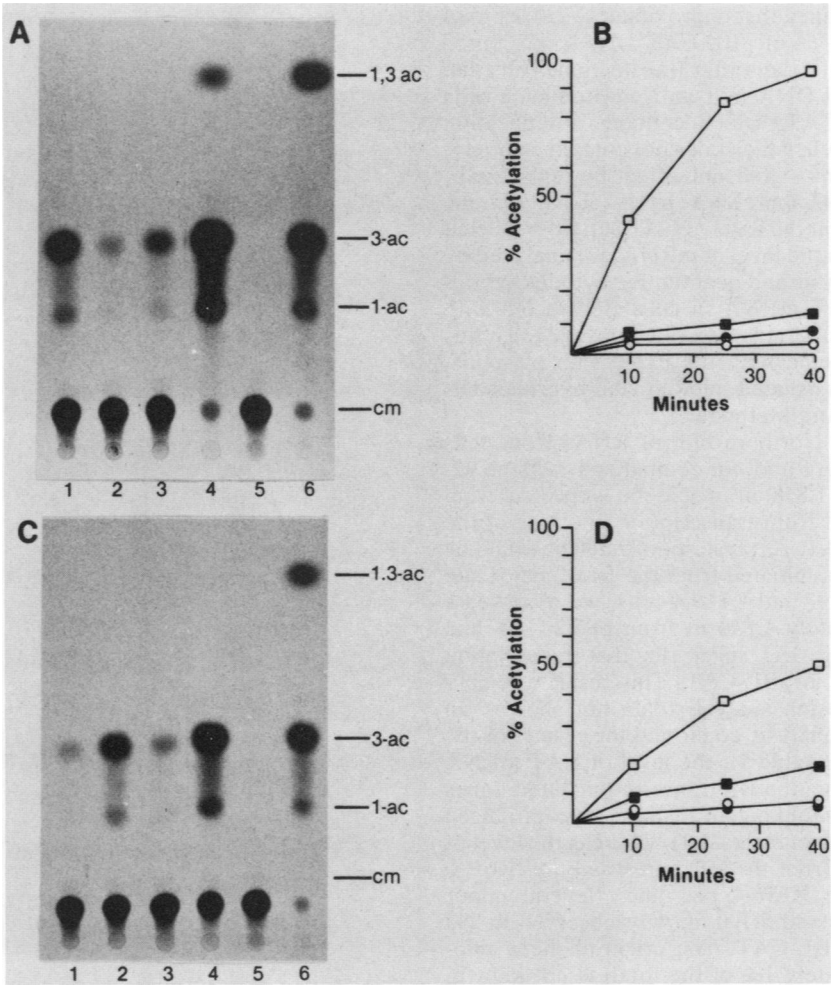

FIG. 2. Activity of CAT expression plasmids in human kidney cells expressing Ad5 E1A proteins (293 cells) or the SV40 T antigen (K816-4 cells). The plasmid DNAs (10 μg, as a calcium phosphate precipitate) were applied to cells plated the day before at a density of $1.3 \times 10^4/cm^2$. After 48 h a cell extract was prepared and assayed for activity as described in Materials and Methods. Untreated [^{14}C]chloramphenicol (cm) and its acetylated forms, chloramphenicol 1-acetate (1-ac), chloramphenicol 3-acetate (3-ac), and chloramphenicol 1,3-acetate (1,3-ac) were separated on thin-layer silica gels and detected by autoradiography. (A) Level of CAT activity in the 293 cell line with expression plasmids pSV2-CAT (lane 1), pBK-CAT (lane 2), pLP-CAT (lane 3), and pBL-CAT (lane 4). Mock-transfected cells and purified CAT (PL Biochemicals) controls are shown in lanes 5 and 6, respectively. (B) Kinetic analysis of CAT activity in 293 cells with pSV2-CAT (■), pBK-CAT (○), pLP-CAT (●), and pBL-CAT (□). (C) Level of CAT activity in the K816-4 cell line with expression plasmids pLP-CAT (lane 1), pSV2-CAT (lane 2), pBK-CAT (lane 3), and pBL-CAT (lane 4). Lanes 5 and 6, mock-transfected cells and purified CAT, respectively, as controls. (D) Kinetic analysis of CAT activity in the K816-4 cell line with the expression plasmids. The symbols for each plasmid are the same as described for panel B.

