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Comeasurement of Simian Virus 40 Early and Late Promoter Activity in HeLa and 293 Cells in the Presence of T Antigen

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Transcription of the simian virus 40 (SV40) late promoter is strongly activated by SV40 T antigen. We were interested in examining this process in relation to other T-antigen functions such as replication and repression of early transcription. To quantitate the various T-antigen effects, we used a vector which has a promoterless β-globin gene flanking the early and late sides of the SV40 promoter region. Following cotransfection with a plasmid encoding T antigen, transcription from the two promoters can be measured with a single S1 mapping probe and replication can be assayed by Southern blot analysis of DNA recovered in Hirt extracts. In this study, transactivation was examined in HeLa and 293 cells, since these cells differ in their ability to support SV40 replication. The strength of the late promoter relative to the early promoter was approximately three- to fourfold higher in 293 cells. Replication in 293 cells was also more efficient, by the same margin. In both cell lines, late promoter transactivation was barely detectable on replication-defective templates. Taken together, the results suggest that T-antigen activation of late transcription occurs only on replicated, or replicating, DNA. T antigen also activated the late-early start sites, and while in HeLa cells they were seen to be only 30% as strong as the late promoter, in 293 cells late and late-early activities were almost equal.

The DNA tumor virus simian virus 40 (SV40) has a single transcription control region contained within approximately 350 base pairs (bp). Early in infection, transcription initiates predominantly in one direction, from the early-early start sites (EES), giving rise to a transcript which encodes the large T and small t antigens (37). The appearance of T antigen in the cell causes at least four events to occur on the viral DNA. First, there is an activation of the late starts (LS), which proceed in the opposite direction from the early transcription unit (5, 24). Second, the viral origin of DNA replication lies within the promoter region on the early side and becomes active when T antigen binds to specific sites which surround it (10, 11, 35). Third, repression of early transcription also occurs when T antigen binds to the template, presumably because the attachment of RNA polymerase becomes sterically hindered (21, 26, 31, 32, 36). Finally, a new set of start sites, the late-early starts (LES), are used to maintain the production of some early transcript (14). These late-early cap sites are located 5′ of those used early in infection. It has been suggested that there is a competition between the EES and LES for the transcription machinery and that the LES can be more active when the EES are blocked by T antigen (30, 39).

Recently, there have been a number of attempts to examine the relationship and interdependence of these various T-antigen effects, not only to better understand the viral life cycle but also in the hope of elucidating the way in which T antigen can alter cellular gene expression during transformation. One question in particular has concerned the influence that replication has on early and late transcription. Using human 293 cells in which the SV40 origin of replication functions efficiently, some workers have found that early transcription is repressed and that the prevention of replication by either mutations or chemical inhibitors relieves the repression (27, 28). With regard to late gene expression, a number of studies with similar mutants and chemical agents in permissive monkey cells have suggested that replication is not required for T-antigen-mediated transactivation of the late promoter (5, 6, 24, 25). There is, however, some discrepancy between laboratories as to the importance of the T-antigen-binding sites at the replication origin for this effect, with some data suggesting an almost total requirement for them (6, 7) and other results indicating that only about 30% of the late promoter activity requires an intact origin (25).

Most of the studies on DNA sequences which control SV40 early or late transcription have been done with vectors that permit the assessment of one or the other. For example, late promoter function is often assessed in COS cells, which constitutively express T antigen, and the early promoter has been extensively characterized in HeLa cells. We have been interested in developing a vector system which will permit us to examine simultaneously all the effects of T antigen on SV40 transcription and replication and to quantitate at the transcription level the relative strengths of the early and late promoters. This vector has been used to look at late promoter transactivation in HeLa and 293 cells, to determine whether concomitant with the repression of early transcription reported to occur in 293 cells (27, 28) there is an enhancement of late transcription. Also, since it had previously been shown that SV40 replicates more efficiently in 293 cells than in HeLa cells (27, 28, 34), we wanted to use these cell lines to examine the role of replication in T-antigen transactivation without having to use mutants or inhibitors. Surprisingly, we found that in both cell lines early expression is largely unaffected by an *ori* mutation, either with or without T antigen, whereas both late and late-early activities are markedly reduced in the mutant. By our best estimates, both replication and T-antigen transactivation of the late promoter on wild-type templates are severalfold more efficient in 293 cells, and taken together the results suggest that in these cell lines, T-antigen-mediated late gene expression occurs primarily on newly replicated DNA.

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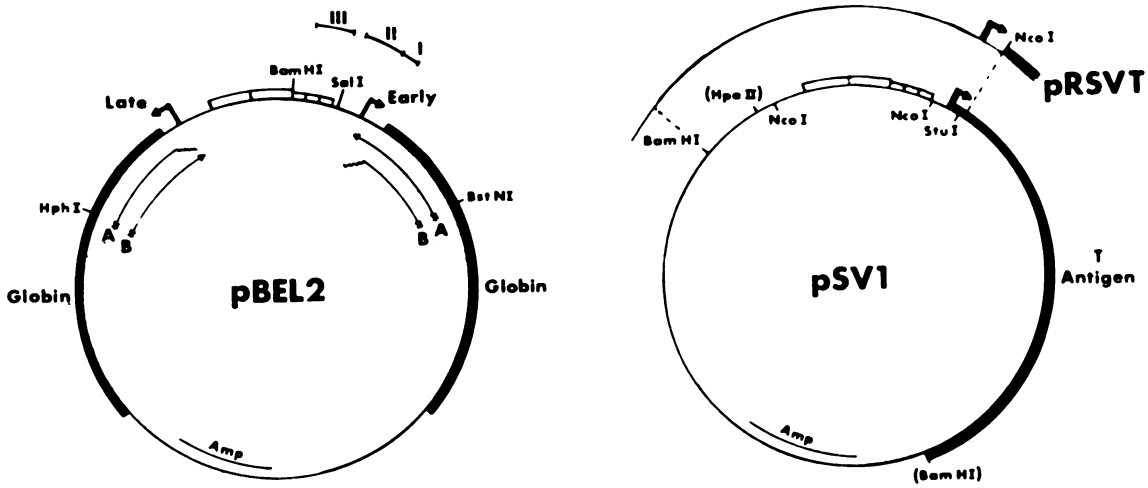

