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Simian Virus 40 T Antigen Activates the Late Promoter by Modulating the Activity of Negative Regulatory Elements

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Late promoter activity measured before viral DNA replication results from a complex involvement of negative and positive *cis*-acting elements located both in the enhancer and in the 21-bp repeats. GC motifs located within the 21-bp repeats act in cooperation with sequences overlapping the early TATA box to down-regulate the late promoter activity. Analysis of insertion mutants indicates that the late promoter might be negatively regulated at least partially by the early promoter machinery. The GTI motif located within the enhancer as well as the GC motifs lose the ability to down-regulate the late promoter in the presence of T antigen. Results obtained with *ts*A58 protein indicate that two different domains of T antigen are involved in the negative autoregulation of the early promoter activity and in the release of the down-regulation of the late promoter by the GC motifs.

Negative regulation is a common mechanism for eucaryotic regulation of promoters, especially for promoters functioning in specific cells or for those which are activated in a sequential order (for reviews, see references 24 and 33). The simian virus 40 (SV40) late promoter, which is expressed at a low level before viral DNA replication in the absence of T antigen, is a good model for studying the negative regulation of eucaryotic gene expression.

The regulatory region of the SV40 genome contains multiple elements within the enhancer and the 21-bp repeats that regulate both early and late gene expression in a sequential order. The 21-bp repeats comprising six GC motifs down-regulate the constitutive expression of late gene in the absence of T antigen but stimulate late transcription after viral DNA replication (1, 17, 38). The GC motifs involved in the negative regulation of late genes are motifs I and II, which are located at the early proximal end of the 21-bp repeats, whereas GC motifs IV, V, and VI are located at the early distal end of this element, where they contribute to the efficiency of late transcription after viral DNA replication (16).

The enhancer region accounts for the constitutive late promoter activity that is observed before viral DNA replication in the absence of T antigen (15, 38, 45). The enhancer region is also required for the full expression of late genes after viral DNA replication (17, 28). Moreover, the sequence motifs defined within the enhancer element for early promoter activity (57) also constitute sequence motifs required for efficient late transcription before and after viral DNA replication (22, 35, 55). Recent results indicate that enhancer motifs are involved in both positive and negative regulation of the constitutive late promoter activity. Positive activation is mediated by the motifs Sph and/or octamer and, to a lesser extent, the motifs GTI and P, while the motif GTII overlaps elements that negatively regulate expression of the late promoter (45).

In addition to the enhancer and the 21-bp repeats, the late promoter sequence includes a 60-bp segment extending upstream from the enhancer to the major late initiation site (nucleotides [nt] 332 to 273) (38). This element contains five of the late initiation sites mapped by Ghosh et al. (20) and Haegeman and Fiers (27) as well as the sequences which specify the positions of the majority of these initiation sites (2, 11, 38, 46). When the 60-bp element is present, the minor initiation sites at upstream locations are utilized sparingly (39, 46).

To account for the late promoter efficiency observed after DNA replication, it has been proposed that T antigen indirectly activates late gene transcription by autoregulating negatively early transcription and by amplifying the SV40 genome (6, 43). Moreover, an effect of large T antigen on late transcription in the absence of, and independent of, DNA replication or genome amplification has been reported (8, 30). According to Brady and Khoury (9), two regions of the SV40 template are required for efficient T-antigen-mediated late transcription, both the 72-bp repeats and the region which encompasses T-antigen binding sites I and II. It is suggested that T antigen acts indirectly on late promoter activity through protein-protein interactions or through activation of cellular transcriptional factors (10, 19, 37, 40, 48).

The regions of T antigen required for this activity have been determined recently by Zhu et al. (58). These authors showed that sequences in the N-terminal region of large T antigen play a role in transactivation. In the context of a full-length T antigen, mutations within the DNA binding domain also affect transactivation.

We and others (26, 45) have found that mutations within the enhancer motifs, especially within the motifs GTI, Sph, and P, cause a loss in the ability of the late promoter to be activated by T antigen. On the other hand, T antigen releases the negative regulation mediated by the GTII motif (45). These data indicate that T-antigen transcriptional activation of the late promoter is mediated at least partially through factors that bind to the enhancer motifs. Here, we have examined the effect of T antigen on the down-regulation of the late promoter conferred by the GC motifs.

