Role of the SV40 enhancer in the early to late shift in viral transcription

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ABSTRACT

Simian virus 40 large tumor antigen is a multifunctional protein, with two of its roles being the promotion of viral DNA replication and replication-independent activation of viral transcription. Replication leads to a shift in transcription from the early-early to the late and late-early cap sites, through mechanisms poorly understood. The viral transcription enhancer contains sequences important for both early and late transcription, and we therefore have carried out experiments to evaluate its role in these events. We find that the ability of replication to lead to a shift diminishes when early-early transcription is made increasingly stronger by multimerizing the enhancer, and suggest that replication might lead to the shift by interfering with the ability of the enhancer to direct initiation to those sites. The natural situation in the virus of having two copies of this element might represent a compromise between maximizing both T antigen expression early in infection and late gene expression after replication begins. We also show that replication-independent transcription activation by T antigen is bidirectional and involves at least in part elements to which the factor TEF-1 binds.

INTRODUCTION

The DNA sequences controlling transcription of the early promoter of simian virus 40 (SV40) include a TATA box, an upstream sequence element containing binding sites for the factor Sp1, and an enhancer. The SV40 enhancer has been a prototype for studying how this class of regulatory sequences functions. Mutational analyses, combined with *in vivo* and *in vitro* transcription assays, have demonstrated that the enhancer consists of multiple enhansons, each of which appears to bind a specific regulatory protein (1–18). These enhansons can be distinguished functionally based on whether they generate enhancers either when individual enhansons are multimerized, when properly spaced tandem repeats of the enhanson are multimerized, or when tandem repeats of different enhansons are multimerized (4,8). Some of the elements within the enhancer that bind these different

proteins exhibit cell- and promoter-specificities. For example, the Sph enhansons, to which the factor TEF-1 binds, overlap the octamer motif that can bind the proteins OCT-1 and OCT-2. The octamer motif is active in lymphoid cells, while the Sph motif is active in non-lymphoid lines such as Hela cells (11).

Most analyses of the SV40 enhancer have used either the SV40 early promoter or a heterologous promoter to assess activity. In the virus the enhancer also contains sequences involved in late transcription, both in the presence and absence of the early gene product large tumor antigen (SVT; 19–24). SVT can affect initiation from the late start sites (LS) in two ways. First, in the absence of replication it can activate late viral transcription (22,24). Second, when replication occurs in response to the appearance of SVT in a permissive cell, there is a large increase in late transcription (22,25). Replication also causes early transcription initiation to be shifted upstream to a new set of start sites, termed the late-early start sites (LES; 26). Transcription from the original early start sites (the early-early start sites, EES) is repressed when SVT appears and replication begins. The mechanism whereby the shift in transcription occurs is not well understood, but there are several pieces of information germane to the problem. First, we have observed that the replication-independent transcription activation by SVT affects early transcription as well as late, and occurs even if an SVT that is restricted to the cytoplasm is used (27). Second, there are several suggestions in the literature to explain the autoregulation of EES seen on replicating templates. SVT is able to bind to sites in the origin of replication, that overlap the start sites for early-early transcription. *In vitro* it has been demonstrated that this binding can inhibit transcription, presumably by sterically interfering with RNA polymerase II (28,29). In addition, *in vivo* the replication process itself has been shown to autoregulate early transcription, by an unknown mechanism (30,31). Third, mutations in the TATA box, which diminish activity from the EES, result in an elevation of late-early transcription, leading to the hypothesis that there is a competition between the early-early and late-early promoters (32).

Since sequences within the enhancer are involved in both early and late transcription, we have carried out experiments to examine the role that this promoter element plays in transactivation by

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SVT and in the early to late shift. Activation of late and late-early transcription was found to diminish as early-early transcription was strengthened by multimerization of the 72 bp enhancer sequence. It is speculated that the mechanism whereby replication leads to the shift might be through interference with the ability of the enhancer to direct transcription to the early-early start sites, an ability that is lost when the enhancer is multimerized. Data are also presented which indicate that the replication-independent activation of SV40 transcription by either wild type or non-nuclear SVT is bidirectional and involves the TEF-1 binding sites in the enhancer.

