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The SV40 enhancer influences viral late transcription *in vitro* and *in vivo* but not on replicating templates

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We have examined transcription from the SV40 late promoter *in vitro* and *in vivo*. In HeLa whole cell extracts, late transcription is efficient in the absence of T antigen but is impaired by enhancer specific point mutations. *In vivo*, when replication is prevented, transcription from the late promoter requires T antigen as well as a functional enhancer. However, enhancer sequences fail to potentiate late transcription from replicating templates although, under such conditions, enhancer binding factors do not become limiting. It appears that the SV40 late transcription unit is refractory to enhancer-mediated activation when it is located on a replicating template.

Key words: in vitro transcription/SV40 late promoter/replication/transient expression/viral enhancer

Introduction

The SV40 early promoter, like most RNA polymerase II promoters, contains a TATA box equivalent (Goldberg, 1978), upstream elements consisting of a GGGCGG motif repeated six times, also referred to as 21 base pair (bp) repeats (Everett *et al.*, 1983), and a 72-bp repeated sequence identified as a strong enhancer of transcription (Banerji *et al.*, 1981; Moreau *et al.*, 1981). Each of these promoter elements participates in transcriptional regulation, at least in part, by binding specific factors. A TATA box binding factor has been partially characterized (Davison *et al.*, 1983) as well as the Sp1 factor that binds to the repeated GGGCGG sequences (Dynan and Tjian, 1983a, 1983b). The enhancer region has also been shown to bind cellular components (Schöler and Gruss, 1984; Wildeman *et al.*, 1984; Sassone-Corsi *et al.*, 1985). In addition, SV40 early transcription is repressed by binding of one of its own products, large T antigen, to specific sequences in the early promoter (Tjian, 1978; Rio *et al.*, 1980; Myers *et al.*, 1981). Apart from T antigen, all these factors and RNA polymerase II, whether or not they interact directly with each other, are apparently required for optimal SV40 early transcription.

The late promoter largely overlaps the early promoter sequences (see Figure 1 and Buchman *et al.*, 1981). The Sp1 factor and sequences to which it binds have been shown to influence SV40 late transcription (Everett *et al.*, 1983; Rio and Tjian, 1984). Sequences located between the early TATA box and the Sp1 binding sites have also been shown to be involved in regulating late transcription (Contreras *et al.*, 1982; Fromm and Berg, 1982; Gheysen *et al.*, 1983). The late promoter, however, lacks a conventional TATA box, which may explain why the heterogeneous late initiation sites are scattered over nearly 300 nucleotides (Ghosh *et al.*, 1978). Sequences located downstream from the major late initiation site at nucleotide 325 (numbering according to Tooze, 1981) have also been described as influencing the relative abundance of transcripts initiated at that position (Piatak *et al.*, 1983). Furthermore, unlike SV40 early transcription, late transcription is stimulated by T antigen in the absence of DNA replication (Brady *et al.*, 1984; Keller and Alwine, 1984).

The SV40 enhancer is capable of raising the level of transcription from many promoters in *cis*, in a manner independent of its orientation and distance from the initiation sites (Banerji *et al.*, 1981, 1983; de Villiers and Schaffner, 1981; Benoist and Chambon, 1981; Gruss *et al.*, 1981; Levinson *et al.*, 1982). In view of its bidirectional nature, one might imagine that the enhancer also controls late transcription. However, the information available is either contradictory or inconclusive (Gheysen *et al.*, 1983; Fromm and Berg, 1983; Hartzell *et al.*, 1984; Ernoult-Lange *et al.*, 1984).

We, and others, have previously reported that some of the effects of the SV40 enhancer can be reproduced *in vitro* (Sassone-Corsi *et al.*, 1984; Sergeant *et al.*, 1984). A 5- to 10-fold activation of transcription from different transcription units (rabbit β-globin, SV40 early and adenovirus major late genes) by the SV40 enhancer was detected in various HeLa cell extracts.