The results were obtained by analyzing a series of deletion and substitution mutants. We observed that in addition to the 21-bp repeats, the early promoter sequences overlapping the TATA box are involved in the GC motif-mediated negative regulation of the constitutive late promoter activity. T antigen relieves the negative regulation through the same early promoter sequences, whereas the results obtained with

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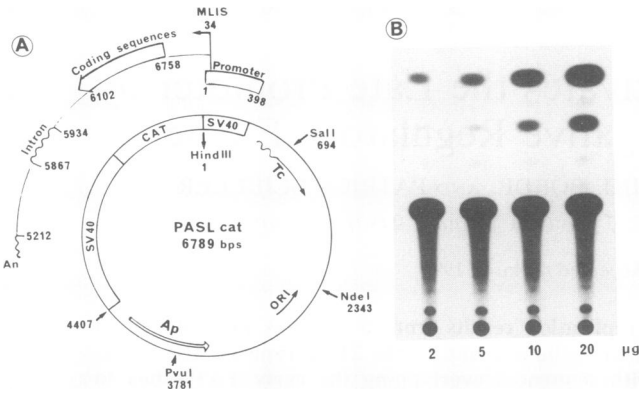

FIG. 1. (A) General structure of plasmid pASLcat. Numbering of the nucleotide sequences begins within the unique *Hin*dIII site, at the junction between the SV40 late promoter and the CAT gene. Nucleotide numbering continues toward the CAT gene. The late promoter sequences correspond to the fragment located in the SV40 genome from nt 332 to 5172 in the counterclockwise direction. An additional 22-bp SV40 fragment (from nt 5171 to 5192) separates the CAT gene from the late promoter sequences. The different elements of the promoter region are depicted in Fig. 2. MLIS, major late initiation site. (B) Representative autoradiograph of CAT assays showing a linear response to 20 μg of transfected CAT recombinant. CV1-P cells were transfected with 2, 5, 10, or 20 μg of pASLcat. After overnight incubation, the precipitate was removed. Fresh Dulbecco modified medium plus 2.5% fetal calf serum and 2.5% newborn calf serum was added, and incubation at 37°C was continued for an additional 30 h. Cell extracts were then prepared, and extracts from 3.5×10^6 cells were assayed for CAT activity for 30 min at 37°C as described in Materials and Methods.

the *ts*A58 protein show that the function of T antigen involved in the activation of the constitutive late promoter activity is different from that involved in the negative autoregulation of the early transcription.

MATERIALS AND METHODS

Recombinant plasmids. The T-antigen-encoding recombinant pAS is a nonreplicative plasmid constructed by Benoist and Chambon (4). This plasmid contains SV40 early-gene sequences from HpaII (nt 346 in SV40) to *Bam*HI (nt 2533 in SV40) sites with a 9-bp deletion at the unique *Bgl*I site in SV40, forming a *Stu*I site at nt 2. The pASEcat recombinant was constructed from pAS by replacing the fragment spanning the SV40 early genes from nt 5171 to 2533 (numbering in the SV40 system) by the *Hin*dIII-*Eco*RI fragment isolated from pSV2-cat (25) and spanning the CAT gene, the SV40 small T-antigen intron, and the polyadenylation signal of SV40 early mRNA. The late promoter recombinants pSVL-cat, pASLcat, pASΔ21Lcat, pSVL380cat, and pSVL1130cat were constructed from pASEcat by replacing the early promoter by the late promoter sequences isolated from pSVori, pASori, pASoriΔ21, pEMP-LCAP-38, and pEMP-LCAP-113, respectively (Fig. 1 and 2). pSVori, pSVoriΔ21, pEMP-LCAP-38, and pEMP-LCAP-113 were described previously (15, 16, 38). Plasmids pASori and pASoriΔ21 are the nonreplicative counterparts of pSVori and pSVoriΔ21, respectively. They were constructed by replacing the SV40 fragment containing the wild-type (WT) origin by the corresponding origin-mutated fragment isolated from pAS (see above). PSVL20cat was derived from pASLcat by deleting the 43-bp fragment located between the two StuI sites located within the regulatory region of SV40 at nt 2 and 5193.

The puASLGcat and pASLGcat series were derived from the pSEG series described by Barrera-Saldana et al. (3). The pSEG recombinants harbor a *Sal*I site at nt 32 in the SV40 regulatory sequence. The puASLGcat series was constructed by replacing the regulatory region of puASLcat from *Nco*I (nt 37 in SV40) to *Kpn*I (nt 294 in SV40) sites by the corresponding *Nco*I-*Kpn*I fragment isolated from pSEGO, pSVEG12, and pSVEG456, respectively. The pASLGcat series was constructed in a similar manner, but the *Nco*I-*Kpn*I fragment of pASLcat was replaced by the *Sal*I (nt 32 in SV40)-*Kpn*I (nt 294 in SV40) fragment isolated from the pSEG recombinants. The *Sal*I and *Nco*I sites were converted to blunt ends before ligation, leading to a 9-bp insertion (Fig. 3).

Plasmid pSVA58 encodes the temperature-sensitive SV40 A gene. The early gene fragment from *Kpn*I (nt 294) to *Bam*HI (nt 2533) sites was isolated from the *ts*A58 mutant (56) and inserted between the *Kpn*I and *Bam*HI sites of pUC18. This plasmid has the WT replication origin.

These plasmids were constructed and isolated by standard recombinant DNA techniques. Their partial linear maps are shown in Fig. 2 and 3.