found that it had no effect. In contrast, this promoter (in pSL-CAT) was approximately 20 times stronger than the MLP alone in each of the additional cell lines tested (data not shown). Thus, the MLP activity was stimulated by the SV40 enhancer in the non-E1A-producing cell lines. These data further contrast the ability of the E1A products to repress transcription from promoters containing the SV40 enhancer (2, 6, 61; this report) while apparently stimulating transcription in the presence of the BKV enhancer.

We examined the plasmid content of cells transfected with the various expression vectors to ensure that the differences in CAT protein and mRNA levels observed within each cell line were not due to differences in the amount of plasmid retained in the cells. The amount of DNA in each cell was determined at 72 h after transfection by hybridization analysis of either total cellular DNA or DNA isolated by the Hirt procedure (see Materials and Methods). Within each individual cell line tested, we did not observe any significant differences in the amount of each transfected plasmid. Thus, for any one cell, differences in copy number were not responsible for the differences in CAT activity from the expression plasmids. However, we did observe differences in the amount of plasmid DNA retained between the different cell lines. Depending on the cell line, two to five times more DNA was retained in the 293 cell line than in the other cells used in this study. This level of stabilization is far lower than that reported by Alwine (2). However, in a report by Lebkowski et al. (38), stabilization of plasmid DNAs was not observed in the 293 cell line. We also examined *Mbo*I-digested DNAs from the T-antigen-producing cell lines by Southern blot analysis to determine how much replication had occurred (replicated DNA can be distinguished by its ability to be cut with *Mbo*I; methylated input plasmid DNA is refractory to cleavage with this enzyme). In repeated

experiments, we determined that approximately 22% of total pSV2-CAT DNA and 17% of pBL-CAT DNA represented newly replicated DNA at 2 days after transfection. Thus, the total amount of plasmid DNA in T-antigen-producing cells transfected with pBL-CAT, which contains a replication origin, and with pLP-CAT, which does not contain an origin, was not influenced to any significant extent by replication.

Analysis of transcripts and RNA levels. To determine whether the differences in the level of CAT activity reflected equivalent differences in the level of mRNA, we analyzed by both Northern blot analysis and quantitative cytodot hybridization the level of CAT mRNA in cells transfected with each of the expression plasmids described in Fig. 1. To this end, cells were transfected with the expression plasmids, and 72 h later RNA was isolated, blotted, and hybridized as described in Materials and Methods.

In Fig. 3 we show a Northern blot of RNAs from cells transfected with pBL-CAT and, for comparison, with pSV2-CAT. A transcript of 1,838 nt would be expected from pSV2-CAT and 1,847 nt from transcription initiating at the MLP cap site in pBL-CAT. A transcript of 2,280 nt would be expected if transcription initiated from the BKV major late start site (52). In both 293 and K816-4 cells, we observed a transcript of approximately 1,840 nt from pSV2-CAT and also from pBL-CAT (Fig. 3A), suggesting that transcription initiated from the MLP in pBL-CAT. This result was confirmed by primer extension analysis (data not shown). In addition, the Northern analysis confirmed the results of the CAT assays (Fig. 1 and Table 1); the level of CAT mRNA produced in 293 cells from the BL transcriptional unit (lanes 2) was approximately 10-fold higher than the level produced from the SV40 early promoter (lanes 1), whereas the level of CAT mRNA produced from the BL unit was only two- to threefold higher in the K816-4 cell line. Several minor mRNA species of 3,500 and 5,100 nt were observed in 293 cells transfected with pBL-CAT. The origin of these additional bands (approximately 1% of the total) is not known, but they may represent aberrantly processed transcripts. In Fig. 3B we show quantitative analysis of RNA levels in transfected cells by the cytodot method. Again, the relative level of CAT mRNA from pSV2-CAT and pBL-CAT as well as pLP-CAT correlated exactly with the relative level of CAT activity (Table 1). The relative level of CAT mRNA from pBK-CAT was approximately fivefold higher than the relative level of CAT activity. The reason for this difference is probably the presence of an ATG codon in the restriction fragment of BKV used to construct pBK-CAT. This ATG specifies the initiating methionine codon of the putative agnoprotein of the virus and would precede the authentic AUG codon for the initiating methionine of the CAT protein. Based on the results of Kozac (35), the additional AUG would probably reduce the level of initiation at the CAT AUG, explaining the discrepancy between the relative CAT and mRNA levels. It should be noted that the lower translational efficiency of mRNA transcribed from pBK-CAT was observed in all cell lines examined. Also shown in Fig. 3B is the relative level of CAT mRNA from 293 cells transfected with plasmid pdBL-CAT. This expression plasmid, which produced approximately 10-fold less CAT mRNA than pBL-CAT, is a deletion derivative of pBL-CAT that contains only one copy of the BKV enhancer sequence and will be described in more detail below.

