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Transactivation of both early and late simian virus 40 promoters by large tumor antigen does not require nuclear localization of the protein

(transcription/tumor virus/gene regulation)

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ABSTRACT **The early gene product of simian virus 40, large tumor antigen (T antigen), is required for the onset of viral replication. This protein has also been reported to transactivate viral late gene expression, independently of replication. In this study I have used a vector that permits simultaneously a precise quantitation of simian virus 40 early and late promoter activity with a single nuclease S1 mapping probe. The results show that T antigen can activate the early promoter as well as the late promoter and that only on replicating templates does a shift occur in the ratio of late-to-early transcription. This simultaneous transactivation of early and late promoters occurs in human (HeLa) and monkey (CV-1) cells but does not occur in mouse embryonal carcinoma cells. It is seen with either wild-type T antigen or with a T antigen protein that carries a mutation in the nuclear localization signal. The mutant protein cannot bring about an early-to-late shift, consistent with its inability to support viral replication.**

Expression of the early and late genes of the simian virus 40 (SV40) requires the transcription machinery of the host cell. The viral early gene product, large tumor antigen (T antigen), plays a key role, however, in modulating the relative amounts of early and late transcription. In addition to its ability to transform cells, T antigen has been reported to repress early transcription (1–5), to promote viral replication (6–8), and to activate the late genes that encode virion proteins (9, 10). Although late-gene transcription becomes maximal only after the onset of viral replication, several laboratories have demonstrated that T antigen can activate late-gene expression in the absence of replication (9, 10). This activation has been thought of as a model for the study of how proteins such as T antigen modify cellular gene expression during cell transformation. Transforming proteins from other DNA tumor viruses, for example, the adenovirus early region 1A (E1A) protein, also can affect transcription from specific viral promoters (11).

Much data has been obtained concerning the viral sequences important for SV40 early transcription. Controlling elements include a TATA box, an upstream element consisting of six -CCGCCC- motifs clustered within three repeats of a 21-base pair (bp) sequence, and an enhancer (for review, see ref. 12). The enhancer spans about 160 bp and contains two copies of a 72-bp sequence; functionally it is made up of a number of specific motifs that contribute to its activity (13). The DNA sequences controlling late transcription are less well defined, although studies in several laboratories indicate that the GC-blocks and enhancer motifs defined for early transcription may be important (14, 15). Considering that all sequences that control the divergent early and late transcription units are present within ≈350 bp, it is not surprising that some control elements should overlap.

When analzying the effect that T antigen has on SV40 transcription, direct comparison of early and late RNA levels is difficult because (*i*) different probes must be used to measure each, and the mRNAs may have different stabilities in the cell; and (*ii*) viral replication can alter the amount of transcription by changing template copy number. To overcome these problems we constructed a vector that enables total SV40 transcription activity and the early-to-late transcriptional shift to be measured with a single nuclease S1 mapping probe (16). With this vector both early and late transcripts are globin mRNAs and should have similar stabilities in the cell. Data obtained with this system and presented here indicate that in human (HeLa) and monkey (CV-1) cells T antigen transactivates the SV40 early promoter as well as the late promoter on replication-defective templates; this transactivation is not accompanied by any early-to-late gene shift. Transactivation was also seen when a T antigen that cannot be transported to the nucleus, but still can transform cells, was used. The mutant T antigen does not support replication and was seen not to allow any early-to-late gene shift on wild-type templates. These results suggest that T antigen may be modifying in the cytoplasm some component(s) of the transcription machinery that is used by both early and late promoters.

Finally, the results presented here bear directly on the question of early gene repression (autoregulation) by T antigen binding to the origin region. Some recent studies have indicated that *in vivo* early gene expression may be repressed by the replication process and not simply by T antigen binding and sterically interfering with RNA polymerase (17, 18). Indeed, only with *in vitro* transcription systems where replication is not an issue in the interpretation of data has autoregulation been clearly demonstrated (1, 19). As will be discussed, the results from analyses in different cell lines with wild-type and mutant T antigens provide evidence that autoregulation, as described from *in vitro* studies, can be detected *in vivo*, but that, in addition, the replication process may contribute to early gene repression in a cell-specific manner.