Cell growth and DNA transfection. CV-1P and COS-7 cells were seeded at 10^6 cells per 90-mm-diameter petri dish in Dulbecco modified Eagle essential medium supplemented with 2.5% fetal calf serum and 2.5% newborn calf serum. After 24 h, the cells were transfected by the calcium phosphate precipitation technique as previously described (17, 38). Unless otherwise stated, 10 μg of promoter CAT plasmid and either 10 μg of T-antigen-coding plasmid (pAS) or control plasmid (pASori) was added to each petri dish. The total amount of DNA was adjusted to 30 μg by addition of thymus DNA. When the experiments were carried out at 40.5°C, cells were transferred to this temperature immediately after addition of calcium-precipitated DNA. At 8 h (40.5°C) or 16 h (37°C) after addition of DNA, dishes were washed twice and incubated with fresh medium containing 2.5% fetal calf serum and 2.5% newborn calf serum for another 36 h (40.5°C) or 30 h (37°C).

CAT assay. The chloramphenicol acetyltransferase (CAT) assay was performed according to Gorman et al. (25). Cell extract prepared from one petri dish was incubated with 0.1 μCi of [^{14}C]chloramphenicol (50 mCi/mmol; New England Nuclear Corp.) and 80 nmol of acetyl coenzyme A (Boehringer GmbH, Mannheim, Germany) in 250 mM Tris-HCl (pH 8) at 37°C for 30 min. The products of the cell reaction were chromatographed and quantitated by scintillation counting. When the percentage of acetylation exceeded 40%, a reduced amount of cell extract was used. Each activity was normalized to reflect the activity generated by 10^5 transfected cells; cell counts were performed from parallel mock-transfected cultures.

RESULTS

These studies were performed to analyze the effects of T antigen on the transcriptional activity of the late promoter in the absence of genome amplification. This analysis was performed with CAT transient-expression vectors. The parental nonreplicative recombinant (pASLcat) having a 9-bp deletion at the *Bgl*I site is presented in Fig. 1A. Recombinant plasmids were transfected in permissive CV-1P cells. Cell extracts were prepared 44 h after transfection and tested for the resulting CAT activity. To allow for differences in transfection efficiencies from one experiment to another, at least eight independent transfection experiments using a