Despite the fact that, in such *in vitro* assays, SV40 DNA does

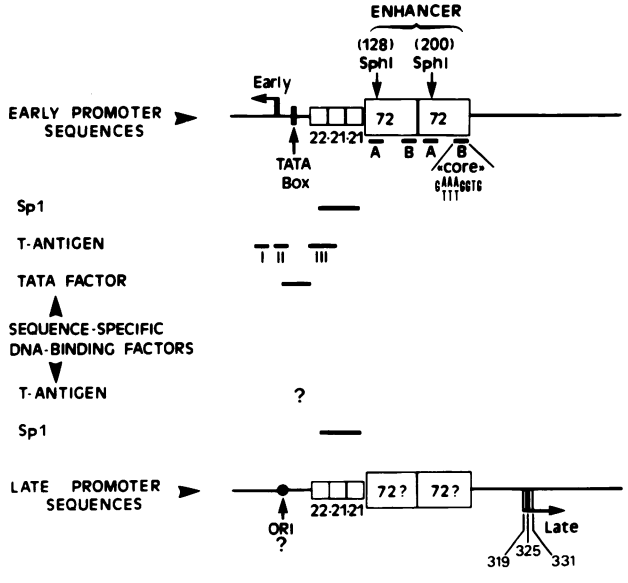

Fig. 1. Control elements and specific binding factors affecting transcription from the SV40 early and late promoters. The early and late promoter elements are diagrammed at the top and bottom of the figure respectively. Between these, to the left, known factors are listed that are involved in expression from either promoter and their sites of interaction with the DNA are indicated, where identified, by horizontal bars above and below the different motifs.

not replicate and no T antigen is present, we and others (Dynan and Tjian, 1983a; Rio and Tjian, 1984; Natarajan *et al.*, 1983) also detect accurate and efficient transcription from the SV40 late promoter *in vitro*. In this report we show that late transcription is activated by the viral enhancer *in vitro* and that this activation can also be seen *in vivo* but only when template replication is suppressed.

Results

SV40 enhancer mutations affect late promoter activity in vitro

To demonstrate the effect of the enhancer on late transcription *in vitro*, SV40 DNA and several different plasmids were used. Plasmids p280, p281 and p282 (Weiher *et al.*, 1983) contain the complete SV40 genome minus one of the 72 bp repeats. Within the 94 bp between the single *Sph*I and *Kpn*I sites, p281 and p282 have 12 and 9 point mutations respectively, whereas p280 retains the wild-type sequence. One further difference between the viral DNA and plasmid templates is that the latter all contain an additional *Bam*HI site inserted at the *Hpa*II site of SV40 at position 346 (Figure 2a).

In vitro transcription assays were carried out in whole cell extracts of HeLa cells under conditions described previously (Sergeant *et al.*, 1984). Analysis of late transcripts made *in vitro* on viral templates by primer extension shows that a number of initiation sites are used, the major one being at nucleotide 325 (Figure 3, lane 1). The plasmid templates all produce transcripts from the same sites but the major product initiates at position 331 (Figure 3, lanes 2–4, and see Figure 1). The reverse transcripts of the latter are six nucleotides longer than normal due to the inserted *Bam*HI linker. Reduced levels of late transcripts are produced by the enhancer mutants (lanes 3 and 4, Figure 3) in agreement with the effect of these mutations on SV40 early gene transcription *in vitro* and *in vivo* (Sergeant *et al.*, 1984) and their analysis by the chloramphenicol acetyl transferase (CAT) assay (Weiher *et al.*, 1983).

S1 analysis of these late transcripts gives the same result except that only a single protected fragment is seen (Figure 3, lanes 5–10). This is also the case with RNA transcribed from wild-type templates (Figure 3, lane 11).

