Regulation of Polyomavirus Late Promoter Activity by Viral Early Proteins

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To assess the effect of the polyomavirus (Py) early proteins, the large T (LT), middle T (MT), and small T (ST) antigens, on gene expression from the Py late promoter, replication-defective plasmid constructs with the bacterial chloramphenicol acetyltransferase (*cat*) gene linked to this promoter were cotransfected into mouse or rat cells with plasmids capable of producing either LT, MT, or all three early proteins. When target CAT plasmids contained a truncated early region and thus had the coding potential for MT and ST, base-line CAT activities were low, whereas cotransfection with an LT plasmid resulted in up to 70-fold stimulation of CAT activity that was also reflected in similar increases in the level of steady-state mRNA. Studies with target plasmids with deletions within the Py regulatory region indicated that at least the major LT-binding site C and a functional enhancer region were both required for maximal stimulation of CAT activity. However, although enhancer deletions totally suppressed the ability of target plasmids to be *trans* activated, a consistent two- to fourfold stimulation of CAT activity by LT was still observed with a plasmid in which all three major LT-binding sites were deleted. Of four mutant LTs incapable of binding Py DNA but retaining immortalization potential, only one showed a low but significant *trans*-activating ability. When the early coding region was completely eliminated from the target plasmid, base-line CAT activity was increased 10-fold. LT failed to stimulate CAT activity to the same levels observed with the target plasmid containing the truncated early region, but this limited response could be enhanced by supplying, in addition, MT and ST. Our results suggest that LT *trans* activation may involve the formation of a complex of transcriptional factors which interacts with the enhancer, an interaction that is facilitated both by the binding of LT to the Py regulatory region and by the presence of MT or ST or both, and that a significant portion of LT stimulation of late gene expression is a result of the removal of the competing early transcriptional unit via autoregulation. In addition, our results suggest that LT *trans* activation involves a second indirect component acting independently of LT binding and that the immortalization and *trans* activation functions of LT can be dissociated.

A feature common to the proteins synthesized early in the course of many viral infections is their ability to stimulate expression from promoters used at later times. These proteins include the large T (LT) antigen of simian virus 40 (SV40) (1, 5-7, 30, 42) and the proteins encoded by the E1A gene of adenovirus (4, 38), the immediate-early genes of herpes simplex virus (17, 53, 67), cytomegalovirus (18), varicella-zoster virus (17), and pseudorabies virus (20, 29, 35, 36), and the E2 gene of bovine papillomavirus (61). *Trans*-acting proteins that stimulate the viral promoter situated within the long terminal repeats of human T lymphotropic (9, 58), Rous sarcoma (8), and bovine leukemia (15, 56) retroviruses have also been described. Another feature of some of these proteins is their ability to stimulate cellular promoters with an apparent lack of sequence specificity (46). This has led to the suggestion that another property of many of these proteins, the ability to immortalize primary cells, might be related to their ability to *trans* activate promoters.

The early region of the polyomavirus (Py) genome encodes three identifiable proteins, the small T (ST), middle T (MT), and LT antigens. Relatively little is known of the function of the ST and MT proteins during the course of lytic

infection. These proteins are required only in certain cell types that presumably lack as yet undefined cellular permissivity factors, and it has been hypothesized that they may induce the expression of these factors (27, 62). In contrast, LT is the viral DNA replication protein and thus is absolutely required for productive infection (23). LT binds specifically to DNA sites containing repeats of a pentanucleotide recognition sequence located in the origin of viral DNA replication and in the early promoter region (16, 25, 52). Three major binding sites that bind LT with high affinity, as well as three minor binding sites that bind with less affinity, have been described (11). Binding of LT to the major binding sites located within the early promoter region results in the subsequent repression of early gene expression (13, 19, 21). Although it appears that autoregulation can still occur when the binding site located nearest the TATA box and cap sites is deleted (19), the precise contribution of each of the individual binding sites remains to be determined.

Py LT also possesses an immortalization function that has been localized to the N-terminal portion of the protein (54). In addition, at the protein level Py LT is homologous with much of the LT of SV40 (59). Given the ability of the latter protein to stimulate SV40 late transcription (1, 5-7, 30, 42), we sought to determine whether the same situation is true for Py. We therefore tested the ability of wild-type and mutant LTs to stimulate the expression of a chloramphenicol acetyltransferase (*cat*) gene linked to the Py late promoter in a transient assay system (28). Using a stable transformation assay, we had previously shown that the expression of

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foreign genes under the control of the Py late promoter did not require viral DNA synthesis or early gene expression but was dependent on the presence of functional enhancer elements (43–45). We show here that LT can significantly stimulate the expression of genes under the control of the Py late promoter and that the effect can be observed in the absence of viral DNA replication but requires a functional enhancer region. A significant portion of the LT-mediated stimulation appears to be due to binding to the major LT-binding sites and the subsequent down regulation of early region transcription. In addition, the increased CAT activity observed with target plasmids that lack the major LT-binding sites suggests that LT can also have an indirect effect on late gene expression.

MATERIALS AND METHODS

Cell culture and transfection. Mouse NIH 3T3 cells and rat F2408 fibroblasts were maintained in Dulbecco modified Eagle medium containing 10% calf serum and 10% CO₂. The cells were plated at a density of 2×10^5 per 100-mm dish 24 h before transfection. The procedures for the preparation and application of calcium phosphate precipitates have been described (43). Transfection cocktails generally contained approximately 1 pmol of the target plasmid DNA (~4 to 5 µg) and an equimolar amount of the cotransfected plasmid (~4 to 6 µg), with high-molecular-weight rat liver DNA or F2408 fibroblast DNA added as a carrier to give a final DNA concentration of 20 µg/ml. Similar trends were observed when pEMBL8 plasmid DNA (14) was used as the carrier. However, in this case the levels of CAT activity were greatly reduced in all samples. No effect of cotransfection with LT on the intracellular levels of target plasmid DNA was observed, as judged by Southern blot analysis of Hirt supernatant DNAs (34) prepared at the time the cells were harvested, 40 h after removal of the precipitate. Comparison of the activity obtained with plasmids either single or double banded in CsCl-ethidium bromide gradients did not show appreciable differences in CAT activity.