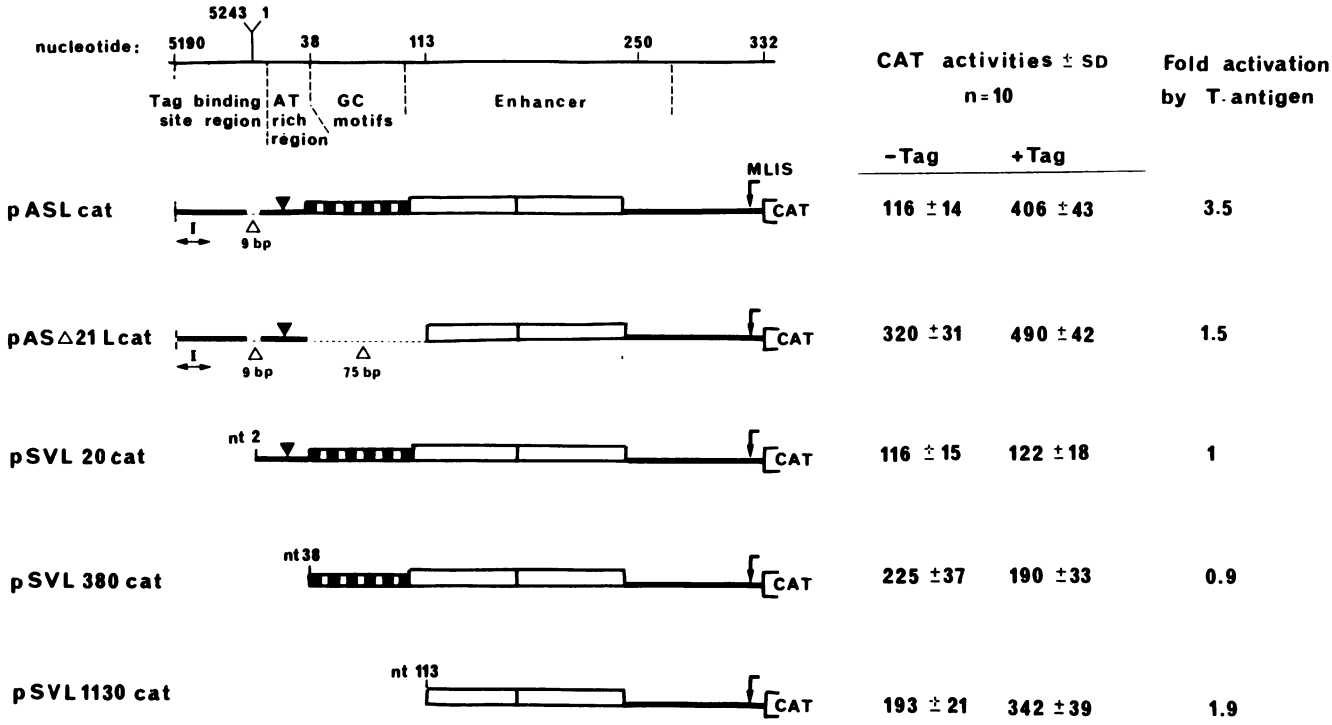

FIG. 2. CAT activities of extracts of cells transfected with various deletion mutants. A schematic representation of the promoter elements present in the different plasmids is shown. Nucleotide numbers are those of the authentic SV40 DNA. The following features are indicated: T-antigen (Tag) binding site I, the 9-bp deletion at the origin of replication characteristic of the AS mutation, the Golberg-Hogness TATA sequence of the early promoter (▼), the six CCGCCC motifs present within the 21-bp repeats (six black boxes), the tandem 72-bp repeats (open boxes), and the position of the major late initiation site (MLIS). CV1-P cells were transfected with 10 μg of CAT plasmid and 10 μg of pASori (−Tag) or pAS (+Tag). The cells were harvested 46 h after the addition of calcium-precipitated DNA. The data are normalized to reflect the activity of 10^5 transfected cells, expressed in arbitrary units; n represents the number of independent experiments.

minimum of three different plasmid preparations were performed for each series of the plasmids tested. To ensure that none of the experiments were carried out under conditions of excess input DNA, we first determined the dose-response relationship for pASLcat. Results presented in Fig. 1B show that transfecting up to 20 μg of CAT plasmid did not result in saturation of the system. Usually transfections were performed with 10 μg of CAT plasmid.

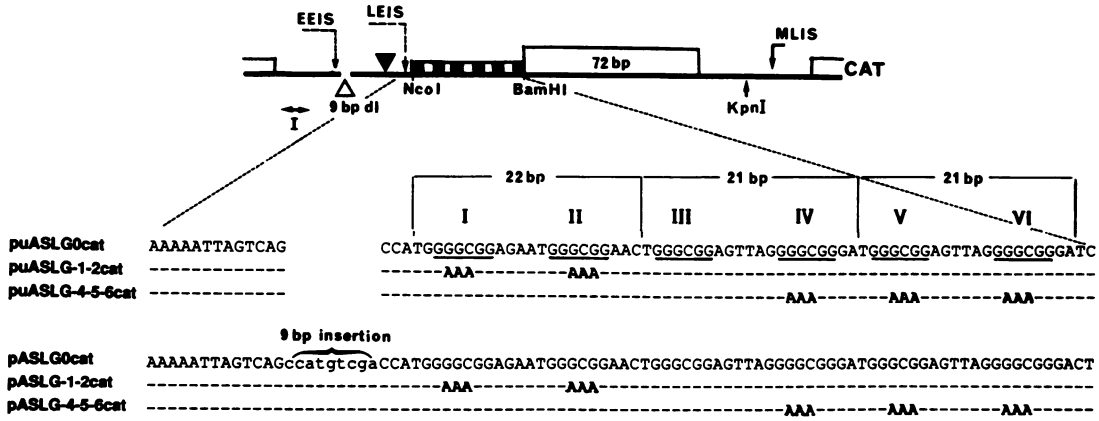

FIG. 3. Promoter organization of puASLGcat and pASLcat series and position of the point mutation clusters introduced in the GC motifs. The following features are indicated in the diagram at the top: the position of T-antigen binding site I, the 5′ ends of early-early (EEIS) and late-early (LEIS) mRNAs, the 9-bp deletion (dl) characterizing the AS mutation, the Golberg-Hogness early TATA box (▼), the six CCGCCC motifs present within the 21-bp repeats (six black boxes), the single 72-bp sequence (open box), and the position of the major late initiation site (MLIS). The second line shows the sequence of puASLGOcat (noncoding strand). This sequence corresponds to the WT SV40 sequence between nt 24 and 105. The sequence mutated in the puASLG series is indicated below the puASLGO sequence. The next line shows the position of the 9-bp insertion introduced in pASLGOcat. This insertion has created a *Sal*I site. The next two lines indicate the mutations present in recombinants pASLG-1-2cat and pASLG-4-5-6cat.

TABLE 1. Effects of point mutation clusters within the GC motifs on the late promoter activity in the absence of T antigen

Recombinant	Mutated GC motifs	CAT activity (mean ± SD)[a]
puASLG0cat	None	100 ± 18[b]
puASLG-1-2cat	I, II	300 ± 27[b]
puASLG-4-5-6cat	IV, V, VI	136 ± 9[b]
pASLG0cat	None	100 ± 19[c]
pASLG-1-2cat	I, II	154 ± 16[c]
pASLG-4-5-6cat	IV, V, VI	272 ± 25[c]

[a] Results are means of four independent experiments.
[b] Relative to puASLG0cat.
[c] Relative to pASLG0cat.