MATERIALS AND METHODS

Plasmid vectors

Transcription from the SV40 promoter was measured using the vector pBEL. The construction of it and its ori⁻ derivative have been described previously (25,27). Derivatives carrying multimers of the 72 bp sequence or point mutations within the enhancer were constructed by transferring into pBEL the appropriate *Kpn* I to *Bam* HI fragment from a series of plasmids previously used in a systematic site-directed mutagenesis study of the SV40 enhancer (1). Both *Kpn* I and *Bam* HI are unique in pBEL or pBELori⁻, and flank the enhancer region. Standard cloning procedures were used for these steps (33).

The 4 bp insertion between the 21 and 72 bp sequences of pBEL1 and pBEL2 was made by digesting the plasmids with *Bam* HI, filling in the sticky ends with Klenow polymerase, and reclosing the plasmids by blunt-end ligation. The 12 bp insertion was made by filling in the *Bam* HI site, and inserting into the blunt ends an 8 bp *Xho* I linker (CCTCGAGG). The 21 bp sequence was inverted by digesting these 12 bp insertion plasmids with *Sal* I (which cuts between the 21 bp sequence and the TATA box) and *Xho* I, and religating the mixture. Transformants which had the 21 bp region re-inserted in the inverse orientation were selected.

Wild type SVT was provided by the plasmid pRSVT (25), which has the Rous sarcoma virus promoter driving expression of the early region of SV40. Non-nuclear SVT was provided by pBM11, which carries a mutation that converts Lys128 to isoleucine in the gene for large T antigen (34). The plasmid pRSVT⁻ (25) is identical to pRSVT, except for a frameshift mutation in the SVT gene. It does not produce T antigen and was used to standardize the amount of DNA used in each dish of cells during the transfection experiments. The plasmid pβ(244+)β encodes a globin transcript under the control of its own promoter, and as previously described (25,27) was used in the transfection experiments as a control against which transcription from the pBEL templates could be standardized.

Cells, transfections, and RNA analysis

Hela cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. Calcium phosphate transfections (35) were carried out in 10 cm dishes at 60–70% confluency. In every experiment, each dish received 10 μg of pBEL test plasmid, 15 μg of the plasmid encoding SVT (pRSVT or pBM11, or pRSVT⁻ in control dishes), and 15 μg of pβ(244+)β. After 16 hours the DNA precipitates were removed, fresh medium was added, and the cells left for an additional 32 hours. Cytoplasmic RNA extractions, nuclease S1 mapping, and polyacrylamide gel electrophoresis were carried out as previously described (1). The S1 mapping probe was 5'-end

labeled with polynucleotide kinase and γ-³²P ATP at a *Bst* NI site in the globin gene on the early side of pBEL (25). This probe detects the EES and LES directly, and the LS are seen as a break-point band on the autoradiogram corresponding in position to where the globin gene on the late side is fused to the late leader region. Transcription levels were determined by densitometric scanning of several exposures of the autoradiograms, and different samples within an experiment were compared by standardizing them to the pβ(244+)β globin control band.

RESULTS

Multimerization of the 72 bp repeat prevents an early to late shift

We began a study on the role of the enhancer in the shift from EES to LES and LS activity by asking how the shift was affected by multimerization of the 72 bp sequence. The virus naturally contains an enhancer with two copies of this sequence, although variants have been found that contain insertions, or deletions of either whole or part of one 72 bp segment, within the enhancer (1,41–44). An enhancer with a single 72 bp sequence retains approximately 30% of the wild type activity, and in the past has provided a simpler system for site-directed mutagenesis and functional dissection of protein-binding sites (e.g. 1,10).