CAT assay. Extracts of cells were prepared by freeze-thaw and assayed for CAT activity, as described by Gorman et al. (28), with 2.6 nmol (0.12 to 0.150 µCi) of [¹⁴C]chloramphenicol (~50 mCi/mmol; New England Nuclear Corp., Boston, Mass.). Acetylated forms were separated from nonacetylated substrate on thin-layer chromatography plates, and the amount of radioactivity present was determined by cutting out the spots and subjecting them to liquid scintillation counting. Values were determined by using an amount of extract such that the percent [¹⁴C]chloramphenicol converted was in a range that was linear with respect to the time of incubation. Values are expressed as picomoles converted per minute per 100-mm dish, although similar trends are indicated if the values are expressed per cell or per microgram of protein. Generally, a 100-mm dish contained 2×10^6 to 3×10^6 cells at harvest. As is frequently observed in such transient assays, absolute values occasionally varied from experiment to experiment. However, transfections were repeated multiple times, after which the general trends reported here were obvious.

Recombinant plasmids. The construction of plasmids p48.19BECAT, pBE32CAT, pBE21CAT, and pBEPyCAT have been described (45). Plasmids pNB10/6BECAT and pB27BECAT were constructed in a similar fashion by using the 1.6-kilobase (kb) *BclI*-*EcoRI* fragment of plasmids pNB10/6 (49) and pB27 (13), respectively. Plasmid pNB10/6BEXCAT contains an *XhoI* linker insertion at the

origin and was constructed by ligating a 2.2-kb *ApaI* fragment of pNB10/6BECAT to a 4.1-kb *ApaI* fragment of p48.19BECAT. Plasmid pL1 was constructed by isolating a 0.7-kb *EcoRI*-*BstXI* origin fragment from p48.19BECAT, filling in the termini with T4 DNA polymerase, adding *HindIII* linkers, and inserting the resulting 0.44-kb *HindIII* fragment into *HindIII*-cut pSVOCAT vector DNA. The orientation of the insert is such that the *cat* gene is under the control of the Py late promoter. The construction of p48.19BBCAT has been described (45). pB32BBCAT was constructed in an analogous fashion by using a 0.66-kb *BamHI*-*BstXI* fragment of plasmid pB32 (13).

The construction of pBLTwt12 (45) has been described. pBLT-3 is identical except that it contains the carboxy-terminal portion of the *tsa* LT (50). The construction of plasmids 91023LT, LTd1300, LT300P, LT293, and LT297 is described elsewhere (A. Cowie, J. de Villier, and R. Kamen, submitted for publication). p48.19 was provided by R. Kamen (Genetics Institute, Cambridge, Mass.).

RNA preparation and Northern blot analysis. For the analysis of transiently expressed neomycin resistance and *cat*-specific total cellular RNA produced in transfected NIH 3T3 cells, a procedure developed by Z. Krawczyk and C. Wu was used. A pellet of $\sim 2.5 \times 10^6$ cells was suspended in 0.5 ml of a lysis solution consisting of 10 mM Tris hydrochloride (pH 7.4), 2.0 mM MgCl₂, 1.0 mM CaCl₂, 10 mM vanadyl ribonucleoside complex, and 1.5 mg of heparin per ml. DNase I (300 U; DPRF; Worthington Diagnostics, Freehold, N.J.) was added, and digestion proceeded for 20 min at room temperature. The sample was then repeatedly extracted with phenol–0.1% hydroxyquinoline and ethanol precipitated. The procedures for Northern blot analysis, using denaturing agarose-formaldehyde gels, transfer to nitrocellulose filters, and hybridization to nick-translated probes have been described (43).

RESULTS

LT stimulation of late promoter function in the absence of viral DNA replication. Since LT is the Py replication protein, any stimulatory effect on late gene expression could result either from an increase in the number of templates available for transcription or from an independent effect of this protein on the promoter itself. To distinguish between these possibilities, we constructed replication-competent and replication-incompetent plasmids that contained the bacterial *cat* gene linked to the Py late promoter via a *HindIII* linker at the viral *BclI* site at nucleotide (nt) 5022. The prototype pBEPyCAT plasmid is depicted in Fig. 1 and was constructed with a viral restriction fragment that has a wild-type replication origin. Plasmid p48.19BECAT is identical except that it has an *XhoI* linker inserted between nt's 35 and 37 (Fig. 2). This linker insertion makes plasmids replication defective in permissive mouse cells constitutively producing Py LT (13), as determined by an *MboI* sensitivity assay (our unpublished results; G. Veldman and R. Kamen, personal communication). The SV40 ST intron and early polyadenylation signal are 3' to the *cat* gene. The plasmids also contain a truncated Py early coding region ending at the *EcoRI* site at nt 1560 and therefore include an early transcriptional unit consisting of the complete coding sequences for ST and MT but lacking those for the C-terminal portion of LT. The alternate early polyadenylation signal at 99 map units is also contained in the early transcriptional unit. In our initial experiments we also used plasmid pBE32CAT, which contains a single deletion of nt's 5246 to 127, removing the Py

FIG. 1. Schematic representation of prototype target plasmid used to study Py late promoter activity. Plasmid pBEPyCAT contains the bacterial *cat* gene linked to the Py late promoter at the viral *BclI* site via *HindIII* linkers (45); 3' of the *cat* gene are the SV40 sT intron-splicing signals and the SV40 early region polyadenylation processing signals present in the pSVOCAT vector (28). The plasmid also contains a truncated Py early coding region ending at the *EcoRI* site at nt 1560. L and E, Directions of late and early transcription, respectively.

major LT-binding sites A and B and minor sites 1 and 2 and thus making this plasmid replication defective (Fig. 2). These three plasmids were transfected alone or cotransfected with plasmid pBLTwt12 into both mouse NIH 3T3 cells and rat F2408 fibroblasts. pBLTwt12 codes only for the Py LT. This plasmid contains the same origin region deletion as that in pBE32CAT and is therefore also replication defective. Protein extracts were prepared from the transfected cells and assayed for CAT activity.