MATERIALS AND METHODS

Plasmid Vectors. The vector pBEL2 is shown in Fig. 1 (*Upper*). Details of its construction have been described (16). Briefly, it contains SV40 sequences from the *Hpa* II site at position 346 to the *Hin*dIII site at position 5171, flanked on each side by a rabbit β-globin-coding sequence. The nuclease S1 mapping probe is made by end-labeling the *Bam*HI–*Bst*NI fragment from the early region (Fig. 1). Early-early start sites (EES) and late-early start sites (LES) are distinguishable with this probe, and late start gene activity (LS) is detected as a

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen; EES, early-early start sites; LES, late-early start sites; LS, late start sites; EC, embryonal carcinoma; AP-1 and -2, activator protein 1 and 2, respectively; nt, nucleotide(s); E1A, early region 1A protein.

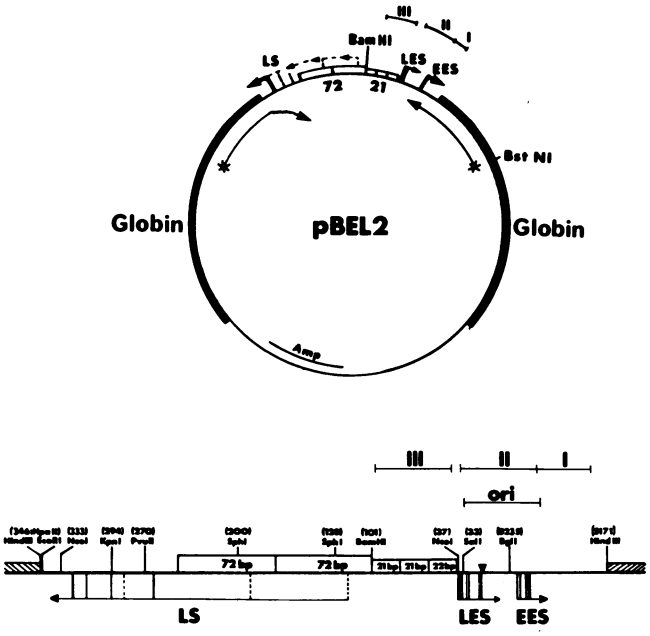

FIG. 1. The expression vector pBEL2. Below the plasmid is a diagram showing the main features of the SV40 sequences. The positions of the EES, LES, and LS are shown. Identical globin coding sequences (hatched lines in lower drawing) flank the early and late sides of the transcription control region, which extends from *Hpa* II on the late side (position 346) to *Hin*dIII on the early side (position 5171). The small arrows within the plasmid indicate the S1 mapping probe, which was made by end-labeling the *Bst*NI to *Bam*HI fragment from the early region. T antigen binding sites I, II, and III are shown. pBEL2ori$^-$ has a 6-bp deletion at the *Bgl* I site at position 5235, within T antigen binding site II. Nucleotide numbers are after Tooze (20).

break point where the globin gene on the late side is fused to the late leader sequence (Fig. 1 *Lower*). Individual late gene cap sites used on pBEL2 have been shown to be the same as those used on SV40 virus (16). pBEL2ori$^-$ is similar to pBEL2 but has a 6-bp deletion at the *Bgl* I site, which blocks replication (21).

Several different plasmids were used to supply T antigen. pPVU-0 (22) contains the entire early region of SV40, with its own promoter. pRSVT uses the Rous sarcoma virus long terminal repeat promoter and lacks the SV40 origin of replication; pRSVT$^-$ is identical to pRSVT except for a frameshift mutation deleting eleven and one-third codons from the 5′ end of the T antigen-coding sequence (described in ref. 16). pBM11 (22) is similar to pPVU-0, except for a mutation in the T antigen-coding sequence that changes lysine at position 128 to isoleucine. This mutation blocks transport of the protein into the nucleus and, consequently, SV40 replication; the mutation has little effect on the ability of the protein to transform cells (22). The plasmid pβ(244$^+$)β encodes a globin transcript under the control of its own promoter and the polyoma virus enhancer (23). With the nuclease S1 mapping probe this plasmid generates a band 9 nucleotides (nt) shorter than the band for LS; the globin genes in pBEL2 were fused to SV40 sequences using a *Pvu* II site at -9 in the globin gene. This plasmid serves as a control for the transfection and RNA extraction efficiencies.