SV40 enhancer mutations appear to affect late promoter function in HeLa cells

To confirm the validity of the activation of late transcription by the viral enhancer *in vitro*, we attempted to demonstrate a similar effect *in vivo*. Initially, we co-transfected HeLa cells with either p280, p281, p282 or SV40 DNA and p9511 as an internal control (see Materials and methods) and analysed RNA isolated 48 h after transfection by primer extension. An effect similar to the one seen *in vitro* is observed with the plasmid templates (Figure 4, lanes 1–3). However, three initiation sites are used equally on all these templates whereas with the wild-type template, as *in vitro*, there is one major start site (Figure 4, lane 4). This difference is probably due to the *Bam*HI linker inserted downstream from the major late start sites (Piatak *et al.*, 1983). As a control for the enhancer effect, early transcription from the test plasmids was also monitored (Figure 4, lanes 8–10) and globin transcription was used as a control for the transfection efficiency (Figure 4, lanes 8–10).

Unfortunately, there is an inherent conundrum in this experi-

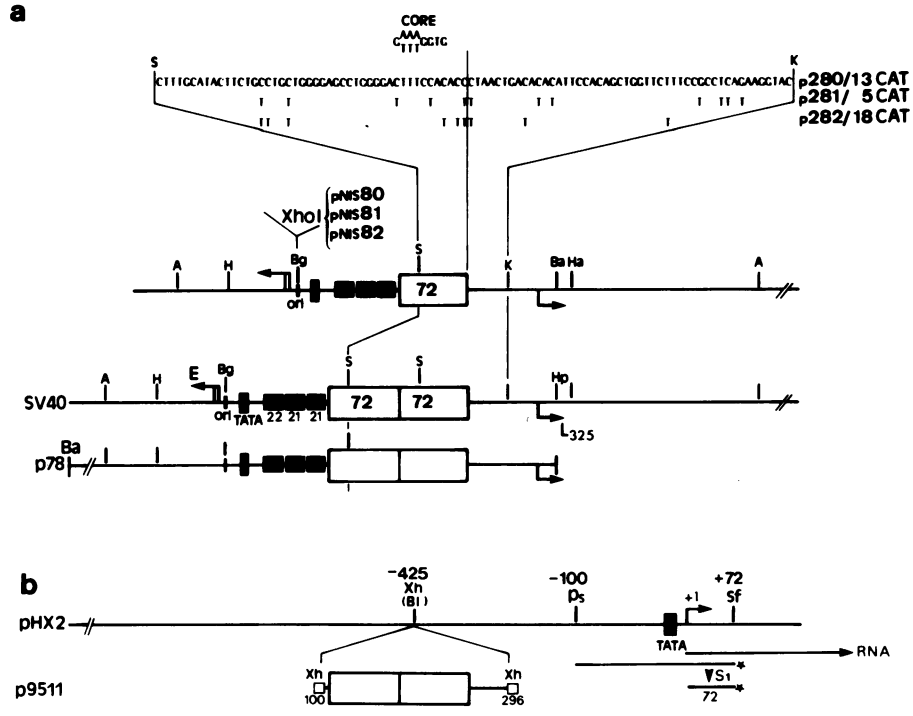

Fig. 2. DNA templates used for *in vitro* transcription and transient expression assays. (**a**) Three series of test clones were used, all of which are modified from wild-type SV40 DNA, strain 776 as follows: all clones contain a single copy of the 72 bp repeat with or without several point mutations; pCAT DNAs contain the CAT gene in place of the gene for T antigen fused to the early promoter at the *Hind*III site at position 5171; the pNIS plasmids all contain a *Xho*I linker at the partially deleted origin *Bgl*I site and are replication defective and, finally, all the clones have a *Bam*HI linker inserted into the *Hpa*II site at SV40 position 346. p78 contains the SV40 early region, early promoter and enhancer intact, but the late region is truncated at the *Hpa*II site (position 346). Late transcripts from this template are not detected by the primer extension assay used here. (**b**) pHX2 consists of the rabbit β-globin gene and upstream sequences cloned into pJ1 and p9511 is a derivative thereof with the SV40 enhancer on a 196 bp fragment inserted at position −425 (de Villiers *et al.*, 1982).
Restriction site abbreviations: S = *Sph*I; K = *Kpn*I; A = *Ava*II; H = *Hind*III; Bg = *Bgl*I; Ba = *Bam*HI; Ha = *Hae*III; Hp = *Hpa*II; Xh = *Xho*I; Ps = *Pst*I; Sf = *Sfa*NI; Bl = *Bgl*II.