The results presented in Fig. 3 and Table 1 show that in permissive mouse cells, the basal level of CAT activity for pBEPyCAT, p48.19BECAT, and pBE32CAT was low but detectable. Cotransfection of the replication-competent pBEPyCAT plasmid with pBLTwt12 resulted in an approximate 70-fold increase in the level of CAT expression. Increases of 15- to 20-fold in CAT expression due to cotransfection with LT were also observed with plasmids p48.19BECAT and pB32BECAT, which are incapable of replication. The same general trend was observed with the rat F2408 fibroblast cell line except that base-line activities were much higher and consequently the increases resulting from cotransfection with LT were lower. Experiments to be described elsewhere indicated that the difference in base-line activity between the NIH 3T3 and F2408 cell lines was not due to differences in the uptake of transfected DNA, as judged by the proportion of cells staining positive for LT by immunofluorescence or by the amount of plasmid DNA present in the Hirt supernatants (34) of transfected cells.

To confirm that the increases in CAT activity were indeed

FIG. 2. Schematic representation of deletions present in plasmids used to map the sequences involved in LT stimulation of the Py late promoter. The deletions are indicated by open spaces. The nt numbering system is that described by Soeda et al. (59). pNB10/6BEXCAT also contains a duplication of nt's 5045 to 5135. All plasmids contain the *cat* gene linked to the Py late promoter at the *BclI* site by a *HindIII* linker. Except for plasmid pL1, all plasmids also contain a truncated early coding region extending to the viral *EcoRI* site at nt 1560. At the top of the figure is a representation of the Py A-2 strain regulatory region, showing the location of the major and minor LT-binding sites (11), the early region TATA and CAACT boxes (59), sequences constituting the minimal origin of replication (40, 48), and the reiterated late mRNA leader sequence generated during lytic infection (65). An area that can form a hairpin loop and the locations of DNase-hypersensitive regions (DHSR) (33), an 8-base-pair direct repeat, and a G-C-rich inverted repeat are also shown, as are sequences with homology to the SV40 core enhancer sequence (68), the mouse immunoglobulin heavy-chain enhancer (2, 32), and the adenovirus 5 E1A enhancer consensus sequence (31). Arrows indicate the locations of the 5' termini of early (39), late-early (22), late lytic (12, 65), or late direction transcripts observed after transfection with chimeric plasmids (43).

FIG. 3. Stimulation of Py late promoter activity by LT. NIH 3T3 cells were transfected with 5.0 μ g of the indicated plasmids or cotransfected with 5.0 μ g of pBLTwt12 (45) with rat liver DNA as the carrier. After overnight incubation, the precipitate was removed, fresh Dulbecco modified Eagle medium plus 10% calf serum was added, and incubation at 37°C was continued for an additional 40 h. Protein extracts were then prepared, and the extract from 5×10^5 cells was assayed for CAT activity for 30 min at 37°C, as described in Materials and Methods.

due to the presence of a functional LT, NIH 3T3 cells were transfected at 39°C with the p48.19BECAT plasmid plus or minus the pBLTwt12 plasmid or its pBLT-3 derivative that codes for a temperature-sensitive (*ts*) LT. After removal of the calcium phosphate precipitate, the cells were incubated at either 39°C, the nonpermissive temperature for the *ts* LT, or 33°C for an additional 40 h before the preparation of protein extracts. Increases in CAT activity at 39°C were only observed after cotransfection with the plasmid encoding the wild-type LT; CAT expression remained at the base-line levels if the plasmid encoding the *ts* LT was used (Table 2). In contrast, at 33°C cotransfection with either plasmid resulted in increased CAT activity.

To determine whether the increases in CAT activity were related to increases in the steady-state level of mRNA and whether similar increases could also be observed when coding sequences other than those for CAT were placed downstream of the late promoter, Northern blot hybridization was performed. Total cellular RNA was isolated from NIH 3T3 cells after cotransfection with plasmids p48.19BECAT and either 91023(B) or 91023LT. 91023(B) is a

TABLE 2. Effect of a *ts* LT on Py late promoter activity at permissive and nonpermissive temperatures^a

Cotransfected plasmid (LT coding capacity)	CAT activity (pmol/min per 100-mm dish)		Ratio of activity (33°C/39°C)
	33°C	39°C	
None	17.0	6.1	2.8
pBLTwt12 (wild type)	170.0 (10.0)	85.0 (14.0)	2.0
pBLT-3 (<i>tsa</i>)	165.0 (9.7)	5.2 (0.8)	31.7

^a NIH 3T3 fibroblasts were transfected with 5.0 μ g of the p48.19BECAT target plasmid alone or together with 5.0 μ g of the LT-containing plasmids at 39°C for 8 h. The precipitate was removed, and the cells were collected and assayed for CAT activity after incubation at the indicated temperatures for an additional 40 h. The numbers in parentheses represent the fold increase due to LT.

cloning vector that allows for the expression of eucaryotic coding sequences placed under the control of the adenovirus major late promoter. It also contains the SV40 origin and enhancer region and the adenovirus VA genes to facilitate the transcription and translation of these sequences (41, 69). 91023LT contains the coding sequences for Py LT inserted into this vector. Hybridization with a *cat*-specific probe revealed LT-dependent increases in the steady-state levels of CAT mRNA that we estimate to approximate the increase in enzyme activity observed with the same cultures. Cotransfection of p48.19Neo, a replication-defective plasmid containing the Tn5 gene for neomycin resistance (*neo*) under the control of the Py late promoter (45), with 91023LT also resulted in increased levels of *neo*-specific mRNA (Fig. 4).