Cell Culture, Transfection, RNA and DNA Analyses. All cells were maintained in Dulbecco's minimal essential medium (Flow Laboratories) supplemented with 10% fetal bovine serum. Human (HeLa) and monkey (CV-1) (laboratory stocks) cells were routinely plated into 100-mm dishes for transfection. P19 mouse embryonal carcinoma (EC) cells (24) were maintained in an undifferentiated state by passaging every 48 hr, and were seeded into 60-mm dishes for transfection.

Cells were transfected when they were 50–60% confluent with the calcium phosphate precipitation procedure (25). The DNA was left on the HeLa cells for 18 hr and on CV-1 and P19 cells for 7–8 hr. Fresh medium was then added, and cells were harvested 48 hr after transfection. Extraction of cytoplasmic RNA and nuclease S1 mapping were done as described (13, 26) with 300 units of enzyme (Boehringer Mannheim). Samples were then run on 8% polyacrylamide sequencing gels, and the intensity of various bands was quantitated by densitometric analysis of the autoradiograms.

Plasmid DNA was extracted from transfected cells after 48 hr using the procedure of Hirt (27). Nonreplicated input DNA was detected by digesting aliquots (one-tenth of the DNA recovered from a dish of transfected cells) with *Dpn* I, which cuts only DNA that is methylated by the *dam* methylase present in *Escherichia coli*. Newly replicated plasmids were detected with the enzyme *Mbo* I, which cuts only unmethylated DNA. Finally, total plasmid DNA was measured by digestion with *Sau*3A, which cuts methylated and nonmethylated DNA. After digestion, the DNA samples were electrophoresed on 0.8% agarose gels, transferred to nitrocellulose, and probed with a nick-translated fragment of the SV40 DNA control region present in pBEL2.

RESULTS

T Antigen Activates Both Early and Late SV40 Promoters. To study T antigen effects on transcription from the SV40 promoter region, either pBEL2 or pBEL2ori$^-$ were cotransfected into HeLa cells with the plasmid pRSVT. Also included in each transfection was the plasmid pβ(244$^+$)β as control. Fig. 2 shows that both pBEL2 and pBEL2ori$^-$ respond to T antigen. With pBEL2 (WT) a dramatic increase occurs in both late and late-early transcription, and some increase in early-early transcriptional activity. Densitometric

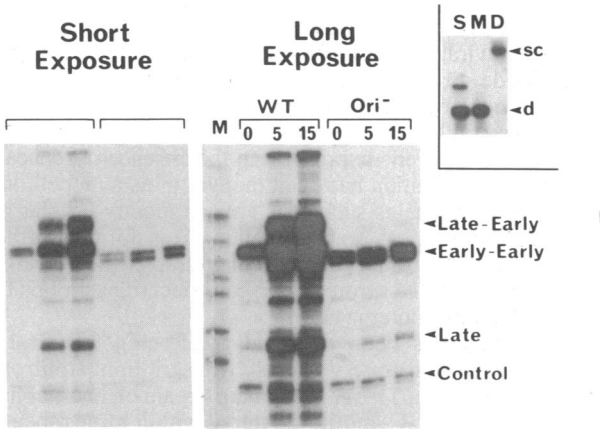

FIG. 2. Activation of early and late transcription by SV40 T antigen in HeLa cells. The autoradiograms show a short and long exposure of RNA levels after nuclease S1 mapping when 10 μg of pBEL2 (WT) or pBEL2ori$^-$ (Ori$^-$) was transfected with 0, 5, or 15 μg of pRSVT. The control band is generated from the plasmid pβ(244$^+$)β (15 μg per dish). The amount of DNA in each dish was equalized by use of plasmid pRSVT$^-$. The marker lane (M) is an *Msp* I digest of pBR322. The early transcripts from pBEL2ori$^-$ are shifted 6 nt downstream from those on pBEL2 because the deletion in the ori$^-$ plasmid is between the EES and TATA box (Fig. 1). The probe to measure transcripts from this plasmid was made using the *Bst*NI–*Bam*HI fragment of pBEL2ori$^-$. (*Inset*) Replication assay for pBEL2 in HeLa cells. DNA recovered from Hirt extracts was digested with *Sau*3A (S), *Mbo* I (M), or *Dpn* I (D), and was analyzed by Southern blotting as described. Uncut supercoiled DNA (sc) and digested fragments (d) detected by the probe are indicated.