ment. It has been reported that T antigen substantially stimulates SV40 late transcription in the absence of DNA replication and that this stimulation is associated with the ability of T antigen to bind DNA (Brady *et al.*, 1984; Keller and Alwine, 1984). The extent of this effect is demonstrated when the CAT gene is substituted for the SV40 early gene on the test plasmids. Although the late promoter is intact, essentially no transcription can be detected from these plasmids when they are transfected into HeLa cells (Figure 4, lanes 5 – 7). The globin controls are shown below (lanes 5 – 7). Since SV40 early RNAs are transcribed with different efficiencies from p280, p281 and p282, varying amounts of T antigen will be present in the cells as a consequence. The decreased levels of late transcription from the enhancer mutants may therefore simply reflect less stimulation due to smaller amounts of T antigen. For this reason, another series of experiments was performed in which T antigen was provided from a second plasmid co-transfected into HeLa cells along with the various test plasmids.

Raised levels of T antigen expression in HeLa cells do not override the effects of enhancer mutations on SV40 late transcription

HeLa cells were co-transfected with either plasmid p280 or p281, p1-3CAT or p5CAT, and an excess of a plasmid that expresses T antigen (p78). Expression of early RNA was analysed in all cases and the levels were found to be constant and therefore independent of the test plasmids (Figure 5, lanes 1 – 4). Under such conditions, the effect of the enhancer mutations on late transcription can be seen (Figure 5, lanes 5 – 8). On the other hand, when no T antigen is provided in *trans*, late transcription can still be detected from p280, which expresses T antigen, but from p1-3CAT, which does not, there is only a very low level of transcription that cannot be detected without over-exposing the autoradiographs (Figure 4, lanes 1 and 5). Templates carrying mutated enhancer sequences produce similarly low levels of late RNAs regardless of the presence of T antigen (Figure 5, lanes 6 and 8). Thus, expression of T antigen in the cells is essential to stimulate SV40 late transcription but the extent to which this stimulation is reflected in late transcription depends on the integrity of the enhancer.

SV40 enhancer mutations affect late promoter activity on replication defective templates in cos cells

As a further means of demonstrating the effect of enhancer sequences on late transcription, a comparable series of transfections was carried out in cos cells, which produce high levels of T antigen constitutively. It should be noted that, although cos cells produce some endogenous SV40 late RNA (Figure 6a, lane 4), primer extension analysis distinguishes between this and late RNA from transfected templates because the latter all have a linker insertion as discussed above. The 6 bp deletion at the origin of the SV40 DNA in cos cells (Gluzman, 1981) results in the endogenous early RNA being shorter than, and therefore also distinguishable from, early RNA transcribed from transfected templates (not shown).