FIG. 1. Plasmid vectors used to study T-antigen transactivation of the SV40 promoter region. The plasmid pBEL2 was constructed as described in Materials and Methods. Probes A or B can be used to measure transcription by S1 nuclease mapping; the breakpoint of homology with each probe is shown by the bend in the arrow. Probe A measures early cap sites, and probe B measures late cap sites (see text). Only the major early and late cap sites are shown to simplify the diagram. A more detailed drawing of the SV40 promoter is shown in Fig. 2. T antigen is supplied from pSV1 or PRSVT (see Materials and Methods for their construction). T-antigen-binding sites I, II, and III are also shown on pBEL2.

MATERIALS AND METHODS

Cell lines. HeLa cells were maintained in Dulbecco minimal essential medium supplemented with 10% fetal calf serum. Human 293 cells, which are transformed by adenovirus type 5 DNA and constitutively express the E1a and E1b genes of the virus (1, 18), were grown in the same medium and periodically verified by confirming their ability to support the replication of an SV40 T-antigen-producing adenovirus vector, AdSVR112 (gift from Y. Gluzman), that is defective for its own early gene products.

Construction of plasmids. The expression vector (Fig. 1) used in this study, pBEL2, contains the SV40 sequences from *Hpa*II (position 346) to *Hin*dIII (position 5171) flanked on each side by a promoterless rabbit β-globin gene (*Pvu*II fragment extending from −9 to +1650, relative to the mRNA initiation site [38]). It was constructed by placing a *Hin*dIII linker at the *Eco*RI site of pAW2 (formerly the *Hpa*II site referred to above; see references 40 and 41 for a description of pAW2) and replacing the fragment extending from this new *Hin*dIII site to *Pst*I (positions 3609 to 4363 of pBR322) with the *Hin*dIII-to-*Pst*I fragment from pSEG0 (3) carrying another promoterless globin gene. The *Bam*HI sites in both β-globin genes had previously been destroyed by digestion and repair of the sticky ends with Klenow polymerase. The resulting plasmid, therefore, had a unique *Bam*HI site at the junction of the 72-bp enhancer sequence and the 21-bp repeat upstream sequence element. This site had previously been introduced into pA0, the parental plasmid for pAW2, by site-directed mutagenesis (41). A unique *Sal*I site, also previously engineered between the 21-bp repeats and the TATA box (3), was transferred into pBEL2 by standard recombinant DNA techniques. pBEL2ori was constructed by replacing the wild-type origin in pBEL2 with one containing a 6-bp deletion at the *Bgl*I site, at position 5235, which blocks replication (16), again by standard procedures.

Two plasmids which encode T antigen were used in this study (Fig. 1). One, pSV1, has been described previously (4) and consists of the entire SV40 early region in pBR322. The second, pRSVT, was constructed by digesting pSV1 with *Stu*I, treating with BAL 31 for a limited time, digesting with *Nco*I (which cuts at positions 37 and 331 in SV40), and reclosing the vector with DNA ligase. Colonies which had a restored *Nco*I site were selected. Several were sequenced, and one was found where the BAL 31 had digested to within 9 bp of the ATG codon of T antigen. An *Nco*I linker was then placed at the *Hin*dIII site of RSV-CAT (17), followed by a *Bgl*II linker at the *Nde*I site so that a *Bgl*II-to-*Nco*I fragment containing the Rous sarcoma virus long terminal repeat promoter could be excised. This fragment was then inserted by using *Bam*HI and *Nco*I into the pSV1 derivative with the new *Nco*I site. The ability of this new plasmid to express T antigen was confirmed by Western blot (immunoblot) analysis of proteins extracted from transfected cells. Another plasmid, pRSVT$^-$, was also constructed in the same way by using a clone where BAL 31 had removed the first eleven and one-third codons from the T-antigen-coding sequence. This plasmid does not produce T antigen, but is essentially identical to pRSVT.

DNA transfection, nuclease S1 mapping, and Western blot analysis. Transfections were done by the calcium phosphate procedure (19). Cells at 50 to 60% confluency in 100-mm dishes were fed with fresh medium 4 h before transfection. The medium was again changed 6 h (293 cells) or 18 h (HeLa cells) after transfection. Cells were harvested after 48 h, and cytoplasmic RNA was extracted by lysis with Nonidet P-40. Nuclease S1 mapping was done with 300 units of S1 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 4 h at 25°C as previously described (3). The probes were prepared by 5′ end labeling and strand separation of appropriate DNA fragments as described in the text. Following electrophoresis on 8% polyacrylamide gels containing 8.3 M urea, the RNA levels for EES, LES, and LS were measured by densitometry of the autoradiograms. Late promoter transactivation was quantitated as an increase in the ratio of late to early transcription in the presence of T antigen. In some experiments, a second plasmid which produces a globin transcript from a polyomavirus enhancer-driven globin promoter was cotransfected with the test template. This transcript serves as a control against which the EES, LES, or LS can be

standardized. The method of quantitation is discussed further in the Results and figure legends.

T-antigen levels in cells 48 h after transfection were determined by Western blot analysis as previously described (33). Monoclonal anti-T antigen antibody pAB419 (22) was used, with an alkaline phosphatase-conjugated anti-mouse immunoglobulin G antibody (Promega Biotec, Madison, Wis.) used for detection.