TABLE 2. Effects of T antigen on the activity of late promoter sequences including or not including the 21-bp repeats

Plasmid	Cells	CAT activity (mean ± SD, n = 9)[a]		Fold activation by T antigen
		−T antigen	+T antigen	
pASLcat	CV-1P	79 ± 10	269 ± 27	3.4
	COS-7		670 ± 47	
pASΔ21Lcat	CV-1P	210 ± 27	500 ± 34	2.4
	COS-7		1,220 ± 47	

[a] Data are normalized to reflect the activity of 10^5 transfected cells, expressed in arbitrary units. n, number of independent experiments. CV-1P cells were cotransfected with pASori (control) (−T antigen) or pAS (recombinant coding for T antigen) (+T antigen).

Down-regulation of the late promoter by the GC motifs requires additional elements of the early promoter region. Permissive CV-1P cells were transfected with 10 μg of the following nonreplicative plasmids: pASLcat, which retains the entire late promoter sequence, including the early promoter elements; pASΔ21Lcat, identical to plasmid pASLcat except for a deletion from nt 37 to 113 removing the six GC motifs; pSVL20cat, deleted for the T-antigen binding sites I and II and the early initiation sites; pSVL380cat, which has an additional 35-bp deletion removing the early TATA box; and pSVL1130cat, which retains only the enhancer element (Fig. 2). In the absence of T antigen, expression of the CAT gene under the control of the late promoter deleted for the 21-bp repeats (pASΔ21Lcat) is threefold higher than the CAT activity obtained with extracts of cells transfected with pASLcat. This result indicates that GC motifs down-regulate the constitutive expression of late genes, confirming our previous published data (17).

In addition, comparison of CAT activities obtained by transfecting mutants pASLcat, pSVL20cat, and pSVL380cat (Fig. 2) indicates that the down-regulation of the late promoter is maximized when sequences upstream of the late promoter region extend to nt 2, including the GC motifs and the early TATA box, indicating that these two early promoter elements cooperated to down-regulate the late promoter.

Identification of the GC motifs involved in down-regulation of the late promoter: spacing between the 21-bp repeats and the core origin sequences leads to a switch from motifs I and II to motifs IV, V, and VI. We previously analyzed the contribution of the different GC motifs to the late promoter efficiency by measuring the transcriptional activities of a series of clustered point mutations generated within the GC motifs. The motifs located at the early proximal end of the 21-bp repeats (motifs I and II; Fig. 3) are the motifs involved in down-regulation of the late promoter in the absence of T antigen (17). The data presented here confirm these results. The nonreplicative recombinants puASLGOcat, puASLG-1-2cat, and puASLG-4-5-6cat (Fig. 3) are constructs derived from pSVEG-type recombinants described by Barrera-Saldana et al. (3). The SV40 regulation region of these recombinants has one copy of the 72-bp repeat. An additional 9-bp deletion has been introduced at the SV40 *Bgl*I site (AS mutation) to inhibit DNA replication. The activity of the late promoter was monitored by measuring the CAT activities of cell extracts prepared 44 h after transfection. Results presented in Table 1 indicate that the various mutations result in an overall stimulation of the constitutive late promoter efficiency. However, the effect obtained by mutating GC motifs I and II (proximal from the early promoter) differs clearly from those obtained by mutating GC motifs IV, V, and VI (distal from the early promoter). Double mutations within motifs I and II (puASLG-1-2cat) result in a consistent increase (threefold) in the activity of the late promoter compared with the wild-type plasmid puASLGO cat, while the activity of the late promoter directed by plasmid puASLG-4-5-6cat is only slightly stimulated. Published data have shown that the requirement of motifs I and II appears to be unique for initiation of early transcription from early-early sites, whereas motifs IV, V, and VI are important for initiation of transcription from both early-early and late-early sites (3). Results presented here and elsewhere (16) demonstrate that GC motifs I and II, which are essential for initiation from early-early sites, down-regulate the constitutive expression of late genes.

To further investigate the effects of GC motif mutations on both early and late promoter activities, we altered the distance between the 21-bp repeats and the TATA box by inserting 9 bp between these two regions. Results obtained with the insertion recombinants depicted in Fig. 3 are presented in Table 1. Moving the 21-bp repeats away from the TATA box by 9 bp leads to a higher stimulation of the late promoter activity by mutating motifs IV, V, and VI than by mutating the motifs I and II. It has been reported by Takahashi et al. (50) that an insertion of one DNA turn at this location leads to a decrease in early transcription from the early-early sites, whereas early initiation from the late-early sites is concomitantly increased.

Results published on the early promoter in combination with our results on the constitutive late promoter suggest that the nature of the GC motifs involved in the down-regulation of the late promoter depends to some extent on the selection of the sites used to initiate the early transcription. This finding supports the assumption that the early transcriptional complex might be involved in the down-regulation of the late promoter.