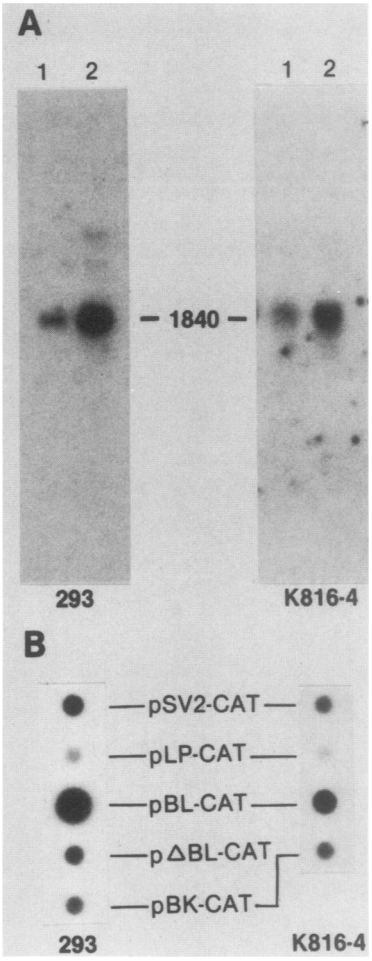

FIG. 3. Level of transcription from the CAT expression vectors in human kidney cell lines 293 and K816-4 by Northern blot (A) and cytodot (B) hybridization analyses. For Northern blot analysis, RNAs (10 μg) were isolated from cells transfected with the expression plasmids, fractionated on formamide-formaldehyde agarose gels, and, after transfer to nitrocellulose, incubated with a ^{32}P-labeled probe as indicated in Materials and Methods. (A) Lanes 1, RNAs from pSV2-CAT; lanes 2, RNAs from pBL-CAT. The major transcript at 1,840 nt is indicated. (B) RNAs were isolated from transfected cells, denatured with formaldehyde, and, after filtration onto nitrocellulose, incubated with the labeled probe (see Materials and Methods).

TABLE 1. CAT levels from expression plasmids with different regulatory regions in human and monkey kidney cell lines

Plasmid	Relative level[a] of CAT in cell line:				
	293	K816-4	COS-1	MK2	SS78
pLP-CAT	0.17	0.16	0.18	0.06	0.07
pSV2-CAT	1	1	1	1	1
pBK-CAT	0.07	0.37	0.40	ND[b]	ND
pBL-CAT	12.4	2.7	1.4	1.3	1.5

[a] The level of CAT from pSV2-CAT as assigned a value of 1 in each cell line, and the levels from other plasmids are expressed relative to that value. Results are the averages of from three to eight individual determinations for each data point.

[b] ND, CAT protein not detected.