DNA replication measurements. Low-molecular-weight DNA was isolated from cells 48 h after transfection by the Hirt extraction procedure (23). Samples of DNA (20% of the DNA from a 100-mm dish) were digested with *Mbo*I (which cuts newly replicated unmethylated DNA) or *Sau*3A (which is insensitive to DNA methylation) and electrophoresed through 0.8% agarose gels. After blotting to nitrocellulose, the membranes were probed with a nick-translated β-globin gene fragment (*Hin*dIII to *Eco*RI from pBEL2). The ratio of newly replicated DNA to transfected input DNA was determined by scanning densitometry, taking the ratio of *Mbo*I-generated fragments to *Sau*3A-generated fragments.

RESULTS

Description of experimental system. The vector pBEL2 (Fig. 1) was constructed so that a single S1 mapping probe could be used to measure early and late transcription, permitting a quantitation of their relative strengths. The probe used can be made homologous to either the early or late transcript; the early probe is end labeled at a *Bst*NI site at position +138 in the β-globin-coding sequence, and the late probe is labeled at an *Hph*I site at position +108 (indicated as probes A and B in Fig. 1 for the early and late sides, respectively). When probe A is used, late promoter activity appears as a breakpoint band where the late globin gene is fused to the SV40 late promoter and thus is a summation of the activities from all the late cap sites. Longer protected fragments reveal the early cap sites. Conversely, if probe B is used, all early activity is summarized at a breakpoint band where the early globin gene meets the early promoter, and individual late promoter cap sites become visible. An example of how these two probes can be used is shown with 293 cells in Fig. 2. When pBEL2 is transfected alone and RNA is analyzed by using the early probe (probe A), the multiple EES at positions 5230 to 5237 are readily seen. If pBEL2 is cotransfected with a plasmid encoding T antigen, such as pRSVT, transactivation of the late promoter results in a large increase in the intensity of the band at the position corresponding to the point where homology between the probe and the late transcript is broken. When the late probe (probe B) is used to analyze portions of these same RNA samples, the major band in the absence of T antigen (−T) represents the breakpoint where the early SV40 leader sequence begins. When pRSVT is present in the transfection, LS appear at a number of positions previously noted (15) to be used in viral infection. In particular, strong sites are observed at nucleotides 325 and 264, with lesser activity seen around positions 315, 294, 282, and 192. These results indicate that the pBEL2 recombinant is accurately indicating the transactivation of the late promoter by T antigen. Except for slightly reduced efficiency at positions 264 and 192, HeLa cells exhibit essentially the same pattern of late promoter cap site utilization as 293 cells (Fig. 2).

Concomitant with the activation of late promoter activity by T antigen, there is an increase in utilization of the LES. Their appearance can be readily detected with probe A (Fig. 2), and with probe B they result in an increase in the intensity of the band at the breakpoint. For the majority of experiments, probe A was used because it enables an examination of late activity, late-early activity, and early-early activity simultaneously.

The advantages of using pBEL2 as an expression vector are several. First, a quantitation of the activity of each promoter relative to one another is facilitated by the fact that only one probe is used. There is no error introduced by having to use different probes with potentially variable specific activities for each promoter. Also, the β-globin transcripts produced from the early and late sides of the SV40 promoter should have identical stabilities in the cell, except for any differences in the stabilities of the segments of SV40 leader sequences in each transcript. Third, there is an assurance that the amount of early and late template in the cell is identical, regardless of how much DNA is taken up by the cells or whether replication occurs.

Quantitative comparison of transactivation efficiency in HeLa and 293 cells. Because of the reported inhibition of SV40 early promoter activity in 293 cells as a result of DNA replication (27, 28), it was of interest to first compare quantitatively the effect of T antigen on pBEL2 expression in these cell lines. The method used to assess this was to cotransfect pBEL2 with pSV1, since both vectors use the same promoter and thus in any one cell type should be expressed with more or less equal efficiencies. Calcium phosphate precipitates of the DNAs to be transfected were prepared, and equal amounts of the precipitate were applied to HeLa and 293 cells. In these transfections, a third plasmid, pβ(244$^+$)β (13), was included as a control. This plasmid uses the polyomavirus enhancer and produces a globin transcript from its natural promoter. With the S1 probe it generates a unique band 9 nucleotides shorter than the breakpoint because in pBEL2 the globin genes are fused with a *Hin*dIII linker at a *Pvu*II site 9 bp upstream of the natural globin cap site.

Before discussing the results, it should be pointed out that the design of the pBEL2 vector permits several types of measurement. Early and late promoter activities each can be expressed relative to the activity of a different promoter transfected into the cells on a separate nonreplicating plasmid [e.g., pβ(244$^+$)β] or expressed relative to one another. The latter method gives a novel measurement, i.e., a direct quantitation of how the total transcription activity of a population of molecules is partitioned among the EES, LES, and LS promoters. When comparing different cell lines, it is not valid to base all the quantitations on a different control promoter because that promoter, too, may be expressed with different efficiency in each cell type. For example, we found that the rabbit globin promoter coupled to the polyomavirus enhancer in pβ(244$^+$)β is less active in 293 cells than in HeLa cells. What the external control promoter does is permit, within any one cell, a correction for the amount of RNA recovered and a comparison of the activity of any one promoter within a set of experimental samples. In light of these considerations, we therefore chose to express T-antigen transactivation as an increase in the ratio of late to early gene expression. Such a measure should be an accurate assessment of the phenomenon, since with a single probe we are analyzing a single population of test molecules. While it might be argued that a different approach should be taken, an attempt is being made to quantitate precisely, in a novel way, the effects of T antigen on SV40 gene expression.