A series of 72 bp multimers was inserted into pBEL, generating pBEL1, pBEL2, pBEL3, pBEL4, and pBEL7. They were transfected into Hela cells and the effect of SVT on early and late transcription measured. As shown in Figure 2, there was a progressive loss in the ability of the promoter to undergo an early to late shift as the 72 bp copy number increased. EES, but not LES and LS, activity was increased in the absence of SVT

Figure 1. SV40 promoter region and vector design. The plasmid pBEL indicated at the top has a rabbit β-globin coding sequence (stippled line) flanking the early and late side of the promoter region diagrammed in the lower part of the figure. The arrows within the plasmid indicate the S1 mapping probe used to measure LES, EES, and LS activity. The probe used in this study was complementary to the early SV40-globin fusion, and therefore total transcription activity from the LS was detected as a break-point band (bent arrow) formed where the probe enters the non-homologous late leader region. The positions of the LS, LES, and EES are shown. The origin of replication consists of a core region (solid line) and flanking auxiliary elements (dotted line), and contains three binding sites for TAg (I, II, and III).

by multimerization of the 72 bp sequence, with a peak activity being reached with 4 copies. Both the late and late-early promoters responded in a parallel manner to SVT and replication, and both diminished simultaneously as a result of the multimerization.

We showed previously that an early to late shift in HeLa cells requires replication (25,27). It was possible, therefore, that the multimerization of the 72 bp sequence was inhibiting replication of pBEL. To test this, Hirt extracts from transfected cells were analysed (36,25). A representative assay, with pBEL1 and pBEL4, is shown in Figure 3. All of the plasmids with enhancer multimers replicated with equal efficiency, as indicated by the appearance of Dpn I-resistant DNA. Dpn I cuts only DNA that is methylated by the *dam* methylase of *E. coli*. Virtually all of the DNA in the Hirt extracts could be digested with Mbo I, which cuts only unmethylated DNA that has been synthesized in mammalian cells. Although not included in the experiment shown here, we have previously confirmed, by the use of plasmids with a mutation in the origin, that the appearance of Mbo I-sensitive DNA is dependent upon DNA replication (25). Therefore, the loss of an early to late transcription shift on plasmids carrying multiple copies of the 72 bp sequence was not because replication was inhibited.

LES, but not LS, activation on replicating templates requires correct steric alignment of the 21 and 72 bp sequences

The parallel responses of LES and LS activity on replicating templates suggested that transcription from these sites might be controlled in an identical manner. It has been shown that transcription from the LES requires both the enhancer and the 21 bp repeat (1,32,37). However, the ability of the enhancer to activate early transcription is disrupted by insertion mutations that separate it from the 21 bp by 0.5, 1.5, 2.5, etc, helical turns, whereas insertions of full helical turns are tolerated (38). These data indicated that there is a requirement for proper steric alignment of transcription factors that bind to these sequences. In these studies both the EES and LES were affected similarly by these insertions.

Since the LS and LES were activated almost identically, we asked whether late promoter activity was also affected by insertions between the 21 and 72 bp sequences. The results, shown in Figure 4, demonstrate that they are not. Insertions of 4 bp (approximately a half helical turn) or 12 bp (approximately

a full helical turn) were generated in pBEL1 and pBEL2. In the absence of SVT, the effect of these insertions on EES and LES activity was similar to that reported earlier. That is, relative to the globin control band the 4 bp insertion diminished early transcription to approximately 25% of the corresponding wild type plasmid with no insertion, and the 12 bp insertion restored activity to 90% of wild type. The responses were similar for pBEL1 and pBEL2, and affected the EES and LES but not the LS. Expectedly, the pBEL2 derivatives gave higher levels of early transcription than those from pBEL1. The plasmid that had the 21 bp repeat inverted gave early transcription that was approximately 75% of wild type, for both pBEL1 and pBEL2. The inversion positioned GC-motif I (normally near the TATA box) 17 bp from the 3' border of the 72 bp sequence, thus making its steric alignment relative to the enhancer similar to the plasmids that had the 12 bp insertion (which placed GC-motif VI 18 bp from the enhancer). As with the insertion mutants, the 21 bp inversion had no effect on LS levels.