Effect of deletions in the BL transcriptional unit. We have demonstrated that the BKV enhancer-stimulated activity of the MLP can be further augmented in cells producing the E1A proteins of adenovirus. To further characterize this stimulation of transcription, we created deletions in the BL transcriptional unit and, for comparison, in the SV40 regu-

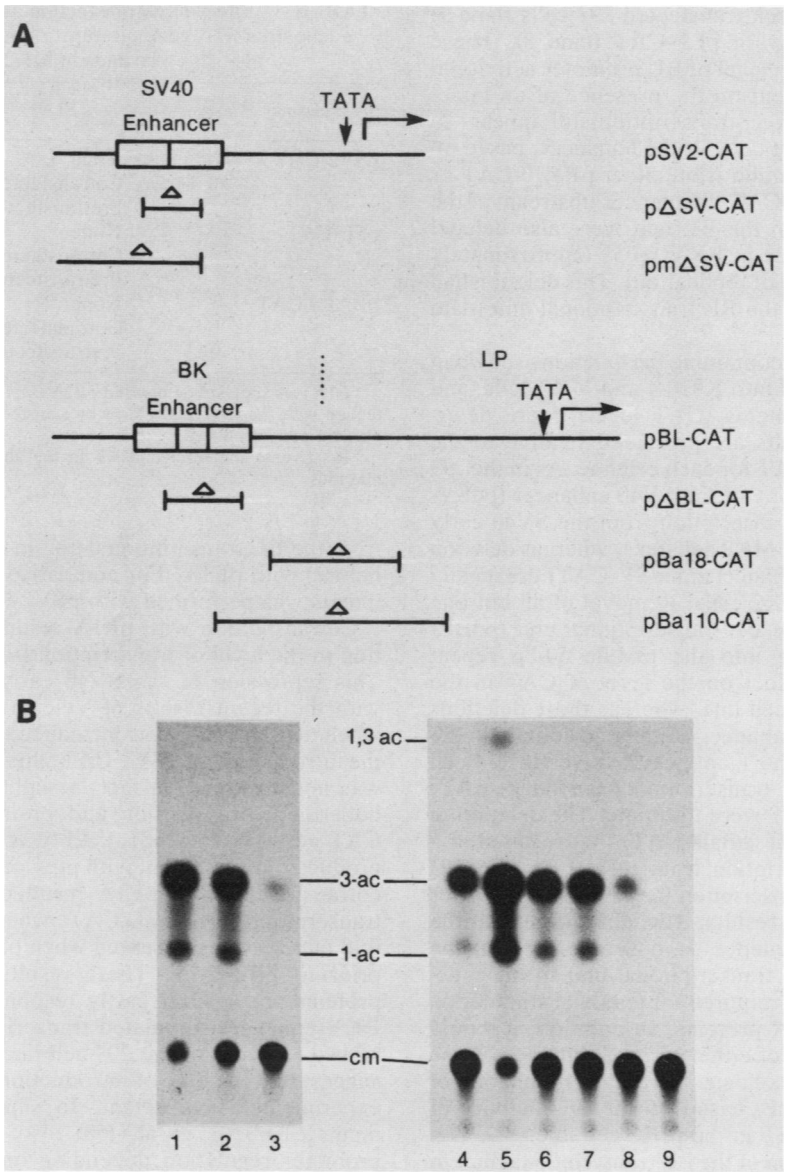

FIG. 4. Effect of deletions in the regulatory regions of pSV2-CAT and pBL-CAT on the level of gene expression. (A) Schematic representation of the deletions in the regulatory regions of CAT expression plasmids produced by removing restriction fragments or by Bal 31 digestion (see text for details). The symbol Δ is used in the schematic instead of the letter d for the plasmid designations. (B) Determination of CAT activity in 293 cells transfected with the plasmids shown in panel A. The assay conditions and abbreviations are described in the legend to Fig. 2. Lane 1, pSV2-CAT; lane 2, pdSV-CAT; lane 3, pmdSV-CAT; lane 4, pSV2-CAT; lane 5, pBal8-CAT; lane 6, pBal10-CAT; lane 7, pdBL-CAT; lane 8, pLP-CAT; and lane 9, mock transfected (no plasmid DNA).