Figure 3 shows the results of one such analysis. The experiment shown was done with different amounts of pBEL2 and a fixed amount of pSV1. In either the presence

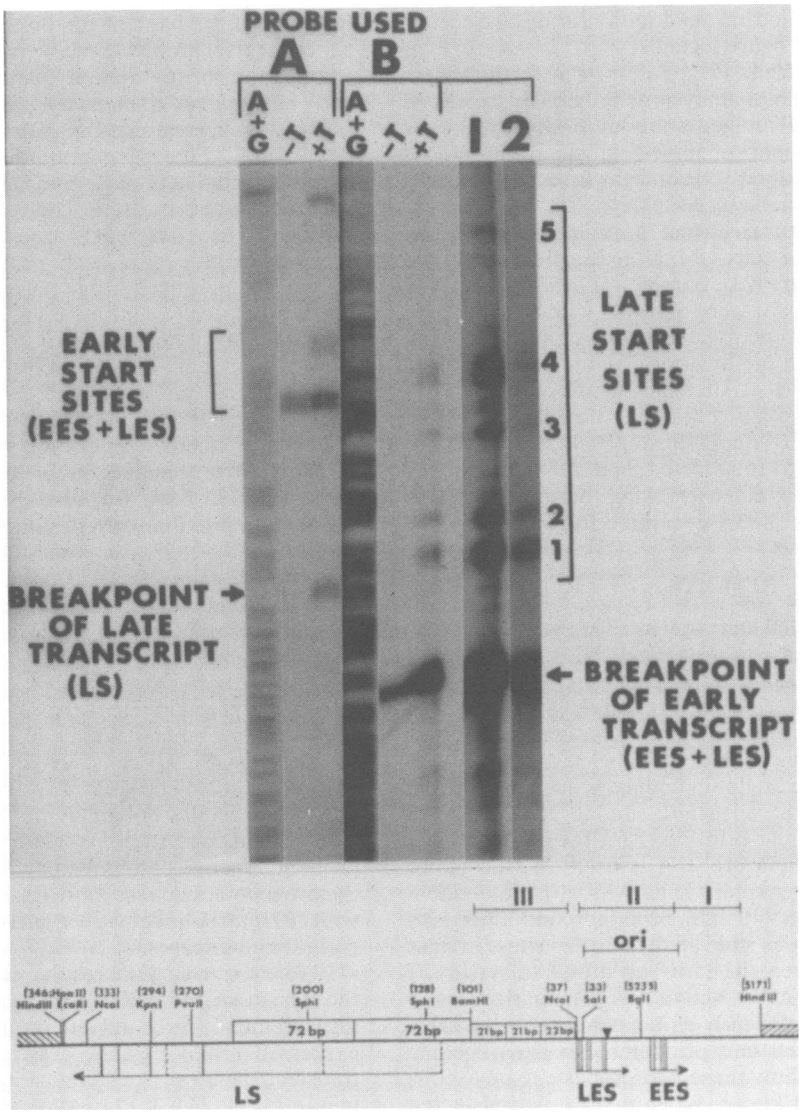

FIG. 2. Measurement of SV40 early and late promoter activities with pBEL2. RNA was extracted from cells transfected with pBEL2 alone (−T) or cotransfected with pBEL2 and pRSVT (+T). The three lanes shown for probe A and probe B are RNA samples from 293 cells, along with an A+G sequence ladder of the appropriate probe. To the right in lane 1 is a longer exposure to the +T sample with probe B, and lane 2 is an RNA sample from HeLa cells cotransfected with pBEL2 and pRSVT and analyzed with probe B. Lanes 1 and 2 are shown to indicate that late cap site utilization is similar in HeLa and 293 cells. Late start sites 1, 2, 3, 4, and 5 are at nucleotide positions 325, 315, 294, 264, and 192, respectively. Below the autoradiograph is a diagram of the SV40 promoter region in pBEL2 with EES, LES, and LS indicated. The origin of replication (ori) and T-antigen-binding sites I, II, and III are indicated. The hatched regions at the borders indicate the globin gene sequences flanking the early and late sides of the promoter.

or absence of T antigen, the SV40 early promoter was expressed in both HeLa and 293 cells (Fig. 2 and 3A). The addition of T antigen to the cells caused a strong activation of both late-early and late transcription. These data were quantitated by scanning the autoradiographs and yielded the results shown in Fig. 3B. Even over a 10-fold range of pBEL2 concentrations, the strength of the late and late-early activity relative to early-early transcription varied by less than a factor of two, with transactivation being slightly less efficient as more pBEL2 was transfected into the cells. Regardless of how much pBEL2 was used, one result was consistent—in 293 cells transactivation of the late promoter was approximately three times more efficient than in HeLa cells, while activation of the LES was about eight times more efficient. Also, over the range of DNA concentrations used here, in 293 cells the late-early activity is about 90% as strong as the late activity, whereas in HeLa cells it is only 30 to 40% as strong.

While in these experiments it was essential to use pSV1 to supply T antigen so that different cell lines could be compared, it was necessary to ensure that the transactivation being observed was due entirely to T antigen, and not because the pSV1 recombinant was competing with pBEL2 for any factors limiting for early expression. This is especially important when quantitating the late or late-early activity relative to early-early transcription. Also, since pSV1 is capable of replication and subject to early gene repression, there would be no certainty that the amount of T antigen in the cell would be directly proportional to the amount of DNA transfected. For this reason, pRSVT was