analysis of autoradiograms from a number of experiments reveals a change in the amount of late transcription relative to early transcription from 3–4% to 40–80%, a 15- to 20-fold shift. The LES respond to replication in a manner similar to the LS, with LES levels ranging from 30 to 100% of LS (in the experiment of Fig. 2 LES and LS are almost equal). With pBEL2ori$^-$ an increase in both late and early activities relative to the globin control occurs. This increase averages 4- to 8-fold in different experiments. However, by being able to measure both activities simultaneously we see that with the ori$^-$ template the ratio of late-to-early transcription is unchanged in the presence of T antigen and is the same as is seen on wild-type pBEL2 without T antigen. That is, on ori$^-$ templates no early-to-late shift occurs.

The increase in EES on replicating templates was surprising, because autoregulation was anticipated to occur as T antigen binds to the origin region. Analysis of pBEL2 plasmid DNA in transfected cells, using Hirt extracts, revealed an increase in template number after replication of ≈50-fold (Fig. 2 *Inset*). The increase in EES activity from pBEL2 was less, being 10- to 15-fold. In most experiments, such a correction for copy number shows that some EES repression may occur on replicating templates. Clearly, though, autoregulation is not readily apparent in HeLa cells. Another plasmid constructed in the laboratory, pCMVT, has the human cytomegalovirus promoter fused to the T antigen gene and expresses about 10-fold higher levels of T antigen than pRSVT or pPVU-0. Even with this plasmid the amount of activation, replication, or autoregulation does not exceed that seen with the other T antigen plasmids (unpublished observations). In other words, within the limits of the assay used here, the system can be saturated for T antigen.

T Antigen Transactivates the SV40 Early and Late Promoters in Monkey Cells, But Autoregulation Is More Apparent. Although much of what is known about SV40 gene regulation has been learned from studies in HeLa cells or HeLa cell extracts, it was important to determine whether a similar phenomenon occurs in viral host monkey cells. Fig. 3 shows that in CV-1 cells the results with the pBEL2ori$^-$ template were identical to those noted in HeLa cells. Both late and early transcription were activated, and, as in the human cells, the ratio of late-to-early transcription was unchanged by the addition of T antigen. The globin control plasmid was noted to express weakly in CV-1 cells.

The main difference between HeLa and CV-1 cells was seen with the wild-type pBEL2 template; in the monkey cells early-early transcription was not increased in the presence of T antigen and, in fact, was even decreased in some experiments (Fig. 3). As in HeLa cells, both late-early and late transcription were activated. Thus, in monkey cells the repression of EES on replicating templates is easily seen. Although the SV40 origin of replication does not function as well in HeLa cells as in monkey cells, nevertheless a significant amount of replication occurred in the human cells (Fig. 2). Thus, T antigen binding and viral replication may not by themselves be sufficient for maximal autoregulation, but the host cell may, in addition, influence the extent to which replication plays a role in early gene repression.

Transactivation by T Antigen That Cannot Enter the Nucleus. Transactivation of early transcription and the absence of an early-to-late transcription shift from a replication-defective template in both CV-1 and HeLa cells suggest that no autoregulation was occurring on this plasmid. Either the activation was overriding autoregulation or the 6-bp deletion at the *Bgl* I site in the ori$^-$ mutation interferes sufficiently with T antigen binding so that autoregulation is prevented. Some studies have shown that this deletion reduces from four to three the number of T antigen tetramers that can bind to the origin region (28), and this may be enough to prevent early gene repression. The question remained, however, whether transactivation could occur independently of replication on templates that do not contain deletions that inactivate the origin. To address this problem the transactivation by a T antigen mutant that cannot enter the nucleus but retains its ability to transform cells was examined. Because replication requires that the protein be in the nucleus (confirmed by Hirt extract analysis and the data in ref. 22), such a mutant should be unable to bring about an early-to-late transcriptional shift. At the same time, if transactivation of SV40 is in any way related to the cell-transforming property of T antigen, the mutant should retain the ability to activate early and late transcription. As shown in Fig. 4, this is what was found; the protein transactivated both early and late activities similarly on wild-type and ori$^-$ pBEL2 templates, and on the ori$^-$ plasmid this activation was similar to that seen with wild-type T antigen (both pRSVT and pPVU-0 were used as sources of wild-type T antigen). Thus, transactivation was not markedly affected by mutations in the origin, and the ori$^-$ template appeared not to be autoregulated by nuclear T antigen. Finally, these data confirm that only when replication occurs is there a shift in the ratio of late-to-early or late-early-to-early transcription.