latory region of pSV2-CAT. Expression plasmids containing the promoter deletions (Fig. 4A) were introduced into 293 cells, and the relative level of expression was determined by assessing the level of CAT produced (Fig. 4B). Compared with the intact SV40 regulatory region (pSV2-CAT, lane 1), removal of all but one copy of the SV40 enhancer (pdSV-CAT) had no effect on the level of transcription. This is consistent with previous reports (4, 25). However, removal of all but 21 bp of the enhancer (up to the *Sph*I site) resulted in a drastic reduction in the level of CAT produced (pmdSV-CAT, lane 3). This is in contrast to previous results in which the SV40 early promoter with or without the enhancer gave equivalent levels of globin mRNA in 293 cells (60). Deletions of various lengths were made in the BL transcriptional unit by Bal 31 nuclease digestion. In plasmid pBal8-CAT, sequences were removed to 27 bp downstream of the enhancer sequence and 127 bp upstream of the MLP cap site. This 232-bp deletion did not reduce the high level of transcription from the BL unit (lane 5). In fact, the level of CAT activity in 293 cells transfected with pBal8-CAT was 1.5- to 2-fold higher than in cells transfected with pBL-CAT (data not shown). However, a larger deletion with endpoints in the middle of the central 50-bp BKV enhancer repeat and 103 bp upstream of the MLP cap site resulted in a significant (five- to sevenfold) reduction in activity (pBal10-CAT, lane 6). A similar decrease in activity from the BL transcriptional unit was observed when all but one copy of the enhancer was deleted (pdBL-CAT, lane 7). Also shown in this figure are

the levels of CAT from mock-transfected 293 cells (lane 9) and 293 cells transfected with pLP-CAT (lane 8). These results indicate that the high level of BL promoter activity in the 293 cell line is dependent on the presence of an intact enhancer sequence. However, transcription still appears to be augmented with only one copy of the enhancer, based on the higher level of transcription from either pBal10-CAT or pdBL-CAT than from pLP-CAT. Sequences upstream of the BKV enhancer sequence in the BL unit were also deleted from the *Stu*I restriction site at BKV bp 51 (approximately 32 bp upstream of the origin of replication). This deletion had no effect on the activity of the BL transcriptional unit (data not shown).

The expression plasmids containing the deletions shown in Fig. 4 were also introduced into K816-4 and MK2 cells, and the relative promoter strengths (CAT levels) were determined (Table 2). The results are presented relative to the level of CAT from pSV2-CAT for each cell line. As in the 293 cell line, removal of one copy of the SV40 enhancer (pdSV-CAT) had little effect on transcription from the SV40 early promoter in the K816-4 and MK2 cell lines, whereas deletion of essentially the entire enhancer (pmdSV-CAT) drastically reduced transcription in MK2 cells. Removal of all but one copy of the enhancer in the BL transcriptional unit (pdBL-CAT) as well as deletion into the middle 50-bp repeat (pBal10-CAT) had little effect on the level of CAT in the T-antigen-positive K816-4 cell line, whereas these deletions essentially eliminated the enhancer-stimulated activity in the MK2 cell line. In fact, in the T-antigen-positive K816-4 cell line, the relative levels of transcription from pdBL-CAT, pBal10-CAT, and pBL-CAT were the same. The deletion in the pBL transcriptional unit (pBal8-CAT) that resulted in a twofold increase in transcription from the BL unit in 293 cells had little effect on transcription in either the K816-4 or MK2 cell line. From these results, it would appear that the intact BKV enhancer sequence is not required for the *trans*-activation of the BL transcriptional unit in the presence of the T antigen but is required for maximal stimulation in the presence of the E1A proteins. In addition, it would appear that in the absence of either *trans*-acting factor, one copy of the enhancer is not sufficient to obtain stimulation of transcription from the MLP, based on the low activity of pdBL-CAT and pBal10-CAT in the MK2 cell line.