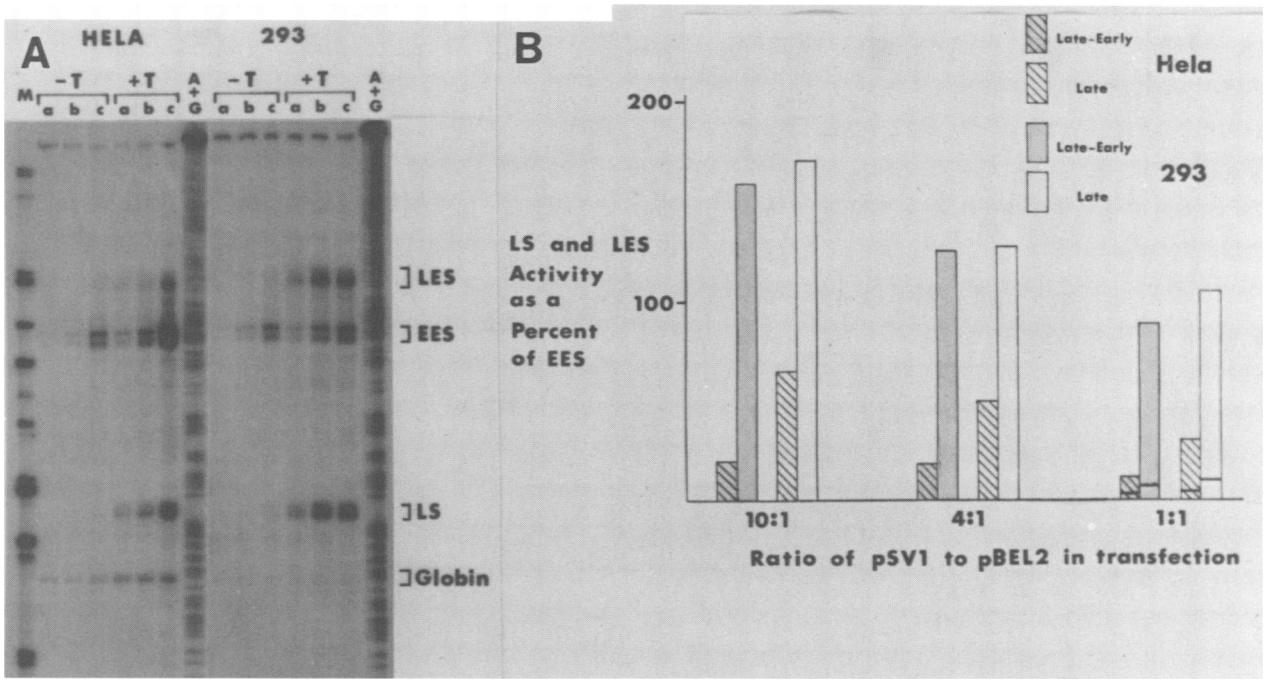

FIG. 3. Transactivation of late and late-early promoter activity by T antigen in HeLa and 293 cells. (A) S1 mapping analysis with probe A of RNA extracted from cells transfected with only pBEL2 (−T) or with pBEL2 and pSV1 (+T). A 4-μg sample of pSV1 was used in each sample with T antigen, and 0.4 (lane a), 1 (lane b), or 4 (lane c) μg of pBEL2 was used in the transfections. A 20-μg portion of pβ(244$^+$)β was included in all transfections as a control, and pBR322 was used to equalize the total amount of DNA in each calcium phosphate precipitate. (B) Results of the experiment after quantitation of the LS, EES, and LES activities in the +T samples by scanning densitometry. The ratios of 10:1, 4:1, and 1:1 correspond to samples a, b, and c in panel A. In the sample with a 1:1 ratio, the results of scanning the c lanes in samples without T antigen (−T) are indicated as bars at much lower positions and are included to demonstrate the magnitude of transactivation over the basal levels of late and late-early activity.

constructed and tested for its ability to express T antigen in HeLa and 293 cells. Western blot analysis with anti-T antigen antibody of cells transfected with pRSVT or pSV1 revealed similar levels of expression of T antigen from each plasmid and indicated that the amount of T antigen was approximately proportional to the amount of pRSVT in the transfection (data not shown). Cotransfection of pBEL2 with increasing amounts of pRSVT resulted in transactivation similar to that seen with pSV1 (Fig. 4A). With either pSV1 or pRSVT, late transcription relative to early-early activity could be pushed further by transfecting more of the T antigen-producing plasmid into the cells. For example, in HeLa cells, with 20 μg of pSV1 or pRSVT (as compared with 4 μg of pSV1 in Fig. 3), late transcription could be made to equal or even exceed somewhat early-early activity (Fig. 4B). The amount of pBEL2 used in Fig. 4A (5 μg) was comparable to the highest amount used in Fig. 3A (4 μg, lane c). As expected, the transactivation observed with a 1:1 ratio of pRSVT to pBEL2 (Fig. 4A) was similar to that seen with the 1:1 ratio of pSV1 to pBEL2 (in each case, LS is 20 to 25% of EES). Taken together, these data demonstrate that the amount of pBEL2 template DNA is not as large a factor as the amount of T antigen in the cell. The plasmid pRSVT$^-$, which encodes a defective T-antigen mRNA, had no effect on pBEL2 expression (Fig. 4B), showing that under our transfection conditions the Rous sarcoma virus promoter does not compete for transcription from the SV40 early promoter. Thus, all the T-antigen effects observed were occurring because of the action of the protein in the cell and not because of competition among transfected DNAs.

In summary, the results of a number of experiments indicate that in our 293 cell line, it is possible to achieve levels of late transcription that are two to three times the level of EES. In the HeLa cells used here, the maximum we observed was a 1.4-fold increase. The data shown in Fig. 3 are typical of the levels of transactivation attainable. These numbers are based on the experimental system used here and serve to highlight the differences between cell lines. While late gene activation by T antigen was readily measurable on the pBEL2 plasmid, it was probably not as efficient as during viral infection.