Figure 3. Replication of pBEL derivatives in HeLa cells. Various pBEL templates representative of the 72 bp multimers (pBEL1 and pBEL4) and the point mutations (pBEL1.4, pBEL1.12c, pBEL1.26, pBEL1.93, and pBEL1.94) were transfected into HeLa cells along with pRSVT. Transfections for replication studies were done the same way as for the transcription studies (see Materials and Methods), with fresh medium being fed to the cells 16 hours after the DNA precipitates were added to the dishes, and cells harvested after a total of 48 hours. Low molecular weight DNA was then extracted using the procedure of Hirt (36), and digested with either Sau 3A (S), Mbo I (M), or Dpn I (D). The samples were then run on a 1% agarose gel, blotted to nitrocellulose, and probed with nick-translated DNA fragment consisting of the SV40 control region of pBEL2. The position of supercoiled (sc) and digested (d) transfected DNAs are shown. Dpn I resistant DNA, and Mbo I sensitive DNA, is derived from replication in mammalian cells. Sau 3A cuts both methylated and unmethylated DNA, and represents the sum of transfected and newly replicated DNA in the Hirt extracts.

Figure 2. Effect of 72 bp copy number on the early to late shift. pBEL templates containing either 1, 2, 3, 4, or 7 copies of the 72 bp sequence were transfected into HeLa cells either alone (-TAG) or with pRSVT (+TAG). RNA was analysed by nuclease S1 mapping as described in Materials and Methods. The position of the LES, EES, LS, and Globin control bands are indicated by arrows, and the marker lane (M) contains an Msp I digestion of pBR322.

Figure 4. Effect of insertions between the 72 bp and 21 bp regions on LS and LES activation. Plasmids with no insertions (pBEL1 and pBEL2), 4 bp insertions (pBEL1.Bam4 and pBEL2.Bam4), or 12 bp insertions (pBEL1.Bam12 and pBEL2.Bam12) between the 21 and 72 bp sequences were transfected into HeLa cells either alone (-TAG) or with pRSVT (+TAG). Also included are templates with the 21 bp region inverted (pBEL1.Inv21 and pBEL2.Inv21).

When T antigen was present, and the plasmids could replicate, late transcription relative to the control plasmid was again unaffected by the insertions or inversion (Figure 4). All plasmids gave LS activity comparable to wild type. In contrast, the 4 bp insertion prevented a concurrent activation of transcription from the LES, for both pBEL1 and pBEL2. The 12 bp insertion restored LES activation, albeit at a slightly reduced level compared to the plasmids with no insertion. The 21 bp inversion also supported some activity from the LES, at a level intermediate between the levels observed with the 4 bp and 12 bp insertions. Finally, it was observed that the 4 bp insertion resulted in almost total autoregulation of EES activity on replicating molecules.

These data indicate that the responses of the late and late-early promoters on replicating templates can be uncoupled. The LES are sensitive to optimal spacing requirements between the 21 and 72 bp sequences, whereas the LS are not. As with the 72 bp multimers, Hirt extracts were analysed to verify that the insertions and inversion replicated normally (data not shown).

Role of the enhancer in transcription activation by large T antigen

The SV40 enhancer contains binding sites for a number of transcription factors. Large T antigen can affect viral gene expression not only through events brought on by DNA replication, but also by replication-independent activation of transcription. We examined the role played by these sites in the enhancer in this activation, and asked whether the activation that can be observed in the absence of replication is also apparent when replication occurs. The approach was to insert into pBEL1 mutations that disrupt binding sites for specific factors, and to determine whether these mutated templates could still be activated by SVT. If the factor that binds to a particular site is involved in the activation, then preventing its binding should interfere with the effect of SVT. The mutations tested were examined both in the original pBEL plasmid, and also in a derivative, pBELori⁻, that cannot replicate because of a 6 bp deletion in the origin of replication. The effects of wild type SVT, and of an SVT that