Effect of WT T antigen on late promoter activity at 37°C. In a preliminary experiment, the CAT activities were measured from extracts of cells transfected with the nonreplicative plasmid pASLcat in the presence or absence of T antigen. T antigen was provided by cotransfecting CV-1P cells with pAS, a nonreplicative plasmid which encodes the SV40 early region. To ensure that limiting transcriptional factors are not competing for the regulatory region of pAS, control cultures were cotransfected with plasmid pASori, which contains the same regulatory sequences as pAS but lacks the early SV40 encoding region (see Materials and Methods). Results presented in Table 2 show that in the absence of replication, cotransfection with a plasmid coding for T antigen results in

TABLE 3. CAT activities of extracts of CV-1P cells transfected with pASLcat and pSVLcat at 37°C in the presence of cytosine arabinosine[a]

Plasmid	CAT activity (mean ± SD, $n = 4$)[b]		Fold activation by T antigen
	−T antigen	+T antigen	
pASLcat	60 ± 6	200 ± 22	3.3
pSVLcat	35 ± 12	360 ± 25	10

[a] Cytosine arabinoside was added to the culture medium 15 min before addition of DNA precipitate at a final concentration of 25 μg/ml.

[b] Data are normalized to reflect the activity of 10^5 transfected cells, expressed in arbitrary units. *n*, number of independent experiments. Cells were cotransfected with pASori (control) (−T antigen) or pAS (recombinant coding for T antigen) (+T antigen).

a three- to fourfold increase in the expression of nonreplicative pASLcat plasmid. These results are representative of more than 20 independent experiments obtained by cotransfecting CV-1P cells with various amounts of pAS (5 to 20 μg per petri dish). We ensured that the amount of T antigen synthesized in cells increased in parallel with the transfected amount of pAS, which indicated that T antigen is not a limiting factor (data not shown). Transcription from late promoter sequences is markedly more efficient in COS-7 cells, which constitutively expressed T antigen, than in CV-1P cells cotransfected with pAS (Table 2). This difference may be due to a higher efficiency of transfection in COS-7 cells than in CV-1P cells. To control the effect of the 9-bp deletion at the pASLcat recombinant origin of replication on T-antigen-mediated late promoter activation, similar experiments were performed with the pSVLcat recombinant which contained the WT origin of replication. Cytosine arabinoside was added to the culture medium to inhibit DNA replication. Data presented in Table 3 indicate that a 9-bp deletion within the T-antigen binding site II at the origin of replication reduced by a factor of 3 the stimulatory effect of T antigen. These results, in agreement with those published by Kelly et al. (31), indicate that the stimulation of late promoter activity in a nonreplicative context is relatively moderate.

Contribution of the proximal sequences of the early promoter to the T-antigen-mediated activation of the late promoter. CAT activities of plasmid pASΔ21Lcat deleted for the 21-bp repeats were measured in the presence of T antigen provided either by transfecting COS-7 cells or by cotransfecting CV-1P cells with pAS (Fig. 2). Comparing pASΔ21Lcat versus pAScat for late promoter activity measured at 37°C, we found that the stimulation resulting from the 21-bp repeat deletion was less efficient in the presence (490 versus 406 arbitrary units) than in the absence (320 versus 116 arbitrary units) of T antigen. These results, representative of 10 independent experiments, suggest that T-antigen-mediated transactivation is partly due to a release of the negative regulation mediated by the 21-bp repeats. Moreover, the results obtained with plasmids pSVL20cat and pSVL380cat indicated that the activity of the late promoter deleted for the sequences covering the early TATA box and the early-early initiation sites is not stimulated by T antigen. However, a twofold stimulation was observed when the deletion extended through these two early promoter elements and the 21-bp repeats (pSVL1130). This value is close to that obtained with the recombinant pASΔ21Lcat deleted for the 21-bp repeat only. These results strongly suggest that the three early promoter elements (the TATA box, the early initiation sites, and the 21-bp repeats) are involved in a cooperative fashion in the T-antigen-mediated release of down-regulation of the late promoter.

Activation of the late promoter by the *ts*A58 protein at 40.5°C. The following experiments were performed to determine the level of late promoter transactivation in the presence of *ts*A58 protein, a T-antigen mutant defective in autoregulation of early gene expression and in viral DNA replication. CV-1P cells were cotransfected either with pAS, which codes for WT T antigen, or with pSVA58, which codes for *ts*A58 protein. At the nonpermissive temperature (40.5°C), the level of *ts*A58 protein is comparable to that of WT T antigen, although *ts*A58 protein was less stable than WT protein (14a). Transfected cells were incubated for 44 h at 40.5°C and tested for the resulting CAT activity. Preliminary experiments presented in Table 4 confirm that under our experimental conditions, *ts*A58 protein is thermosensitive to the autoregulation of the early promoter. At 40.5°C, cotransfection of pASEcat with pSVA58 has no effect on the level of CAT activity, in contrast to what is obtained with WT T antigen.