trans activation of the late promoter requires enhancer elements. We previously described a plasmid, pBE21, bearing a double deletion in the Py regulatory region (44, 45). The first deletion (nt's 5246 to 127) is the same as that in the pBE32CAT plasmid, and the second spans nt's 5055 to 5182 and thus removes a major portion of the Py enhancer elements. When the *neo* gene is put under the control of the late promoter of the pBE21 plasmid, the resulting construct is basically devoid of any biological activity in stable transformation assays (44) but can give rise to a low but significant number of colonies resistant to the drug G418 (10, 60) when cotransfected with a plasmid encoding LT (45). Similarly, the results presented in Fig. 3 and Table 3 indicate that the pBE21CAT plasmid was capable of being stimulated at least somewhat by Py LT. However, since the base-line activity with this plasmid was essentially at background

TABLE 1. Effect of LT on Py late promoter activity in mouse NIH 3T3 and rat F2408 fibroblasts^a

Target plasmid	Replication ability	Origin	Cotransfected plasmid	CAT activity (pmol/min per 100-mm dish)	
				NIH 3T3	F2408
pBEPyCAT	+	Wild type	None	2.1	37.2
			pBLTwt12	135.5 (64.5)	181.3 (4.9)
p48.19BECAT	—	<i>Xho</i> I linker insertion, nt's 35 to 37	None	2.8	30.4
			pBLTwt12	56.5 (20.1)	171.0 (5.6)
pBE32CAT	—	Deletion, nt's 5246 to 127	None	2.4	38.4
			pBLTwt12	37.1 (15.5)	219.2 (5.7)
pSV2CAT	—	SV40	None	47.8	277.5
			pBLTwt12	40.5 (0.8)	216.5 (0.8)

^a Mouse NIH 3T3 or rat F2408 fibroblasts were transfected with 5.0 μ g of the indicated plasmids alone or cotransfected with 5.0 μ g of the pBLTwt12 LT-encoding plasmid (45). Cells were harvested 60 h after transfection, at which point a 100-mm dish contained approximately 2.5×10^6 NIH 3T3 cells and 1.5×10^6 F2408 cells. Extracts were assayed for CAT activity as described in Materials and Methods. The numbers in parentheses represent the fold increase due to LT.

FIG. 4. LT-mediated increase in steady-state levels of mRNA driven by the Py late promoter. NIH 3T3 cells were cotransfected with 5.0 μ g of p48.19Neo (45) and equimolar amounts of either the 91023(B) plasmid vector (69) alone (lane 2) or the 91023LT plasmid that codes for LT (lane 3). Total cellular RNA from 1.5×10^6 cells was separated on a 1.2% agarose-2.2 M formaldehyde gel, transferred to nitrocellulose, and hybridized to a 32 P-labeled 1.1-kb *Bgl*III-*Sall* neo fragment of Tn5 (3). Lane 1 contained 3.0 μ g of poly(A)⁺ RNA isolated from the G418^r cell line L7 (43). Total RNA from 1.2×10^6 cells cotransfected with p48.19BECAT and 91023(B) (lanes 4 and 6) or 91023LT (lanes 5 and 7) was hybridized to a 773-base-pair *Hind*III-*Sau*3A-I *cat*-specific probe isolated from pSV2CAT (28). Lanes 1 through 5 are from an autoradiograph exposed for 24 h with a Cronex Lightning-Plus intensifying screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). Lanes 6 and 7 are from an overnight exposure without a screen.

levels, we were unable to determine the degree of the response. In similar constructs that have the *neo* gene linked to the Py late promoter, we have shown that replacement of the second additional origin region deletion present in this plasmid has the effect of increasing the frequency of G418^r colony formation in a stable transfection assay system (44).

We therefore replaced the origin sequences deleted in the pBE21CAT construct with those from p48.19BECAT and observed that the resulting pBE22XCAT plasmid was capable of directing *cat* gene expression that, although greatly diminished compared with that obtained with the

p48.19BECAT plasmid, was clearly above background levels. The results shown in Table 3, however, indicate that CAT expression driven by the pBE22XCAT plasmid was not effectively stimulated by LT either in mouse or rat cells.

The apparent discrepancy between the results obtained with the pBE21CAT and pBE22XCAT plasmids was likely due to the fact that the small extent of *trans* activation observed with pBE21CAT was detectable only because the base-line activity of this plasmid is essentially zero. With pBE22XCAT, which possesses a higher base-line activity, *trans* activation becomes insignificant. We therefore conclude that deletion of nt's 5055 to 5182, the major portion of the enhancer region, results in the inability of the late promoter to be significantly *trans* activated by LT. Thus, functional enhancer elements are required not only for the basal activity of the late promoter but also for its activation.

Maximal LT-mediated increases in late expression require DNA-binding sites. LT binds to specific sites in the Py DNA regulatory region, and such binding is thought to mediate viral DNA replication and repression of early transcription. Our results with the pBE32CAT plasmid indicated that the major LT-binding sites A and B, as well as the minor binding sites 1 and 2, could be eliminated from a target plasmid (Fig. 1 and 2) without significantly affecting the ability of LT to stimulate its late promoter. To determine the possible contribution of the remaining LT-binding sites to *trans* activation, two additional plasmids were constructed. pB27BECAT is identical to pBEPyCAT except that it contains a deletion of nt's 5 to 242 and thus lacks all three major LT-binding sites, as well as most of minor binding site 1. pNB10/6BEXCAT contains a deletion of nt's 5180 to 5254; minor binding site 3 is therefore deleted. This plasmid also contains a duplication of enhancer sequences contained within nt's 5045 to 5135, which may compensate for the lack of the enhancer sequences contained within the deletion since no decrease in the base-line activity was observed when compared with that of the p48.19BECAT plasmid (Table 4).