Figure 5. Effect of point mutations in the enhancer on transcription activation by SVT and the early to late shift. The plasmid pBEL1 contains a single 72 bp sequence, with no mutations. It and the various pBEL templates carrying mutations in the enhancer were transfected into Hela cells with either no SVT (–TAG), an SVT with a mutation in the nuclear localization signal (+CytO TAG), or wild type SVT (+WT TAG). The templates used had either an intact origin of replication (ori+) or a 6 bp deletion in the origin (ori–). The probe used to S1 map the ori– constructs was made from pBEL1.ori–. It generates protected fragments for the EES and LES that are 6 nucleotides shorter than those from the ori+ plasmids, because the 6 bp deletion lies downstream of these cap sites. Only one exposure of this autoradiogram is shown; shorter and longer exposures were used to quantitate the data for all of the test plasmids, and the effects on early-early transcription of these and other mutations analysed are summarized in Figure 6.

is non-nuclear because of a mutation in the nuclear localization signal, were assessed.

A representative autoradiogram is shown in Figure 5, and the effects of SVT on early-early transcription from these and other mutants tested are summarized in Figure 6. To interpret the data on early and late transcription it was necessary to obtain different exposures of the types of gels shown in Figure 5, since shorter exposures needed for assessment of transcription from more actively transcribed or replicating templates were too faint to permit late transcription to be seen. The pBEL plasmids carrying mutations in the enhancer are designated according to the enhancer mutations described earlier (1), and their positions in the enhancer and the factors whose binding they affect are shown in Figure 6.

In the absence of SVT, the effects of the mutations on early transcription were generally comparable to those published previously, except for pBEL1.12c, which disrupts both the GT-I and TC-II enhancers. In the original study (1), this mutation reduced early-early transcription to approximately 15% of wild type, whereas in our experiments it was reduced to less than 5%. As seen in Figure 5, the mutations had the same affect on transcription regardless of whether they were assessed in pBEL or pBELori⁻.

When the pBEL plasmids were cotransfected with a plasmid expressing a non-nuclear SVT, most exhibited an overall increase in transcription. Both early and late activity was stimulated relative to the globin control plasmid. Quantitation of the effect of SVT on early-early transcription revealed the following. EES levels from both replicating and non-replicating pBEL templates with intact enhancers were increased almost 7-fold (the ratio of transcription levels in the presence of SVT to those levels in the absence of SVT; Figure 6). However, some of the enhancer mutations diminished this activation. Most notably, the mutation within the TEF-1 binding site in the Sph-II enhancer (pBEL1.22; Figure 5) allowed only a 2.5-fold activation by SVT. The

Figure 6. Role of specific enhancers in replication-independent activation of SV40 early transcription by SVT. Enhancers containing clusters of three point mutations were inserted into pBEL, and their effects on transcription in the presence and absence of SVT assessed by transfection into Hela cells. The data are summarized in the boxed table, and represent the averages of at least three independent experiments for each mutant. Transcription levels were standardized to pBEL1 in the absence of SVT, using the RNA levels from the cotransfected globin control plasmid as a reference. In the table, numbers in italics indicate mutants that interfere with the ability of SVT to activate transcription, as evidenced by a reduced ratio of transcription in the presence of SVT to that seen in the absence of SVT.

mutation in pBEL1.4, which lies within the TEF-1 site in the GT-IIC enhanson, also was activated less efficiently than pBEL1 (only approximately 3-fold). A mutation in the Sph-I enhanson, that also binds TEF-1, did not affect transactivation by SVT as much as mutations in the other two TEF-1 sites. Although a mutation in the TC-II enhanson diminished transcription, pBEL1.15 was still activated by SVT as effectively as pBEL1 (i.e. approximately 7-fold). A mutation in the octamer motif, overlapping the early side of Sph-II (pBEL1.24), and in the AP-1 site (pBEL1.30) did not markedly diminish the ability of SVT to activate transcription. The mutation in pBEL1.12c greatly reduced transcription, but long exposures of the autoradiograms indicated that it did not abolish activation by SVT. Finally, as with the EES, mutations in the TEF-1 sites in Sph-II, and to a lesser extent in GT-IIC, interfered with late and late-early activity, both in the presence and absence of SVT.