At 40.5°C WT T antigen stimulates the late promoter activity by 12-fold, which is clearly higher than the level of stimulation observed at 37°C (Table 4). Likewise at 37°C, the WT T-antigen-mediated stimulation of CAT activity is much less effective for pASΔ21Lcat than for pASLcat (3- versus 12-fold). Comparing pASΔ21Lcat versus pAScat for the late promoter activity observed at 40.5°C, we found that the stimulation resulting from the 21-bp repeat deletion was less efficient in the presence (1,278 versus 934 arbitrary units) than in the absence (416 versus 80 arbitrary units) of T antigen, and this finding reinforces the results obtained at 37°C.

TABLE 4. Effects of WT T antigen and the *ts*A mutant on early and late promoter activities at 40.5°C

Promoter	CAT activity (mean ± SD, $n = 12$)[a]		
	No T antigen	WT T antigen	+*ts*A58
Late			
pASLcat	80 ± 9	934 ± 79 (12)	769 ± 64 (10)
pASΔ21Lcat	416 ± 119	1,278 ± 85 (3.1)	1,380 ± 145 (3.3)
pSVL1130cat	283 ± 39	510 ± 54 (1.8)	402 ± 30 (1.4)
Early			
pASEcat	10,450 ± 851	2,770 ± 121	10,860 ± 864

[a] Data are normalized to reflect the activity of 10^5 transfected cells, expressed in arbitrary units. *n*, number of independent experiments. CV-1P cells were cotransfected with pASori (no T antigen) pAS (WT T antigen), or pSVA58 (+*ts*A58). Numbers in parentheses indicate T-antigen activation factors observed in the presence of WT T antigen and *ts*A protein.

When pSVA58 is cotransfected with pASLcat, the CAT activity of cell extracts is stimulated 10-fold (Table 4). These results indicate that the late promoter is still stimulated by *ts*A58 protein, although to a lesser extent (83%) than by WT T antigen. The diminution of the level of activation measured with *ts*A58 protein might be related to the reduced stability of *ts*A protein at 40.5°C (14a, 51). Plasmid pASΔ21Lcat is also stimulated by *ts*A58 protein by factor of 3.3. This factor is close to that obtained with WT antigen at 40.5°C (Table 4). These results demonstrate that activation of the late promoter, measured with mutants deleted for the 21-bp repeats, is not affected by the thermosensitivity of *ts*A58 protein. Moreover, the results presented in Table 4 indicate that the *ts*A58 protein-mediated stimulation is still more efficient with the intact promoter (10-fold) than with the promoter deleted for 21-bp repeats (3.3-fold). These data strongly suggest that the T-antigen function involved in the release of the 21-bp repeat-mediated down-regulation of the late promoter is not thermosensitive, as opposed to that involved in the autoregulation of the early genes.

DISCUSSION

Before viral replication, a complex involvement of negative and positive *cis*-acting elements accounts for the transcriptional activity of the late promoter. Negatively acting sites are detected both in the enhancer element (45) and in the GC motifs (1, 17, 38). Results presented here indicate that the late promoter is negatively regulated by the GC motifs acting in cooperation with *cis*-acting elements located within the sequences overlapping the early TATA box. GC motifs I and II are involved in the down-regulation of the late promoter. Increasing the distance between the 21-bp repeat region and the TATA box by 9 bp leads to a switch from GC motifs I and II to motifs IV, V, and VI.

We previously showed that the negatively acting elements located within the enhancer appeared to restrict the expression of the late promoter only in the absence of T antigen. The results presented in this report extend this observation to the negative regulatory elements located within the 21-bp repeats, since the presence of T antigen diminishes the stimulative effect of the 21-bp deletion. These results suggest that T antigen is also able to modulate the negative regulation mediated by the GC motifs.

It is well known that T antigen autoregulates the level of SV40 early mRNA through direct binding to sequences on the early side of the origin of replication, that is, to T-antigen binding sites I and II (44). However, results obtained with the *ts*A58 protein indicate that two distinct mechanisms might account for the negative and positive regulation of the early and late promoters, respectively. The efficiency of transcription from the early promoter is not negatively regulated in the presence of *ts*A58 protein at the nonpermissive temperature, whereas the late promoter activity is still stimulated by *ts*A58 protein at the nonpermissive temperature. These results strongly suggest that, contrary to the negative regulation of the early promoter, the transactivation of the late promoter mediated by *ts*A58 protein is not thermolabile.

This difference between T-antigen-mediated regulation of early and late promoters leads us to propose a model whereby two different functions of T antigen may be involved in autoregulation of the early promoter and in transactivation of the late gene. The following observations described earlier fit in well with this assumption: (i) cells infected with a temperature-sensitive early mutant placed in nonpermissive conditions for autoregulation of early transcription still synthesize late viral RNA at an elevated level (5, 13, 32, 42), and (ii) a temperature upshift causes the loss of *ts*A58 protein, but not of WT T antigen, from the minichromosome (34).