The results of cotransfection experiments with plasmids encoding LT indicate that minor LT-binding site 3 can be deleted without any detectable effect on the ability of LT to stimulate expression from the late promoter. However, when nt's 5 to 242 are deleted from the target plasmid, cotransfection with LT generally results in only a 2- to 4-fold increase in CAT activity, in contrast to the approximate 15-fold activation observed with the p48.19BECAT plasmid in the same experiments. As indicated above, the pBE32CAT plasmid was *trans* activated to approximately the same extent as p48.19BECAT was (Table 4).

These results indicate the importance of the presence of at

TABLE 3. Effect of an enhancer region deletion on LT stimulation of late promoter activity in mouse NIH 3T3 and rat F2408 fibroblasts^a

Target plasmid	Py sequences (nt's)	Cotransfected plasmid (LT coding capacity)	CAT activity (pmol/min per 100-mm dish)	
			NIH 3T3	F2408
p48.19BECAT	5022-1560; <i>Xho</i> I linker insertion, 35-37	None (-) pBLTwt12 (+)	20.4 148.4 (7.3)	149.0 317.0 (2.1)
pBE21CAT	5022-1560; deletions, 5055-5182 and 5246-127	None (-) pBLTwt12 (+)	<1.0 1.4 (>1.4)	0.8 1.9 (2.4)
pBE22XCAT	5022-1560; deletion, 5055-5182; <i>Xho</i> I linker insertion, 35-37	None (-) pBLTwt12 (+)	3.3 1.6 (0.5)	14.3 9.6 (0.7)

^a F2408 or NIH 3T3 cells were transfected with 5.0 μ g of the indicated target plasmids alone or cotransfected with 5.0 μ g of the LT-encoding plasmid. Plasmid pEMBL8 (14) was used as carrier DNA in the F2408 transfection. The numbers in parentheses represent the fold increase in the presence of LT.

TABLE 4. Effect of deletions of LT-binding sites on late promoter stimulation by LT^a

Target plasmid	Deleted LT-binding site(s)	Cotransfected plasmid (LT coding capacity)	CAT activity (pmol/min per 100-mm dish) ^b	Stimulation by LT (fold)
p48.19BECAT	None	None (-)	13.3 ± 3.5 (16)	
		p1.6BE3 (truncated)	16.8 ± 9.9 (4)	1.3
		pBLTwt12 (+)	226.8 ± 34.6 (12)	17.0
		91023LT (+)	998.8 ± 126.5 (6)	75.1
pBE32CAT	A, B, 1, 2	None (-)	7.0 ± 2.9 (4)	
		pBLTwt12 (+)	86.6 ± 30.8 (3)	12.4
pB27BECAT	A, B, C, 1	None (-)	15.5 ± 4.3 (7)	
		pBLTwt12 (+)	29.1 ± 6.7 (6)	1.9
		91023LT (+)	65.1 (1)	4.2
pNB10/6BEXCAT	3	None (-)	28.6 (2)	
		91023LT (+)	973.9 (2)	34.0

^a NIH 3T3 cells were transfected with 5 µg of the indicated target plasmid alone or cotransfected with an equimolar amount of the indicated plasmid. The cells were collected 48 h after removal of the precipitate, and extracts were assayed for CAT activity.

^b Values are the means ± standard errors calculated from the number of determinations indicated in parentheses.

least one major LT-binding site for maximal activation of late promoter activity by LT. Although our results do not rule out the possibility that binding sites A and B can substitute for the lack of binding site C, these experiments suggest that site C, the only major binding site remaining in the pBE32CAT plasmid, is sufficient to allow full activation of the late promoter by LT. The C binding site is located within the sequences used to initiate early mRNA transcription and may therefore be involved in the autoregulation of early transcription. It is possible that the interaction of LT with binding site C may lead to increased late promoter activity either by altering the DNA conformation in the regulatory region or by diminishing the competition by the early promoter for transcription factors.

Implicit in the above hypothesis is that the presence of binding site C alone on the Py DNA molecule would have been sufficient to produce a full down regulation of early gene expression in the presence of LT. To verify this, plasmids containing the *cat* gene under the control of the Py early promoter were constructed. The control plasmid, p48.19BBCAT, contains the enhancer and origin-spanning *Bam*HI-to-*Bst*XI fragment, with the *Xho*I linker insertion between nt's 35 and 37. The *cat* gene is downstream of the early mRNA cap sites just before the translation initiation codon of Py early mRNAs. The second plasmid, pB32BBCAT, has only the C binding site since it contains the same fragment with an origin region deletion of nt's 5246 to 127. Thus, both plasmids are replication incompetent.

The two plasmids were cotransfected with either the LT-encoding 91023LT plasmid or with plasmid LT300P, which codes for a mutated LT incapable of binding to the Py regulatory region (Cowie et al., submitted). In the experiments shown in Table 5 we used rat F2408 fibroblasts because they give a higher base-line activity than mouse cells do (see above) and therefore facilitate the detection of inhibition of gene expression. The results indicate that in the presence of a functional LT, expression was reduced to approximately 10 to 20% of base-line levels with both target plasmids. Since cotransfection with the mutant LT did not diminish activity, this effect was not due to competition for limiting factors but was instead related to the ability of the LT to bind to the viral DNA. Similar results were obtained in mouse cells, although as discussed above, the base-line activity of both plasmids was lower (data not shown). It can thus be concluded that in both permissive and nonpermissive cells LT-binding site C is sufficient for LT-mediated repression of early transcription.