The role of the TEF-1 sites, and most strikingly of the Sph-II enhanson, in directing late and late-early transcription was also seen when wild type SVT was used (eg. pBEL1.22, ori⁺, +WT TAG; Figure 5). None of the enhancer mutations prevented an early to late shift *per se* (i.e. a change in the ratio of late to early-early transcription). The effect of the mutations confirms that the enhancer continues to function bidirectionally on replicating templates. Plasmids with mutations that do not affect replication-independent transcription activation by SVT (e.g. pBEL1.15 and pBEL1.30) had an early to late shift equivalent to that of the wild type plasmid. In general, the results with the replicating (ori⁺) templates reinforce the earlier observation (25,27) that replication is required for an early to late shift. Replication can be blocked either by a mutation in the origin or by preventing SVT from entering the nucleus. The effects of wild type SVT on pBELori⁻ test templates were the same as those observed with cytoplasmic SVT on either ori⁺ or ori⁻ templates. Finally, Hirt extracts were analysed to confirm that the point mutations in the enhancer did not interfere with the ability of the plasmids to replicate. Several such replication assays with plasmids having point mutations are shown in Figure 3. It has been reported from another laboratory that point mutations in the SV40 enhancer can interfere with DNA replication (47). This result is not in disagreement with our own data, since in those experiments the plasmids being studied had no 21 bp region. It is known that either the 21 bp or 72 bp elements can act as auxiliary elements for DNA replication, and that if the 21 bp element is present (such as in our pBEL plasmids), the 72 bp sequence has no role in replication (reviewed in 48).

DISCUSSION

The data presented here indicate that the SV40 enhancer plays an important role in regulating the shift in SV40 transcription initiation from the early-early to the late and late-early start sites. Although these latter start sites appear simultaneously after the onset of viral DNA replication, and are inhibited in parallel by progressive multimerization of the 72 bp sequence, there clearly are differences in their regulation. The LES have a requirement for a correct steric alignment between the 21 and 72 bp regions, whereas the LS do not.

There are two hypotheses that might explain the suppression of an early to late shift by multimerization of the 72 bp sequence. The first assumes that sequences controlling LS and LES activity are spread throughout regions on the late side of the 72 bp element, within the 72 bp element, and on the early side of the

enhancer. By inserting additional copies of the 72 bp region, a critical spacing requirement between these sequences may be disrupted. For several reasons this does not seem likely. Considering first the late promoter, the additional copies of the 72 bp element are in effect added at the junction between the 72 and 21 bp regions. If this promoter requires sequences on the early side of the enhancer, it might be anticipated that the insertion mutations between the 21 and 72 bp regions would have had at least some effect on late transcription. This was not seen. In addition, attempts to map the sequences required for late transcription *in vivo* have indicated that the sequences on the early side of the enhancer (notably within the origin or replication) play a relatively minor role in late promoter activation (21,22). Finally, in the absence of SVT and DNA replication, the multimerization of the enhancer has no effect on basal levels of late transcription. Considering the late-early promoter, it is equally unlikely that transcription from it is totally dependent upon a DNA sequence upstream of the 72 bp region. Only if that were the case could the effect on the LES of inserting extra copies of the 72 bp region be explained. There is abundant evidence, both in the present study and in work published from other laboratories (1,32,37), that the sequences required for LES activity lie predominantly within the 72 bp and 21 bp regions.