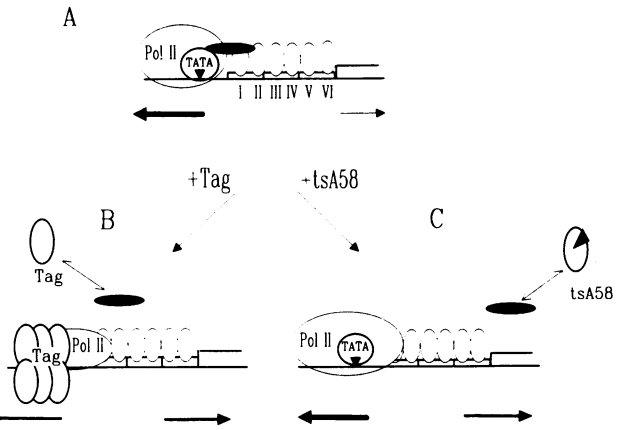

FIG. 4. Model proposed to account for the negative regulation of the late promoter mediated by GC motifs I and II. (A) In the absence of T antigen (Tag); (B) in the presence of WT T antigen; (C) in the presence of *ts*A58 protein. The differently sized horizontal arrows represent the relative level of transcriptional activity initiated from the early promoter (leftward direction) or from the late promoter (rightward direction). Numbers I to VI stand for GC motifs I to VI. The filled oval is the presumed negatively acting factor. T antigen and *ts*A58 might interact with this factor either directly (protein-protein interaction) or indirectly by some posttranscriptional modification. Pol II, polymerase II.

Figure 4 shows a tentative scheme to illustrate the different mechanisms by which T antigen might regulate negatively the early promoter and positively the late promoter through the same sequences. In vitro studies have shown that the 21-bp repeats are able to activate transcription in both directions (21, 36). In vivo, positively acting factors may be bound to the GC motifs, but their potential transcriptional activity in the late direction is masked by association with an inhibitory factor. T antigen might activate transcription in the late direction by neutralizing the supposed inhibitory factor either by protein-protein interactions or by some posttranscriptional modification(s). The two transcriptional factors Sp1 and AP-2 bind to the 21-bp repeat element (see references 29 and 37 and references therein). AP-2 also binds to the origin sequences and to recognition sequences in the SV40 enhancer element. Moreover, binding of AP-2 to these three sites is completely inhibited in vitro by T antigen. On the other hand, in vitro transcription experiments suggest that AP-2 may function in a coordinate fashion with Sp1 to stimulate synthesis of RNA (37). It is tempting to speculate that binding of AP-2 to the 21-bp element regulates both early transcription (positively) and late transcription (negatively) and that T antigen activates late transcription by inhibiting the binding of AP-2 to specific sequences within the 21-bp repeats.

Alternatively, T antigen can induce some posttranscriptional modifications leading to the inactivation of the supposed inhibitory factor. An example of such posttranscriptional modification has been recently described (7). The activation of c-*jun* in HeLa cells treated with tetradecanoyl phorbol acetate is accompanied by specific changes in the phosphorylation pattern of this transcription factor.

An attractive hypothesis would be that the constitutive transcriptional activity of the late promoter is determined by a balance between the activity of the negative and positive cellular factors. In agreement with the results presented here and in other reports (19, 26, 45, 58), the role of T antigen would then be to tip the balance by increasing the activity of positive factors and by decreasing the activity of negative factors. A similar model has been proposed to explain, for example, the activity of the polyomavirus late promoter (12), the down-regulated transcription of the JC virus late promoter (49), the regulation of the expression of the Epstein-Barr viral latent membrane protein (18), the cell type specificity of the polyomavirus enhancer (54), and the regulation of beta interferon transcription (23).

In addition to its implication in the regulation of viral gene expression and viral DNA replication, T antigen induces a variety of cellular processes leading to cell proliferation and cell transformation (41, 47, 52). For example, stimulation of cellular enzymes involved in induction of the S phase has been shown to be correlated with T-antigen synthesis (for a review, see reference 53). However, mechanisms by which T antigen activates cellular genes are not yet understood. Regulation of some of these genes might be similar in some aspect to the regulation of the SV40 late promoter activity. From this point of view, it is interesting to note that the promoter regions of a number of cellular housekeeping genes have been shown to contain multiple copies of the GC hexanucleotide box present on six copies within the 21-bp repeats (reference 14 and references therein). As for the late promoter, T antigen may activate some of these genes by interacting directly or indirectly with a negative regulatory factor(s) specific to GC boxes. Analysis of the effect of T antigen in activating some of these genes should help us to understand the regulation of the expression of cellular genes induced during the T-antigen-induced S phase.

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