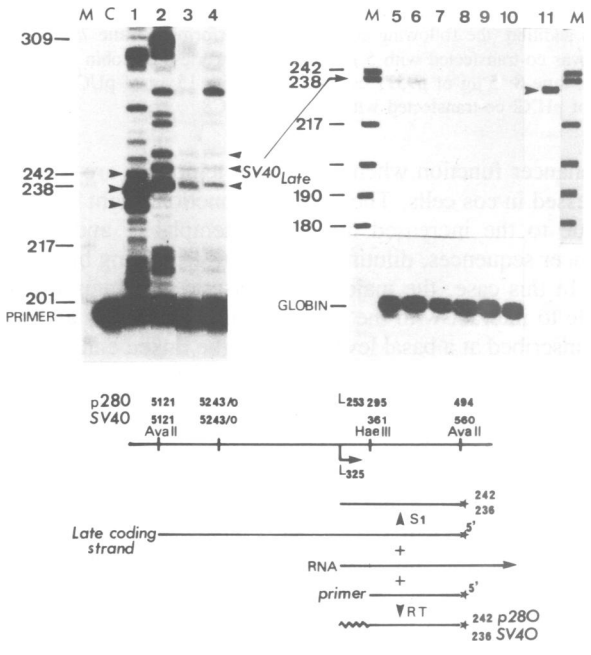

Fig. 3. The SV40 enhancer stimulates late transcription *in vitro*. The left panel shows a primer extension analysis of late RNA transcribed *in vitro*. 40 μg/ml of each uncut template was incubated in a HeLa whole-cell extract (see Materials and methods). **Lane C**, primer alone; **lane 1**, SV40 virion DNA; **lane 2**, p280; **lane 3**, p281; **lane 4**, p282. The right panel shows an S1 analysis of RNA from a comparable experiment. The probe and S1 digestion product are schematically presented at the bottom of the figure. *In vitro* transcription reactions contained 60 μg/ml of pUC8 (Vieira and Messing, 1982) as carrier DNA and 2.5 μg/ml of pHX2 as internal reference. Reactions were performed in duplicate. **Lanes 5** and **6**, p280 (2.5 μg/ml); **lanes 7** and **8**, p281 (2.5 μg/ml); **lanes 9** and **10**, p282 (2.5 μg/ml). Globin reference transcription is shown below each lane. The S1 product of RNA transcribed *in vitro* from SV40 virion DNA is shown in **lane 11**.

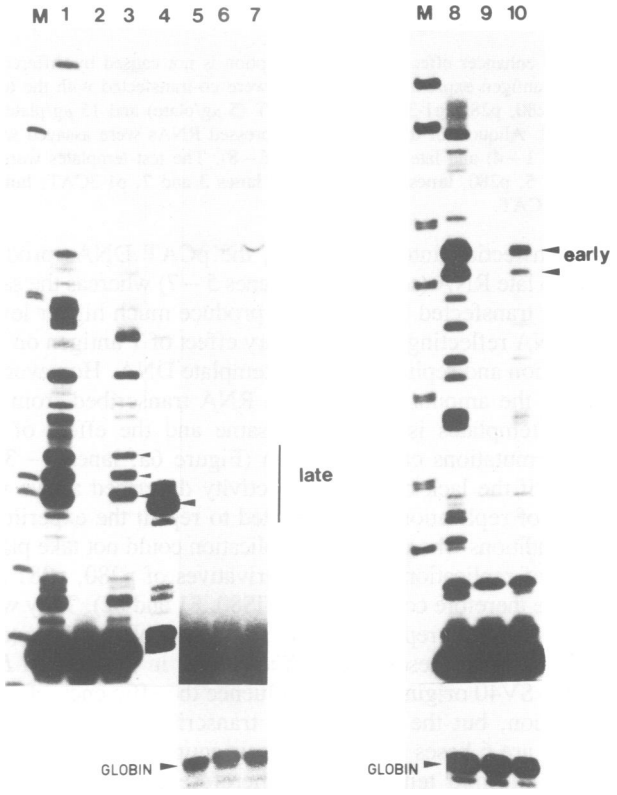

Fig. 4. T antigen and a functional enhancer are both needed for late transcription in HeLa cells. RNA was isolated from HeLa cells transfected with various DNA templates and late transcripts were analysed by primer extension. Transfections were as follows: **lane 1**, p280; **lane 2**, p281; **lane 3**, p282; **lane 4**, SV40 virion DNA; **lane 5**, p1-3CAT; **lane 6**, p18CAT; **lane 7**, p5CAT. **Lanes 8 – 10** show an analysis of SV40 early RNA from the same transfections analysed in **lanes 1 – 3** respectively. Globin controls are shown below the corresponding lanes (controls for **lanes 1 – 3** are the same as for **lanes 8 – 10**). 15 μg of each test plasmid was transfected along with 5 μg of p9511 per plate.