The results presented above suggested the hypothesis that one component of the LT-mediated activation of the Py late promoter involves down regulation of early transcription. We therefore wished to determine the effects of LT on a target plasmid that retained the LT-binding sites but lacked an early transcription unit. A replication-defective plasmid was constructed that contained the *cat* gene linked to the late promoter at the *Bcl*I site but extended in the early direction only to the *Bst*XI site at nt 168. The plasmid, designated

TABLE 5. Down regulation of early transcription by Py LT in rat F2408 fibroblasts^a

Target plasmid	Py sequences (nt's)	LT-binding sites	Cotransfected plasmid (DNA-binding capacity)	CAT activity (pmol/min per 100-mm dish)
p48.19BBCAT	5022-168; <i>Xho</i> I linker insertion, 35-37	A, B, C, 1, 2, 3	None (-)	294.7
			91023LT (+)	57.2 (0.19) ^b
			LT300P (-)	286.0 (0.97)
pB32BBCAT	5022-168; deletion, 5246-127	C, 3	None (-)	109.0
			91023LT (+)	13.4 (0.12)
			LT300P (-)	90.9 (0.83)

^a Rat F2408 fibroblasts were transfected with 1.0 pmol (~4.0 µg) of the indicated target plasmid containing *cat* sequences linked to the Py early promoter at the *Bst*XI site either alone or together with an equimolar amount of the plasmid encoding wild-type or mutant LT. The cells were harvested 60 h after transfection, and extracts were assayed for CAT activity.

^b The numbers in parentheses indicate the fraction of the activity observed in the absence of the cotransfected plasmid.

TABLE 6. Effect of deletion of early coding sequences on base-line and LT-stimulated late promoter activity^a

Target plasmid	Py sequence (nt's)	Cotransfected plasmid (LT coding capacity)	CAT activity (pmol/min per 100-mm dish)	
			Expt 1	Expt 2
p48.19BECAT	5022-1560; <i>Xho</i> I linker insertion, 35-37	None (-)	21.1	54.3
		91023LT (+)	1,289.9 (61.1) ^b	1,268.3 (23.3)
pL1	5022-168; <i>Xho</i> I linker insertion, 35-37	None (-)	219.0	479.5
		91023LT (+)	528.3 (2.4)	665.6 (1.4)

^a NIH 3T3 cells were transfected with 1.0 pmol of the indicated target plasmid alone or cotransfected with an equimolar amount of the LT-encoding plasmid. CAT activity was determined with extracts prepared 60 h posttransfection.

^b The numbers in parentheses represent the fold increase in the presence of LT.

pL1, therefore contains the sequences used to initiate early mRNA transcription but lacks completely the early coding sequences and polyadenylation signals.

pL1 and p48.19BECAT were compared in transfection experiments with and without cotransfection with plasmids coding only for LT. Removal of the early transcriptional unit increased the base-line activity approximately 10-fold (Table 6). Cotransfection with LT increased CAT activity with the pL1 plasmid, but only about two- to threefold. Since the levels of CAT activity reached in the presence of LT were higher with the p48.19BECAT plasmid than with the pL1 plasmid, the decreased response of pL1 to LT was not due to a depletion of necessary transcription factors but appears instead to have been related to the lack of an early transcription unit. Similarly, the lower base line obtained with the p48.19BECAT plasmid does not appear to have been due to a lack of sufficient quantities of transcription factors to support two transcriptional units. This conclusion can be reached from experiments in which the pL1 plasmid was cotransfected with plasmid pCB7, which contains the *neo*

gene under the control of the early promoter (43). In this case, no decrease of CAT activity directed by the pL1 plasmid was observed (data not shown).

Effects of MT and ST proteins. The three plasmids that did not respond to LT to the same degree as did the p48.19BECAT plasmid differ from p48.19BECAT in that they are incapable of producing or can produce only in much smaller amounts functional MT and ST. pL1 completely lacks the coding sequences, pB27BECAT lacks the N-terminal sequences, and pBE22XCAT, because of the *cis*-acting effect of the enhancer region deletion, can produce only greatly reduced amounts of the early mRNAs. To test whether the absence of a full response to LT was due to the lack of these proteins, the plasmids were cotransfected with plasmid p48.19. This plasmid is replication defective because of the *Xho*I linker insertion in the origin region and has the coding regions for all three Py early proteins. In addition, another target plasmid was constructed that is similar to the p48.19BECAT plasmid except that it contains a deletion of an *Sst*I fragment spanning nt's 569 to 1373. This plasmid,

FIG. 5. Effect of supplying MT and ST proteins on LT stimulation of different Py late-promoter CAT plasmids. The indicated target plasmids (1 pmol) were cotransfected with equimolar amounts of the cotransfected plasmids. pBLTwt12 codes for an LT under the control of a Py early promoter with a deletion of nt's 5246 to 127, and pL1.6BE3 encodes a truncated LT antigen ending at the *Eco*RI site under the control of the same promoter (45). 91023LT contains Py LT sequences linked to the adenovirus major late promoter. p48.19 contains Py DNA, with an *Xho*I linker insertion between nt's 35 and 37, inserted into the *Bam*HI site of pXf3 and therefore codes for all three Py early proteins. pPyMT-1 codes only for MT (66). Shown are the results of three individual experiments (A, B, and C).

FIG. 6. Effect of mutant Py LTs and SV40 early proteins on the Py late promoter. NIH 3T3 cells were transfected with 5.0 μ g of the p48.19BECAT plasmid together with an equimolar amount of the indicated plasmid. 91023(B) was the plasmid expression vector (69). 91023LT contains a wild-type Py LT cDNA from nt's 153 to 2962. LTd1300 has a deletion of nt's 1455 to 1487 (amino acids 300 to 310) and a G-to-A base change at nt 1451. LT293 has a C-to-A base change at nt 1435, changing amino acid 293 from proline to histidine. LT297 has an A-to-C base change at nt 1446, changing amino acid 297 from threonine to proline, and LT300P has a T-to-G base change at nt 1456, changing amino acid 300 from leucine to tryptophan (Cowie et al., submitted). pSVRB contains the SV40 4.5-kb *Eco*RI-*Bam*HI fragment cloned into pBR322.

p48.19SstCAT, therefore retains the LT-binding sites but has a deleted early transcription unit coding for nonfunctional proteins.