An alternative hypothesis is that replication-dependent late and late-early activation requires autoregulation of early-early transcription. That is, on replicating templates there may be a competition between the early-early and the late and late-early promoters. In support of this notion, we note that in the absence of SVT multimerization of the enhancer leads to an increase in early-early activity, but has little effect on late and late-early, suggesting that the EES might be preferred targets of enhancer activity. Second, on non-replicating molecules the utilization of the LES has been shown to increase, and of the EES to decrease, when mutations are introduced into the TATA box of the early promoter (32). One model for how replication might autoregulate the EES and activate the LES could be simply that replication impairs the functioning of the TATA box. The multimerization of the enhancer clearly strengthens EES activity, perhaps by allowing the TATA box to continue to function during replication. This model is difficult to test at present, since there is no way of determining what the effect of replication is on DNA affinity and function of the TATA box binding protein TFIID, and the mechanism whereby the enhancer interacts with the basal transcription proteins like TFIID is not known. It is unlikely that LES activation results simply from an interference with EES activity caused by the binding of SVT to the origin of replication. In all of the enhancer multimers tested the T antigen binding sites are unaffected by the distal insertions of the extra 72 bp sequences, yet LES activation is progressively diminished as the enhancer is multimerized. Interestingly, it was noted that EES autoregulation was very efficient when the 72 and 21 bp regions were separated by a half helical turn. The insertion may already compromise the ability of RNA polymerase to initiate at the EES, thereby enabling replication to more easily suppress remaining early-early activity. Finally, while it has been shown that the 21 bp region can act as a bidirectional element to promote transcription, any role that it might play in the early to late shift is also orientation-independent.

The inverse correlation between 72 bp copy number and the magnitude of the early to late shift permits some speculation about the evolution of the virus control region. An enhancer with a single 72 bp sequence has only approximately 30% of the activity

of a wild type enhancer having a 72 bp repeat (1; Figure 2). Although the virus can achieve greater levels of early transcription required for synthesis of the early proteins by having additional copies of the 72 bp sequence, it does so at the expense of late transcription. It is possible that one of the ways in which the virus evolved an appropriate balance between early and late gene products was to multimerize the enhancer sequence until a compromise was reached between early activity and the early to late shift.

It was observed here and previously (1) that multimerizing the 72 bp element up to seven copies resulted in greater early transcription, although the biggest effects are seen in increasing the copy number from one to three or four. Work from another laboratory also indicated that early transcription peaked with four copies, but then declined slightly with six copies, and with beyond eight copies was severely inhibited (45). We have never detected this slight inhibition of transcription with our plasmid having seven copies. We can only speculate that this may be due to differences between cell lines used in the different laboratories, or differences in the amount of DNA used in the transfections. Similarly, we did not observe the inhibition of DNA replication by enhancer multimerization that was also reported by the other laboratory (46), perhaps for the same reasons.

The data presented here indicate that the SV40 enhancer plays a role in bidirectional transcription activation by SVT, and that the sequences within the enhancer that are required for this activation include at least in part those enhansons to which the factor TEF-1 binds. The mutation in pBEL1.22 (Sph-II enhanson) demonstrated this most clearly. This mutation has been shown to specifically disrupt binding of the TEF-I protein (15,39). The other two TEF-1 binding sites in the enhancer, in the Sph-I and GT-IIC enhansons, also appear to be involved, albeit to a lesser extent than Sph-II. Mutations in other enhansons were not observed to affect activation by SVT, regardless of whether they did (e.g. GT-I and TC-II) or did not (e.g. OCT and P motif (AP1)) have an affect on overall levels of early and late transcription.

The role of the Sph region and by inference the protein TEF-1 in mediating transcription activation by SVT has been recognized in other laboratories (21,23,40), although in these earlier studies only the late promoter was examined. One property of the TEF-1 protein is that in Hela cells it represses transcription at high concentrations by a squelching mechanism, presumably because a required factor with which TEF-1 interacts is titrated out (39). This squelching is also observed *in vitro* using Hela cell transcription extracts. One possible mechanism, as yet speculative, for how SVT activates transcription might be through a direct interaction with TEF-1, thereby lowering the concentration of free TEF-1 and thus preventing squelching. This interaction could have the same effect regardless of whether it occurs in the nucleus or the cytoplasm. The recent cloning of the TEF-1 gene (39) should permit a biochemical analysis of whether these two proteins bind to each other.

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