Effect of E1A on expression of the BL transcriptional unit in MK2 cells. Although 293 cells constitutively express the E1A gene products, they also express other adenovirus products, most notably E1B (1). To confirm the involvement of E1A in the stimulation of the BL transcriptional unit, we cotransfected a plasmid coding for the E1A gene of Ad2 (pE1A, described in Materials and Methods) with pBL-CAT into MK2 cells. In addition, we examined the level of expression from the BL transcriptional unit in cells pretransfected (24 h before) with pE1A. For comparison, a similar set of experiments was performed with pSV2-CAT and pSL-CAT.

TABLE 2. Activity of CAT expression plasmids containing deletions in the regulatory region

Plasmid	Relative level[a] of CAT in cell line:		
	293	K816-4	MK2
pSV2-CAT	1	1	1
pdSV-CAT	0.9	1.2	1.4
pmdSV-CAT	0.08	NT[b]	0.02
pBL-CAT	10	2.3	1.75
pdBL-CAT	1.5	2.0	0.1
pBal8-CAT	17	1.8	1.2
pBal10-CAT	2	1.9	0.07

[a] See Table 1, footnote *a*.
[b] NT, Not tested.

TABLE 3. Effect of cotransfection and pretransfection with the adenovirus E1A gene on transcription from CAT expression plasmids in MK2 cells

Test plasmid	E1A[a]	Relative level of CAT[b]
pSV2-CAT	None	1
	Cotransfected	0.35
	Pretransfected	1.55
pSL-CAT	None	1
	Cotransfected	0.12
	Pretransfected	0.46
pBAL8-CAT	None	1
	Cotransfected	1.5
	Pretransfected	4.9

[a] E1A was supplied on plasmid pE1A (Materials and Methods) by cotransfection with the CAT plasmid or by transfecting cells 24 h before the CAT plasmid was introduced.
[b] Relative to the level of CAT in the absence of E1A for each of the plasmids.

Cotransfection with pE1A resulted in a threefold reduction in the level of transcription from pSV2-CAT (Table 3). This repression of the SV40 early promoter is consistent with the recent results of Velcich and Ziff (61). However, when the E1A gene was introduced into the cell 24 h prior to the introduction of pSV2-CAT, this decrease in CAT activity was not observed; in fact, a slight increase was noted. In both the cotransfection and pretransfection experiments, CAT activity from pSL-CAT was repressed by E1A gene products, in agreement with previous results (6). In contrast, cotransfection with pE1A resulted in a slight increase in transcription from pBL-CAT, whereas a reproducible fivefold increase was observed when pE1A was introduced 24 h prior to pBL-CAT. These results suggest that the E1A proteins are at least partly responsible for the increase in BKV enhancer-stimulated transcription from the MLP observed to occur in the 293 cell line. In addition, these data suggest that the time of introduction of the E1A gene in such experiments is important. In similar transfection experiments, Tibbetts et al. (58) also observed differences in promoter regulation depending on when the E1A coding sequence was introduced into the cell. The introduction of the E1A gene prior to the CAT expression plasmid may more closely reflect the situation in the 293 cell line, i.e., the E1A gene is already being expressed when the CAT plasmid is introduced into the cell. While we do not know the fraction of cells that were doubly transfected, it was most likely less than the fraction of singly transfected cells, because successive transfection efficiencies in the MK2 cell line are not 100%. Thus, the fivefold increase in BL promoter activity is striking, considering that not all cells expressing CAT were expressing E1A.