Effect of replication on transcription of pBEL2. As stated above, one of the reasons for examining HeLa and 293 cells is that SV40 replication has been reported to be more efficient in the latter and may in fact cause a repression of early transcription. In our experiments, we did not detect in either cell line a reduction in EES in response to T antigen. We therefore inserted an *ori* mutation into pBEL2, with the prediction that T antigen would again have no effect on early expression. While the *ori* mutation almost totally abolished late and late-early activation by T antigen, it had no effect on EES, except to shift them 6 bp downstream, so that the distance from the 21-bp repeat region and the cap sites remained the same as in the wild type (Fig. 5). In Fig. 5, the probe for the *ori* mutant RNA was made from pBEL2ori to ensure that LES activity could be measured. In HeLa cells, the *ori* mutation supressed transactivation just as in 293 cells. The results of a number of experiments in both cell lines indicated that even with 20 μg of pSV1 or pRSVT in the transfection, late or late-early promoter activity on replication-defective templates did not exceed 3 to 5% of early activity. In the absence of T antigen, both wild-type and *ori*

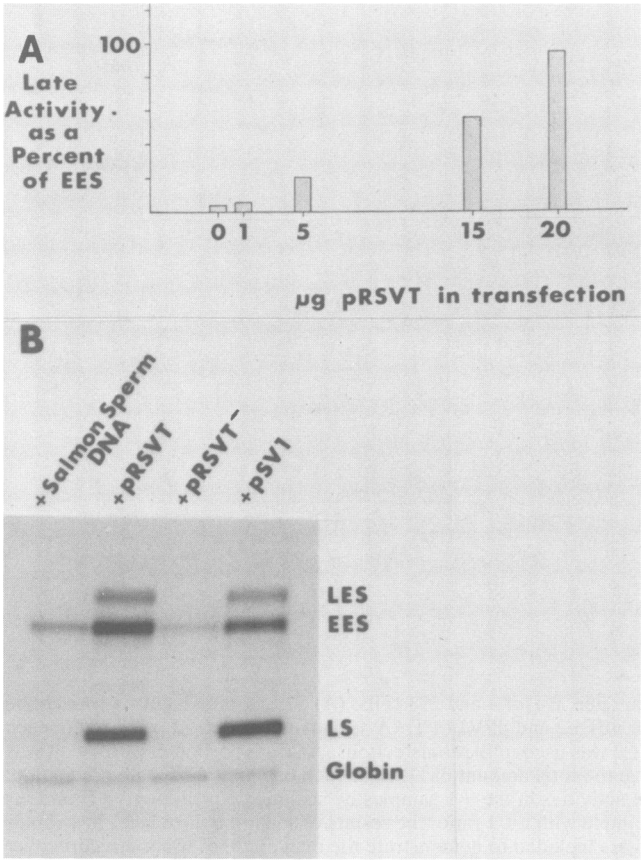

FIG. 4. Comparison of transactivation by T antigen produced from pSV1 or pRSVT. (A) Quantitation of transactivation by pRSVT of the late promoter in HeLa cells, demonstrating increasing amounts of transactivation with increasing amounts of T antigen. A 5-μg portion of pBEL2 template was used per dish. (B) Transactivation in HeLa cells obtained with 20 μg of pRSVT, pRSVT⁻, pSV1, or nonspecific salmon sperm DNA. A 10-μg sample of pBEL2 was used per dish, and the pβ(244⁺)β plasmid was included in this transfection to give the globin control band. Probe A was used for S1 mapping.

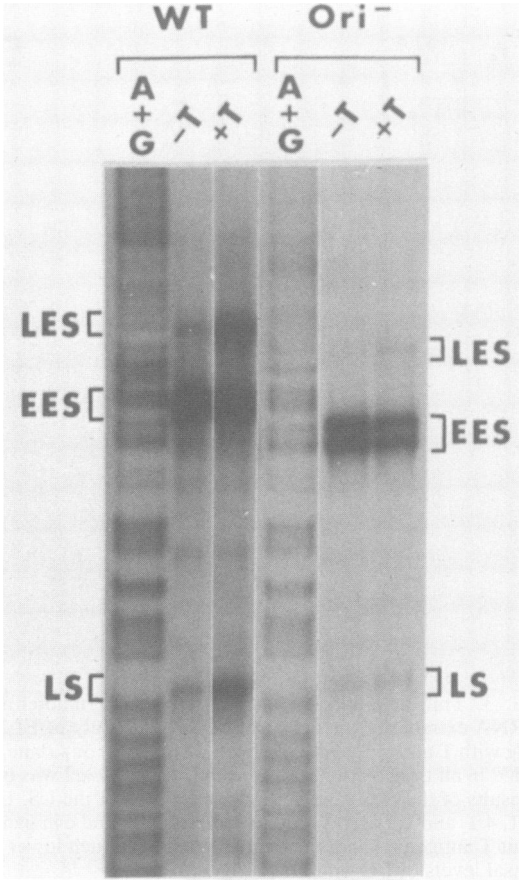

FIG. 5. Comparison of wild-type (WT) and *ori* mutant templates for T-antigen transactivation. 293 cells were transfected with 5 μg of pBEL2 (WT) or pBEL2ori (Ori⁻) and 20 μg of pRSVT (+T) or pBR322 (−T) and analyzed with probe A prepared from pBEL2 in the WT samples or pBEL2ori in the Ori⁻ samples.

templates exhibited late activities that were 2 to 3% of EES. Thus, the shift from early to late expression is almost totally prevented by the *ori* mutation. The late-early transcripts were similarly reduced in the mutant (Fig. 5).