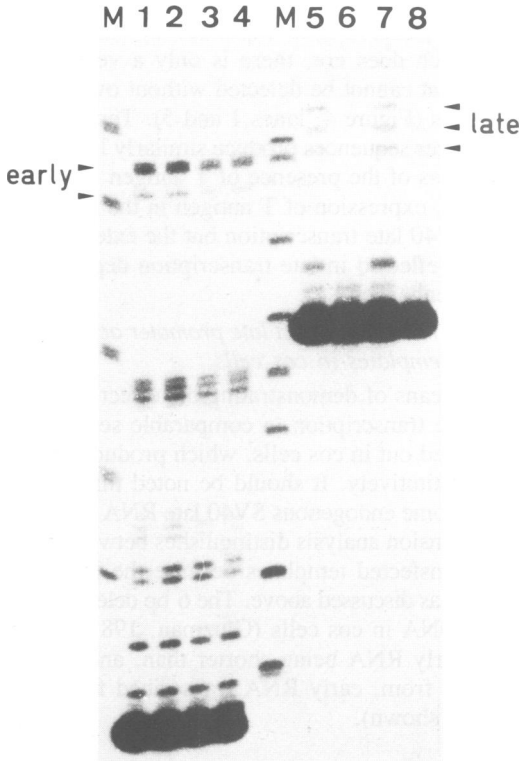

Fig. 5. The enhancer effect on late transcription is not caused by different levels of T antigen expression. HeLa cells were co-transfected with the test templates p280, p281, p1-3CAT and p5CAT (5 μg/plate) and 15 μg/plate of plasmid p78. Aliquots of the transiently expressed RNAs were assayed with early (**lanes 1–4**) and late primers (**lanes 5–8**). The test templates were: **lanes 1** and **5**, p280; **lanes 2** and **6**, p281; **lanes 3** and **7**, p1-3CAT; **lanes 4** and **8**, p5CAT.

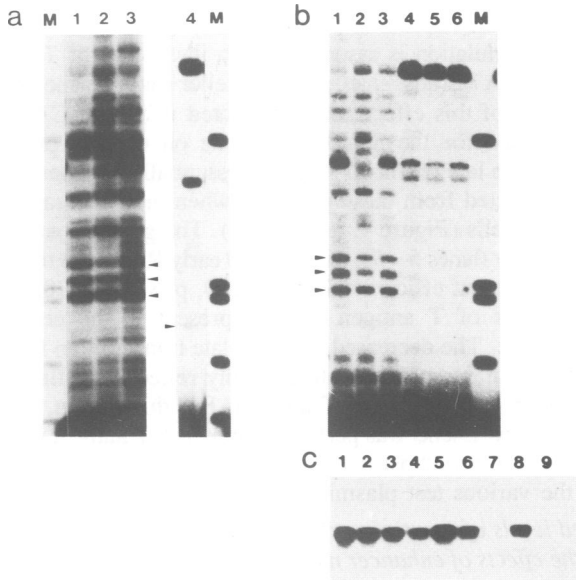

Fig. 6. Replication interferes with enhancer activity in cos cells. (**a**) Primer extension analysis of RNA isolated from cos cells transfected with 10 μg of the following templates: **lane 1**, p1-3CAT; **lane 2**, p18CAT; **lane 3**, p5CAT; **lane 4**, endogenous RNA isolated from cos cells. (**b**) Plasmids p280, p281 and p282 (**lanes 1–3**) and their non-replicating counterparts pNIS80, pNIS81 and pNIS82 (**lanes 4–6**) were transfected into cos cells (10 μg of DNA/plate). RNA isolated after 48 h was analysed by primer extension. (**c**) 5 μg of p9511 was included in each transfection shown in **b**. **Lanes 1–6** of this globin S1 analysis correspond to **lanes 1–6** of Figure 6**b**. In addition, the following controls were performed. **Lane 7:** 10 μg of p280 was co-transfected with 5 μg of the enhancerless β-globin plasmid pHX2; **lane 8**: 5 μg of p9511 co-transfected with 15 μg of pUC8; **lane 9**: 5 μg of pHX2 co-transfected with 15 μg of pUC8.