The results of the cotransfection experiments are shown diagrammatically in Fig. 5A. No target plasmid was stimulated by plasmid p1.6BE3, which encodes a truncated LT. Cotransfection of the pBE22XCAT and pB27BECAT plasmids with the p48.19 plasmid, which contains the complete Py early region, had no obvious effect over that observed for cotransfection with the pBLTwt12 plasmid alone. Thus, the lack of or reduced response to LT observed with these two target plasmids was not due to the absence of MT or ST or both. Instead, these results confirm the conclusion that the target sequences for LT activation of the late promoter are located within the enhancer region and LT-binding sites.

Cotransfection of the p48.19BECAT plasmid with p48.19 resulted in little or no stimulation of CAT activity above that obtained with the pBLTwt12 plasmid and gave activities below that obtained with the 91023LT vector. However, higher values than those obtained with LT alone were observed when the p48.19SstCAT and pL1 plasmids were cotransfected with the complete early region (Fig. 5). Accordingly, a plasmid producing only MT did not stimulate CAT activity in the p48.19BECAT plasmid but caused a significant increase in CAT expression in pL1 (Fig. 5C). It appears therefore that MT contributes in a yet undetermined manner to the stimulation of the Py late promoter by LT.

Effect of mutant Py LTs and SV40 early proteins on the Py late promoter. Since the results presented above suggested that LT binding to Py DNA was important in bringing about a subsequent stimulation of late expression, we were interested in determining the effects of plasmids coding for LT that had mutations interfering with its binding to Py DNA in vitro. It is interesting that despite the inability of these LTs to bind DNA, they are capable of immortalizing primary rat

embryo fibroblasts with a frequency indistinguishable from that of the wild-type LT (Cowie et al., submitted).

The p48.19BECAT target plasmid was transfected into mouse NIH 3T3 cells together with either the 91023(B) vector plasmid alone or the same expression vector containing coding sequences for either the wild-type LT or one of four mutant LTs. With one exception, the mutant LTs failed to stimulate late promoter function to any significant extent. However, plasmid LT293 reproducibly stimulated late expression approximately four- to sixfold (Fig. 6). Whether the reduced stimulation compared with that of the wild-type LT was due to an ability of the protein to bind weakly to the Py control region in vivo or represented a binding-independent component of the LT *trans* activation of late promoter function remains to be determined. These results indicate, however, that the immortalization function of LT can be dissociated from the ability to stimulate late gene expression.

The Py LT-binding sites and the SV40 LT-binding sites share the same pentanucleotide recognition signal (37, 64), and it has been reported that in vitro the SV40 protein can bind with high affinity to the Py LT-binding sites (51, 57). We therefore wished to determine the effects of cotransfection of the Py late promoter CAT construct p48.19BECAT with a plasmid encoding the early region of SV40.

Cotransfection with the SV40 plasmid stimulated late Py expression in mouse cells approximately eightfold, and in the same experiment cotransfection with the Py LT caused a 60-fold stimulation of late expression (Fig. 6). In parallel experiments, SV40 caused an approximately twofold reduction in early gene expression with the p48.19BECAT plasmid, and Py LT caused an eightfold decrease (data not shown). These results suggest a correlation between the degree of repression of early expression and the degree of stimulation of late expression.

DISCUSSION

The regulation of eucaryotic gene expression involves a complex interaction of *cis*- and *trans*-acting factors. The identification of regulatory *cis*-acting elements affecting cellular gene expression is at least in principle fairly straightforward once the gene has been isolated and usually involves deletion or other forms of mutation analysis. In contrast, the identification of *trans*-acting factors involved in the regulation of cellular genes is more problematic. However, recent findings of *trans*-acting regulation of eucaryotic viral gene expression by other viral gene products has provided model systems in which to study how this type of regulation occurs.

In this study we investigated the ability of Py LT to alter the expression of both the early and late transcription units; a transient assay system was used to measure changes in the levels of transcripts directed by the early and late promoters. Our results suggest that a complex series of events is involved in the regulation of late promoter activity by LT. These events include binding to the Py regulatory region, a possible interaction with enhancer-dependent transcription factors, and down regulation of early transcription. In addition, our results suggest that LT exerts an indirect effect, also enhancer mediated, that probably does not involve a specific interaction of the protein with the viral DNA and may not even require a direct DNA-protein interaction. Finally, the results presented indicate that MT by itself and possibly in conjunction with LT can also have a stimulatory effect on late promoter function.

Sequence requirements for LT *trans* activation of the late promoter. The plasmids originally chosen for our studies of

the factors regulating the activity of the Py late promoter were *cis* defective for replication and contained a truncated but otherwise normal early region. The choice of having two transcriptional units present in these target plasmids was made to more closely approximate the physiology of viral infection. With these plasmids, Py LT greatly stimulated late gene expression in both permissive and nonpermissive cells, and such activation required the presence of at least one major LT-binding site and a functional enhancer region. Deletion of minor binding site 3, which is the site most proximal to the late promoter, did not affect the extent of LT stimulation, nor did we observe any significant reduction in the extent of stimulation in a plasmid with a deletion of nt's 5246 to 127 and thus missing minor sites 1 and 2 and major sites A and B (11). A reduction in the extent of stimulation was observed only when all three major binding sites, as well as most of minor site 1, were deleted. While this result implicates the C site as being important, the effect of deletion of only the C site remains to be determined, and it remains a possibility that binding site A or B or both can substitute for the C site.

Further support for the contention that the maximal stimulation of late transcription by LT is closely tied to LT binding comes from our results with plasmids encoding mutant LTs deficient in DNA binding and with the plasmid coding for *ts* LT at the nonpermissive temperature. Cotransfection with these plasmids failed to effectively stimulate late promoter expression to the levels observed when the corresponding wild-type plasmid was used.

The only plasmids whose late promoter was refractory to stimulation by LT contained deletions which removed a major portion of the enhancer elements. Although the deletions we studied do not yet allow a precise definition of the sequences necessary for *trans* activation, the data indicate that the same enhancer elements required for the basal level of expression of the late promoter are also required for LT stimulation of its activity.