Although it had already been observed that 293 cells efficiently replicate SV40 DNA, the fact that we observed some transactivation on wild-type templates in HeLa cells led us to quantitate pBEL2 replication in our cell lines. Cells were cotransfected with pRSVT and pBEL2 or pBEL2ori, and after 48 h they were harvested. From each plate, half of the cells were used for RNA extractions and S1 mapping, while the other half of the cells were used for Hirt extractions to determine the relative proportions of transfected and newly replicated template DNA. Aliquots of the Hirt extracts were digested with *Mbo*I or *Sau*3A. *Mbo*I will cut only unmethylated DNA and not the transfected DNA that had been grown in HB101 and methylated by the *dam* methylase. *Sau*3A, on the other hand, has the same recognition sequence as *Mbo*I, but is insensitive to methylation. Therefore, when these two digests are run in parallel, blotted to nitrocellulose, and probed with a nick-translated fragment of the globin gene, the ratio of replicated to total DNA can be calculated by densitometric analysis. Figure 6A shows one such set of hybridizations and the S1 mapping analysis with the early probe (probe A) of the RNA from those same cells. Quantitation of the results by densitometry revealed that in HeLa cells, 30% of the template DNA retained in the cell after 48 h is newly replicated (i.e., unmethylated) and 70% is original transfected DNA, or in other words, the amount of new DNA was approximately 40% of the original transfected DNA. In 293 cells, 60% of the retained DNA was newly replicated and 40% was input, meaning that the amount of replicated DNA is about 150% of the transfected DNA. A comparison of these 40 and 150% figures yields the final result that in 293 cells the ratio of replicated to nonreplicated DNA is about 3.7 times greater than in HeLa cells. Even allowing for experimental error, this value is surprisingly consistent with the results shown in Fig. 3 (and confirmed for this particular experiment in Fig. 6B), which suggest that transactivation relative to early-early transcription is approximately three times more efficient in 293 cells. It would seem possible, therefore, that T-antigen-transactivated late activity arises only on replicated DNA.

Effect of T antigen on plasmid stability. The effect of T antigen on early expression from pBEL2 was somewhat surprising in that no repression was observed, and in fact more early mRNA was often detected in the presence of T antigen. This enhancement was most evident in HeLa cells,

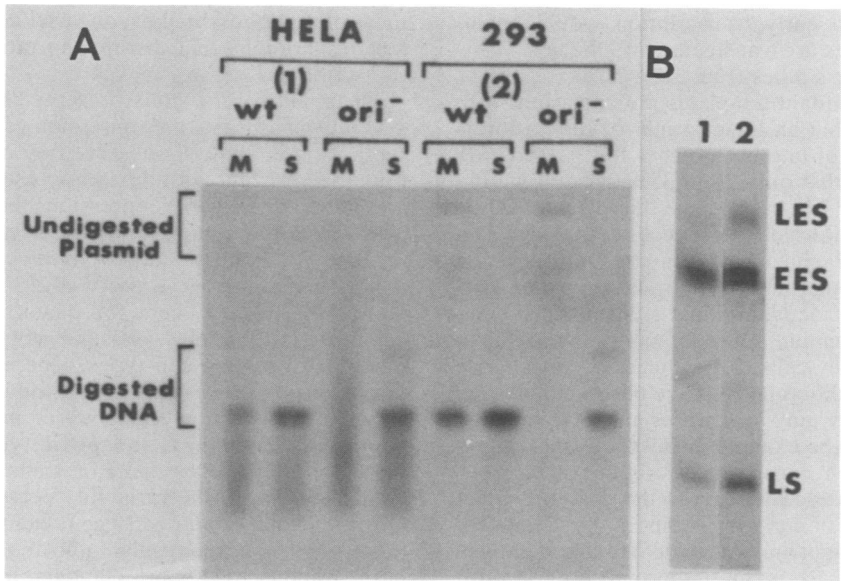

FIG. 6. pBEL2 replication efficiencies in HeLa and 293 cells. Hirt extracts were prepared from half of the cells 48 h after transfection with 10 μg of pRSVT and 5 μg of either pBEL2 (wt) or pBEL2ori (ori⁻). DNA samples were digested with either *Mbo*I (M) or *Sau*3A (S) and analyzed by Southern blotting as described in Materials and Methods (A). Panel B shows the S1 mapping analysis with probe A of RNA extracted from the other half of the transfected cells. Both lanes 1 and 2 (which correspond to the bracketed numbers beside the wild-type samples in panel A) are analyses of pBEL2 samples, with lane 1 being the HeLa cell RNA and lane 2 being from 293 cells.

in which we consistently observed a two- to fivefold increase in the level of EES in response to T antigen (e.g., see Fig. 4). In 293 cells this increase was not noted (Fig. 5). To examine the phenomenon further, we assessed the effect of T antigen on input plasmid stability using *Dpn*I digestion (which cuts only methylated DNA, such as that grown in HB101) of Hirt extracts (Fig. 7). Particularly in HeLa cells, an increase in stability was noted. This increase in retention of transfected templates was also seen with *ori* DNA and may provide an explanation to account for the increased early expression observed. It can, of course, also partly explain the increased late promoter activity, which is why we believed it to be important to use a vector that would enable us to generate from a population of homogeneous molecules early and late transcripts with identical stabilities and measure their abundance relative to one another as an estimate of the early to late switch in SV40 gene expression.

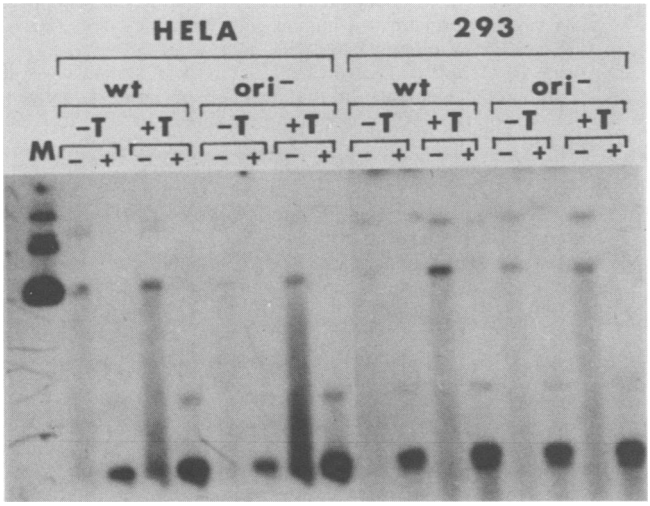

FIG. 7. Stabilization of transfected pBEL2 and pBEL2ori DNA by T antigen. Cells were transfected as described in the legend to Fig. 6 with pBEL2 (wt) or pBEL2ori (ori⁻) with either 10 μg of pBR322 (−T) or 10 μg of pRSVT (+T). DNA samples were analyzed either before restriction enzyme digestion (−) or after digestion with *Dpn*I (+). The fragments of the pBEL plasmid which appear after digestion originate from methylated input DNA and run in the same position as the digested fragments in Fig. 6. The marker (M) is purified undigested pBEL2 plasmid.