Effect of the SV40 T antigen on expression of the BKV and adenovirus LPs and BL transcriptional unit in MK2 cells. To determine whether the T antigen of SV40 could *trans*-activate our CAT expression vectors in the MK2 cell line, each plasmid was cotransfected with pSV3-gpt (42). The presence of the T antigen resulted in an increase in CAT gene expression from each of the promoters tested (Table 4). An 8- and 16-fold increase in BL and LP expression, respectively, was observed. As indicated above, the level of replication of pBL-CAT induced by the SV40 T antigen was not found to influence the copy number to any significant

TABLE 4. Effect of the SV40 T antigen on transcription from CAT expression plasmids in MK2 cells

Expression vector	T antigen[a]	Relative level of CAT[b]
pSV2 CAT	−	1
pLP-CAT	−	0.07
	+	1.12
pBK-CAT[c]	−	ND[d] (0.10)
	+	0.94 (4.75)
pBL-CAT	−	1.2
	+	9.7

[a] The T antigen was supplied by cotransfection with pSV3-gpt (42).
[b] Relative to the level of CAT produced from pSV2-CAT (value of 1).
[c] For pBK-CAT, the values in parentheses represent relative CAT levels based on levels of CAT mRNA. The values were corrected for the five-fold-lower translational efficiency of the mRNA produced by this vector (see text).
[d] ND, CAT protein not detected.

extent at 2 days posttransfection. In fact, the level of activation of the MLP on pLP-CAT, which does not contain a replication origin, was somewhat higher. Very little CAT activity could be detected in MK2 cells transfected with pBK-CAT, although the relative mRNA levels were found to be higher (see above). However, in the presence of the T antigen, significant levels of CAT could be detected; the level of activation was approximately 45-fold, based on relative mRNA levels.

DISCUSSION

The control of transcription in eucaryotic cells is a complex process involving the interaction of cellular protein factors with specific gene sequences. Attempts to elucidate the individual molecular events involved in the initiation of transcription have for the most part relied on expression of viruses. In this report, we have examined the ability of the *cis*-acting enhancer sequences of the human polyomavirus BKV to augment transcription from the Ad2 MLP in the presence and absence of two *trans*-acting factors, the T antigen of SV40 and the E1A proteins of Ad2 and Ad5. We have also examined the effect of these *trans*-acting factors on the expression of the BKV LP. To minimize the potential for effects on transcription due to host cell-specific factors, all of our in vivo expression experiments were performed in kidney cells, the natural host cell for primate papovaviruses.

Expression of the adenovirus MLP was activated by the BKV enhancer sequence in all cell lines tested, although to varying degrees (Fig. 1 and Table 1). The greatest difference between the level of CAT expression by pLP-CAT and pBL-CAT was in the 293 cell line. The level of enhancement of the MLP by the BKV enhancer in the absence of E1A (e.g., SS78 and MK2 cell lines) was approximately 15- to 20-fold, but was approximately 70-fold in the 293 cell line, i.e., the level of enhancement of MLP activity by the BKV enhancer in the presence of E1A proteins was approximately fourfold higher than expected from the level of enhancement observed in other cell lines. In addition, deletions in the BKV enhancer resulted in a five- to sevenfold drop in MLP activity from pBL-CAT in the 293 cells, and a fivefold increase in expression of pBL-CAT was observed in MK2 cells following introduction of the E1A gene. From these results, we suggest that in addition to activation of the MLP (40, 43), which requires sequences around the transcriptional start site (40), the E1A proteins display an enhancer-dependent stimulation in our pBL-CAT expression vector. Leff and Chambon (39) reported only low-level stimulation of the MLP by the 13S E1A product in HeLa cells and suggest that the transcriptional efficiency of the MLP in 293 cells is not necessarily related to E1A-responsive elements. Whether or not E1A products are solely responsible for activation of the MLP in 293 cells, our results clearly show that the BL transcriptional unit can be activated by E1A in cells other than the 293 line.

As indicated above (see Introduction), several reports have indicated that the E1A proteins repress or neutralize the effect of an enhancer on stimulating transcription. In relation to this study, it has been reported that transcription from the MLP, with the SV40 enhancer sequence upstream, is not activated by E1A either in cotransfection experiments with E1A-containing plasmids (6) or in 293 cells (40). As indicated in the text, we also compared the level of transcription between the MLP (pLP-CAT) and a hybrid promoter containing the MLP and an upstream SV40 enhancer (pSL-CAT), and we found no difference in the level of transcription in 293 cells. However, the expression of pSL-CAT was high in all of the non-E1A-producing cell lines tested. Thus, even though BKV and SV40 are highly related, their enhancer sequences function quite differently in the presence of the E1A proteins, being augmented or neutralized (repressed), respectively. This difference may not be too surprising, as the BKV and SV40 regulatory regions, although similar in organization, show little sequence homology (52). Recent evidence also suggests differences in the ability of these two enhancer sequences to interact with certain cellular DNA-binding proteins (44).