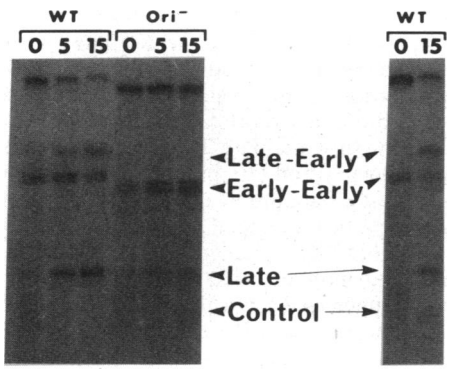

FIG. 3. Activation of early and late transcription by SV40 T antigen in CV-1 cells. (*Left*) RNA levels seen when 10 μg of pBEL2 (WT) or pBEL2ori$^-$ (Ori$^-$) are transfected with 0, 5, or 15 μg of pRSVT. (*Right*) Another experiment showing the extent of early gene repression that can be seen in some trials. The control plasmid [15 μg of pβ(244$^+$)β] expresses weakly in CV-1 cells but normally gives enough signal to verify that the samples were comparable for RNA quantities.

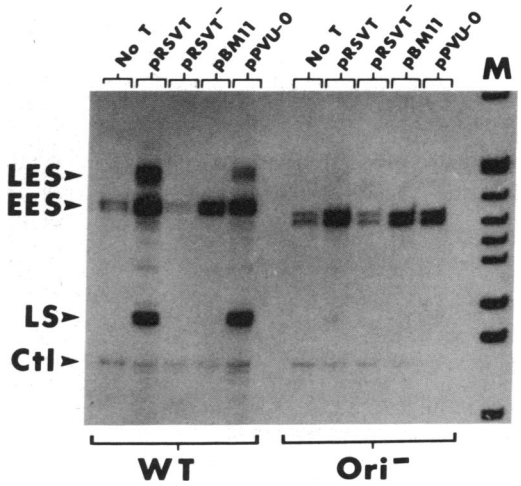

FIG. 4. Comparison of transactivation by wild-type and nonnuclear T antigens. HeLa cells were transfected with 10 μg of pBEL2 (WT) or pBEL2ori$^-$ (Ori$^-$) and either no T antigen plasmid (No T), or 10 μg of pRSVT, pRSVT$^-$, pBM11, or pPVU-0. The control band was obtained from 10 μg of pβ(244$^+$)β.

Autoregulation Is Visible on Wild-Type pBEL2 Template in EC Cells. The data with the wild-type and cytoplasmic T antigens suggested that the wild-type protein had no ability to repress early transcription on ori$^-$ templates. This conclusion was inferred from the observation that both T antigens gave similar amounts of early gene activation (Fig. 4). The model for autoregulation, based primarily on *in vitro* transcription studies using purified protein, contends that T antigen binding to the origin sterically interferes with the initiation of transcription by RNA polymerase (19). As shown earlier in the comparison of HeLa and CV-1 cells, it was difficult to study, much less explain, autoregulation without invoking some additional cell-specific role for replication. The approach to the problem of uncoupling autoregulation from effects of replication was to examine pBEL2 in mouse EC cells. The reason for choosing this type of cell line stemmed from reports that in mouse oocytes the origin of replication played no role in early or late gene expression (29). Although EC cells are clearly distinct from oocytes, the possibility that no SV40 replication occurs in them made them attractive for the present study.