DISCUSSION

The experiments described here employed an expression vector which permits simultaneously a quantitation and comparison of SV40 early and late promoter activity to study the effect of T antigen on SV40 transcription. In particular, it was of interest to compare late promoter transactivation in HeLa cells and 293 cells, since they differ in their ability to support SV40 replication, which like late promoter activation is a process requiring T antigen. The results demonstrate that cell lines can differ in their ability to permit T-antigen transactivation of the late promoter and that total transcription activity of the entire SV40 control region (early and late) shifts toward late transcription in proportion to the percentage of replicated templates in the cell. This, coupled with the observation that replication-defective templates are poorly transactivated by T antigen, suggests that it is the replicated DNA alone that is producing the late transcripts which appear in response to the presence of T antigen in the cell. Why this DNA should be different from nonreplicating DNA is not clear. There is some evidence that a repressor of late transcription binds to the 21-bp region, and as template copy increases during replication, this molecule may be titrated out, thereby liberating plasmids for late transcription (2). If such a molecule exists, it is equally feasible that the replication process simply displaces it. A titration of a limiting amount of a repressor factor would predict that late

transcription relative to early transcription would be increased if more templates are transfected into the cells. This was not observed in our studies (Fig. 3).

Our results with *ori* mutant templates are consistent with previous observations that an intact origin of replication is required for 90 to 95% of late promoter activity (6). Other workers have reported that only 25 to 35% of late function requires the origin (25). The reason for this discrepancy is not clear, but observations on the effect of T antigen on plasmid stability (Fig. 7) must be taken into consideration when attempting any quantitation of transactivation. Furthermore, it should be remembered that studies which demonstrates a lesser requirement for an intact origin of replication were done in monkey cells (25). Thus, it is conceivable that in the same way as differences can be observed between HeLa and 293 cells, there may be features unique to monkey cells which will have to be examined. In the present study, however, the requirement for an intact origin is striking.

In the experiments described here, there is no direct evidence for repression of early transcription by T antigen. A detailed analysis of origin mutants indicated that T antigen binding to site I (Fig. 2) is an important criterion for autoregulation, but only in so much as it facilitates binding to site II and then site III (12, 29). Repression is largely absent when site II is deleted (30). Presumably, early transcription from DNA molecules involved in replication would be repressed since T antigen binding at site II is a prerequisite for replication (29). The *ori* mutant used in our studies contains a 6-bp deletion within site II (16), which although not preventing some T-antigen binding to this site, nevertheless diminishes from four to three the number of T-antigen tetramers that can bind (29). While it is difficult to determine whether the newly replicated templates which appear following transfection also contribute to early transcription, it is not likely that their early activity is very great. In 293 cells, the effect of T antigen on input plasmid stability is not as pronounced as in HeLa cells, and in many transfections, such as the one shown in Fig. 5, similar amounts of wild-type and *ori* mutant DNA were retained and similar levels of early-early transcription were observed from each in the presence or absence of T antigen. Studies are currently directed to significantly increasing the expression of T antigen in the cell lines in an attempt to obtain quantifiable repression on the pBEL vector. If more T antigen is present, a greater proportion of the template molecules in the cell should become bound by the protein. It may also be possible then to test whether the *ori* mutation still permits repression as T antigen becomes bound to it.

In addition to the difference in late promoter transactivation efficiency between the two cell lines used here, the LES are much more evident in 293 cells. Several studies have suggested that late and late-early activities are coordinately activated by the replication process (8, 9), but when measured relative to one another, their activities differed between cell lines (Fig. 3). It has been argued that the late-early and early-early promoters compete for the transcription machinery and that it is only after T-antigen binding has repressed the EES that the late-early promoter can become more active (30, 39). If this model is accepted, it might be hypothesized that T antigen binds the template more efficiently in 293 cells, perhaps aided in its binding by some components of the replication machinery. An alternative explanation is that the LS and LES are both activated by a bidirectional promoter element positioned between them and that its efficiency in each direction varies somewhat between cell lines. While it is not yet possible to distinguish between these or other possibilities, one useful endeavour might be to locate mutations that disrupt the ratio of late-early to late transcription.

The results of the study presented here were surprising in several ways. First, the repression of early transcription by replication in 293 cells observed by others (27, 28) was not found. Consistent with this, the presence of an *ori* mutation had no effect on early expression. Similarly, we found that late transcription in 293 and HeLa cells is suppressed by an *ori* mutation, whereas others have reported that replication is not needed for late activity in 293 cells, but is needed in HeLa cells (20). Except for quantitative differences indicated by our studies, we did not detect this dramatic difference between cell types. One explanation is that different lines of 293 cells may exhibit different properties, either in transfection efficiency or in some aspect of gene control. Alternatively, the pBEL vector, by providing a more thorough assessment of transcription activity, may eliminate some of the variability between cell lines owing to template stability and early gene activity.

In conclusion, the results indicate that cell lines can differ in their ability to support T-antigen transactivation and that the efficiency of transactivation is closely related to the amount of replicated DNA that appears in the cell. In addition, between these cell lines there are subtle differences in late-early promoter utilization. In future studies, it will be interesting to assess how promoter mutations affect the ability of T antigen to activate the shift from early to late gene expression.

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