Mechanism of LT-mediated *trans* activation. The situation described here for Py appears to be similar to that previously described for the enhancement of late-region expression of SV40 by the SV40 LT, i.e., a requirement for LT-binding sites and a functional enhancer region for maximal stimulation to be observed (6, 7, 30, 42). In addition, the data for SV40 indicate that the distance between these elements is critical, leading to the suggestion that SV40 LT binding in some manner either facilitates the formation of or interacts with a complex of positive transcription factors within the enhancer region (7).

Although in principle our data are also amenable to this interpretation, this hypothesis does not account for the approximate 10-fold increase in basal late expression that we observed by simply deleting the early transcription unit. Although the early promoter sequences remain intact in this plasmid (pL1), transcription of the adjacent plasmid sequences in such constructs is not readily detected (L. Lania, personal communication). The removal of a competing transcriptional unit either physically or functionally through autoregulation by LT could then account, perhaps to a significant extent, for the increase in expression from the late promoter. However, the results with pL1 in the presence of LT, as well as the results for the prototype p48.19BECAT plasmid with SV40, indicate that factors in addition to the occlusion of the early promoter or binding to the regulatory region must be involved in the maximal activation of the late promoter by Py LT. The pL1 plasmid contains both of the target regions for LT activation described above, i.e., LT-

binding sites and a functional enhancer region. However, despite having a higher base-line level of activity than p48.19BECAT has, when pL1 was cotransfected with either the pBLTw12 or 91023LT plasmid, both of which code only for LT, it failed to reach the level of CAT activity observed with p48.19BECAT in the presence of either of these two plasmids. It was only when pL1 was cotransfected with p48.19, a plasmid encoding all three early Py proteins, that it reached levels the same as or higher than those for similarly cotransfected p48.19BECAT. Given the nature of the expression vector 91023LT, it is most likely the most potent source of LT used in these experiments (41, 69); it is therefore unlikely that the cotransfection results with p48.19 reflect a requirement for a higher concentration of LT for optimal *trans* activation of pL1.

It is more likely that these results reflect an effect of MT or ST or both. Cotransfection of pL1 with MT alone effectively stimulated late promoter activity; MT can also stimulate transcription from either homologous or heterologous promoters, whereas no such effect is observed with ST (A. Pannuti, G. La Mantia, and L. Lania, submitted for publication; F. G. Kern, unpublished results). We therefore favor the hypothesis that late promoter activation also involves an interaction of LT and MT. In the case of the p48.19BECAT plasmid, MT is supplied by the target molecules themselves, whereas for maximal activation of pL1 by LT, MT must also be supplied by the cotransfected plasmid.

It remains to be determined why, given the apparent stimulatory activity of MT, base-line activity remained low in the p48.19BECAT plasmid. It also remains to be seen whether the stimulation by p48.19 cotransfection observed with pL1 was merely an additive effect of MT and LT or was due to a cooperative effect of these two proteins. What little is known of the role of ST or MT in lytic infection comes from work with host range transformation-defective mutants. It has been hypothesized that the host range transformation-defective function may be involved in the induction of cellular permissivity factors (27, 62). Additional data suggest a failure of nonpermissive cells to accumulate viral DNA and to posttranslationally modify the viral capsid protein (24). It is therefore possible that such induced cellular factors or other posttranslational modification events mediated by MT are also involved in the formation of the complex of positive transcriptional factors hypothesized above.

Finally, it should be noted that late expression directed by the pB27BECAT plasmids, which lack the major LT-binding sites, was still reproducibly stimulated approximately two- to fourfold by LT. The reduction in the extent of stimulation, compared with that observed with the control plasmid, was not due to N-terminal mutations in MT and ST in this plasmid since cotransfection with plasmid p48.19, a nonreplicating plasmid that codes for all three early proteins, also resulted in a reduced response (Fig. 5A). Nor was the reduced stimulation by LT due to a *cis*-acting effect of the deletion on the basal level of late promoter expression (Table 3). Rather, these results indicate that a second component, independent of LT binding, was also involved, although perhaps to a lesser extent, in the stimulation of late transcription by LT.

Although the mutant LT293 plasmid failed to bind to the Py regulatory region *in vitro*, we consistently observed four- to sixfold stimulation of late promoter expression in cotransfection experiments with this plasmid (Fig. 6). Cotransfection with this plasmid can also result in at least some down regulation of our early promoter CAT construct in rat

fibroblasts (unpublished results), and the LT produced by this plasmid may therefore still be capable of weak binding in vivo. However, an alternative explanation is that the LT produced by this plasmid is more efficient than the three other mutant LTs tested in affecting the second indirect component of late promoter activation mentioned above. These mutant LTs are all capable of immortalizing primary rat embryo fibroblasts in vitro, as is a plasmid encoding a truncated LT. Yet, three of the four mutant LTs, as well as the plasmid encoding a truncated LT, failed to *trans* activate the Py late promoter. Similarly, another laboratory recently reported that the adenovirus E1A gene 12S cDNA can immortalize primary cells in vitro (70), but the protein encoded by this cDNA fails to *trans* activate the other adenovirus early promoters (26, 55, 63). Thus, the hypothesis that *trans* activation and immortalization operate via similar molecular mechanisms is not supported by these data.

Other laboratories also recently reported *trans* activation of the human heat shock promoter by Py LT in a manner that also appears to be independent of binding of this protein to the regulatory region (47). In our experiments, Northern blot analysis indicated that the major effect of LT could be seen as an increase in the steady-state levels of either *neo*- or *cat*-specific mRNA when either of these genes was linked to the late promoter. Since we have described target sequences that are located in the Py control region upstream of late mRNA initiation sites that are necessary for a maximal effect to be observed, it is unlikely that the major effect of LT is to increase the stability or translation of late mRNAs. However, our results do not rule out the possibility that the second component of LT-mediated activation involves these types of events.

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