Undifferentiated P19 cells were transfected with various pBEL2 and T antigen recombinants; the results are shown in Fig. 5. Compared with HeLa or CV-1 cells, without T antigen a significantly higher relative level of late gene activity was noted—with the late activity being 15–20% of early-early activity (Fig. 5*A*). Consistently, when pBEL2 was cotransfected with a T antigen-producing plasmid early transcripts were reduced, but no effect was seen on late transcripts. An average of four experiments revealed a reduction of early-transcript synthesis of 3- to 4-fold. Interestingly, late-early transcripts were also reduced when T antigen was present. In Hirt extracts from transfected P19 cells replication of the pBEL2 plasmid could not be detected; consistent with this

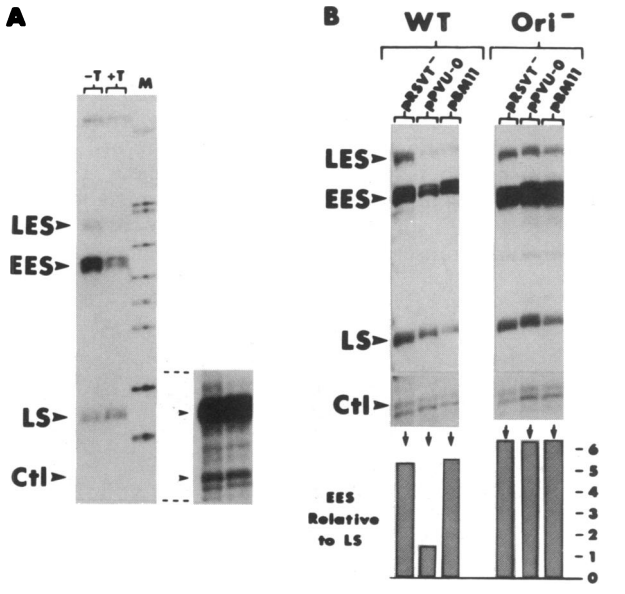

FIG. 5. Repression of SV40 early transcription by T antigen in mouse P19 EC cells. (*A*) Experiment where special care was taken to ensure that the globin control bands were balanced. Cells were transfected with 5 μg of pBEL2 and 5 μg of either pRSVT$^-$ (−T) or pRSVT (+T). Darker exposure of the lower region of the gel shows the control band [from 10 μg of pβ(244$^+$)β] and late activity. (*B*) Experiment comparing the effects of wild-type and nonnuclear T antigens on SV40 gene expression. Five micrograms of either pBEL2 (WT) or pBEL2ori$^-$ (Ori$^-$) was transfected with 5 μg of pRSVT$^-$, pPVU-0, or pBM11. The histogram below the autoradiogram quantitates these results with data obtained by densitometric analysis; EES activity was expressed relative to LS activity because LS is not affected by T antigen.

result was the lack of late or late-early activation. These observations are similar to *in vitro* experiments that looked at the effect of T antigen on SV40 early transcription (e.g., refs. 1 and 19) and are best explained by the model of autoregulation based solely on binding of the protein to the origin region. In support of this hypothesis, in experiments where pBEL2 was cotransfected with the pBM11 plasmid and T antigen was restricted to the cytoplasm no autoregulation of EES was seen (Fig. 5*B*). Finally, by using these cells, it was confirmed that the 6-bp deletion in pBEL2ori$^-$ seriously disrupts autoregulation (compare lanes with pPVU-0 in WT and Ori$^-$ in Fig. 5*B*). This result had been inferred from the data shown in Fig. 4, as described earlier, and the EC cell system provided a direct test of that conclusion.

DISCUSSION

The requirement of SV40 large T antigen for viral replication has been well documented both *in vivo* and *in vitro*. In addition, workers in several laboratories have reported that the protein can activate late viral gene expression (9, 10). The results reported in this paper demonstrate, however, that the ability of T antigen to activate SV40 gene expression is not restricted to late activity but is equally visible on early transcription. This general transactivation also does not appear to require that the T antigen be in the nucleus.

In an earlier study comparing early and late promoter activities in HeLa and 293 cells we also noted that in HeLa cells T antigen could in some experiments stabilize transfected DNA (16), which could then affect the amount of transcription. Careful quantitation with a cotransfected control plasmid is essential to correct for this variable. In all experiments, Hirt extracts were routinely done to ensure that each cell sample contained similar amounts of transfected DNA. The best inherent control for studying the early-to-late transcriptional shift is measuring early- and late-promoter activity simultaneously from a single template so that one can be sure that the copy number of each measured promoter is identical. Recently wild-type and non-nuclear T antigen has been reported to enhance expression from a number of promoters tested, including SV40 (30). These data relied on chloramphenicol acetyltransferase assays, without a control plasmid, and did not permit conclusions about whether the activation was in any way specific for SV40 or about how it related to replication and the early-to-late shift.