We have presented data (Table 2 and Fig. 4) showing that deletion of one copy of the SV40 enhancer in pSV2-CAT had little effect on expression in any of the cell lines examined, as might be expected from previous reports (4, 25). However, even in the 293 cell line, deletion of the enhancer core (pmdSV-CAT) resulted in a drastic drop in the expression level. The loss of transcriptional activity from pmdSL-CAT in 293 cells differs from the results of Treisman et al. (60), who demonstrated that an enhancerless SV40 early promoter linked to the β-globin gene was as transcriptionally active in 293 cells as the intact SV40 early promoter. On the other hand, transient expression studies in HeLa cells (61) suggested that the E1A proteins were incapable of activating an enhancerless SV40 early promoter. The reason for these differences is not clear, but there may be gene- or host cell-specific effects involved.

We have examined the effect of the T antigen of SV40 on the expression of the MLP and the BKV LP and on the BL hybrid transcriptional unit. In these experiments, the effect of T antigen was assessed by transfecting the expression plasmids into T-antigen-producing cell lines as well as by cotransfection with a plasmid coding for the T antigen. Although both the MLP and BL transcriptional unit were activated, the former promoter actually was stimulated to a higher level (Tables 1 and 4). These results suggest that the activation of MLP activity by T antigen is not dependent on the BKV sequences, i.e., the BKV enhancer increases the basal level of transcription but does not appear to influence or at least increase the level of *trans*-activation by T antigen. Moreover, deletions in the BKV sequences that affected expression in the presence of the E1A proteins had no effect on the level of MLP activity from pBL-CAT in the presence of the T antigen (Table 2 and Fig. 4). Thus, *trans*-activation of BL expression by E1A and T antigen appears to involve different mechanisms.

We observed that the level of enhancement of MLP transcription by the BKV enhancer was higher in the T-antigen-positive human kidney cell line (K816-4) than in the analogous monkey kidney cell line (COS). These cell lines produce similar amounts of the SV40 T antigen. BKV is known to infect and persist in the human kidney (24), and its enhancer has been shown to be a homolog of a human cellular enhancer (48). There may be host factors present in the human kidney cells that are required to obtain maximal stimulation from the human BKV enhancer.

Although a number of studies have addressed the role of T antigen in expression from the SV40 LP, the role of the T antigen in regulating the transcription of the BKV LP has not been examined. We have demonstrated that the level of expression of the BKV LP is 45-fold higher in the presence of the SV40 T antigen (Table 4). These results are in agreement with the reports indicating that the T antigen of SV40 is necessary for activating the SV40 LP (8, 32). For the SV40 LP, this activation has been suggested to occur in the absence of viral DNA replication. However, other reports suggest that the T antigen is not directly needed for late gene expression (11, 26). Our results also indicated that the BKV origin of replication was active in the presence of the SV40 T antigen (data not shown), but the role of replication in the observed *trans*-activation of the BKV LP was not assessed.

The ability to regulate the level of transcription of cellular genes, whether in a positive or negative fashion, most certainly plays a role in the dynamics of cell growth and metabolism. The BKV enhancer sequence is a homolog of a cellular enhancer, and our results suggest that it is regulated differently from other viral enhancers, at least by the E1A proteins. It is hoped that further studies to elucidate the mechanisms of *cis*- and *trans*-activation in model viral system such as those described here will aid in our understanding of cellular gene control. Moreover, such studies will yield transcriptional systems useful for high-level expression of heterologous proteins in mammalian cells.

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