One of the most interesting aspects of these results is that even when T antigen cannot enter the nucleus it retains its ability to transform cells (22) and transactivate SV40 gene expression. The existence of many SV40 early promoter mutants (e.g., refs. 13 and 26), which have been used to identify and delineate the binding sites of specific cellular transcription factors, might enable the identification of which promoter motif(s) and factor(s) are mediating the transactivation. One might anticipate that a promoter element(s) shared by both early and late promoters will be involved.

At present, little data are available on SV40 transcription factors that might be influenced by T antigen. Gallo *et al.* (31) have reported that there is an activator protein 1 (AP-1)-like protein present in monkey cells, whose binding properties are altered by T antigen. AP-1 is a HeLa cell transcription factor that binds to the region of the SV40 enhancer, the P motif, proximal to the 21-bp region and to a similar motif in the human metallothionein promoter (32, 33). A 21-bp repeat binding factor, different from transcription factor Sp1, has also been reported to be specific for late transcription *in vitro* (34). Whether these factors are acted on by T antigen remains to be seen. One cellular factor that has been shown to interact with SV40 T antigen is activator protein 2 (AP-2), which has a binding site within the SV40 enhancer near the GT-motif region (35). This protein, too, might therefore be involved in

transactivation. Finally, both early region 1A protein (E1A) and T antigen have been recently shown to interact with the retinoblastoma gene product (36, 37). This anti-oncogene protein may have a suppressive activity on SV40 gene expression and might be inactivated when T antigen binds to it.

The involvement of cytoplasmic events in transactivation is consistent with reports that activation by E1A does not require protein synthesis and may involve modification of a pre-existing cellular protein (38). Two proteins, a TATA box factor (39) and transcription factor IIIC (40), show higher activity in the presence of E1A. Another protein, responsible for the activation of the adenovirus *E2* promoter, also responds to E1A (41). Whether any of these adenovirus results relate directly to the activation by SV40 T antigen is not yet known. It is worth noting that domain 2 of the E1A protein, which is important for transformation, can be functionally replaced (i.e., the transforming function) by a fragment of the T antigen gene (amino acids 101–118) that shows similarity in amino acid sequence (42). There is also evidence that the adenovirus *E2* promoter can be activated by T antigen (43). These data suggest that some properties of these proteins may overlap. Any comparison of E1A and T antigen must, however, also take into account the observation that the activity of the SV40 enhancer is reportedly repressed by E1A (44, 45). Whatever the mechanism of T antigen transactivation, the cellular component(s) involved may not be present, or at least not responsive to T, in EC cells (Fig. 5). Replication-independent transactivation of early transcription also was not detected in human 293 cells, which express adenovirus E1A and E1B proteins (16). Perhaps the E1A protein in 293 cells, or an E1A-like activity reported to be present in EC cells (e.g., ref. 46), has modified the transcription machinery of the cells so that T antigen effects are not detected.

The vector used provided evidence that replication may be needed for the early-to-late gene shift and may play a role (in CV-1 cells) in autoregulation. Repression has also been reported to occur because of replication in certain lines of human 293 cells (17, 18). These cells permit a greater early-to-late shift than HeLa cells and support higher levels of SV40 replication (16). Thus, 293 cells may in this regard more closely reflect the behavior of CV-1 cells. The amount of autoregulation seen in HeLa cells (only after correcting for template copy number) could result primarily from T antigen binding, as in the EC cells. It might be pointed out that the lack of autoregulation or ori$^-$ templates suggests that the binding of the reported enhancer factor AP-2 by T antigen, which was proposed from *in vitro* transcription studies to contribute to autoregulation (35), may not be a significant event for autoregulation *in vivo*, at least with the cell types and experimental conditions used here.

Note Added in Proof. It has recently been reported that SV40 small tumor antigen (t) is a potent activator of the adenovirus E2 promoter, but not the late promoter of SV40 (47). All of the T antigen plasmids in the present study, including the pBM11 mutant, produce t, and a role for this protein in transactivation of expression from pBEL2 cannot yet be excluded.

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