On transfection into HeLa cells, the pCAT DNAs produce almost no late RNA (see Figure 4, lanes 5–7) whereas the same templates transfected into cos cells produce much higher levels of late RNA reflecting the stimulatory effect of T antigen on late transcription and replication of the template DNA. However, in cos cells, the amount of SV40 late RNA transcribed from the different templates is almost the same and the effect of the enhancer mutations cannot be seen (Figure 6a, lanes 1–3).

To see if the lack of enhancer activity described above was an effect of replication, we attempted to repeat the experiment under conditions where template replication could not take place. A series of replication defective derivatives of p280, p281 and p282 were therefore constructed (pNIS80, 81 and 82). They were confirmed as being replication defective by dot blot analysis (data not shown). The presence of a *Xho*I linker in the deleted *Bgl*I site at the SV40 origin does not influence the efficiency of early transcription, but the early RNAs transcribed from the pNIS templates are 6 bases longer than their counterparts made from the other plasmid templates and therefore can also be easily distinguished from endogenous SV40 early RNAs in cos cells (not shown).

Although the level of late transcription from pNIS80 is much lower than that from p280 (Figure 6b, lanes 1 and 4), the enhancer mutations in pNIS81 and 82 impair transcription from the SV40 late promoter in cos cells (Figure 6, lanes 4–6). This clearly contrasts the expression seen with the replicating templates p280, p281 and p282 (lanes 1–3).

There are different possible explanations for the apparent lack of enhancer function when replicating templates are transiently expressed in cos cells. The enhancer function might be abolished due to the increased number of templates, and therefore enhancer sequences, diluting out the corresponding binding factors. In this case, the majority of plasmid templates would be unable to interact with the enhancer binding factors and would be transcribed at a basal level whether the linked enhancer were functional or not. Alternatively, it is possible that templates in the state of active replication are not competent for enhancer function (see Discussion).

Loss of enhancer function on replicating templates in cos cells is not due to limiting enhancer binding factors

As a means of testing the first explanation, non-replicating plasmids carrying the rabbit β-globin gene, either linked to (p9511) or lacking (pHX2) the SV40 enhancer (de Villiers *et al.*, 1982 and Figure 2b), were transfected into cos cells alone or along with either a replication-competent SV40 template containing a functional enhancer (p280), or various mutant derivatives. β-Globin transcription could only be detected in cells transfected with p9511 (Figure 6c, lanes 1–6 and 8). Furthermore, the level of globin expression from templates co-transfected with replicating SV40 templates (lanes 1–3) was the same as from those transfected into cos cells alone (lane 8) or along with non-replicating SV40 templates (lanes 4–6). In no case could transcription be detected from pHX2 (Figure 6c, lanes 7 and 9). This demonstrates that enhancer binding factors are not competed out by a replicating template containing functional enhancer sequences.

Discussion

The results presented here demonstrate that point mutations in the SV40 enhancer sequences which have been shown to impair transcriptional activation of linked promoters *in vivo* (Weiher *et al.*, 1983) and *in vitro* (Sergeant *et al.*, 1984) also reduce the level of RNA synthesis from the SV40 late transcription unit *in vitro* as well as *in vivo*.

It has been shown that, although T antigen stimulates SV40 late transcription *in vivo*, a basal level of transcription from the late promoter can be detected in its absence (Contreras *et al.*, 1982; Brady *et al.*, 1984; Keller and Alwine, 1984). In our *in vivo* experiments we also find a very low level of late transcription in the absence of T antigen, since late transcripts are made from the pCAT series in HeLa cells. Such transcripts are only seen after long exposure of the gels (not shown). For reasons that are presently unclear, the block on late transcription that T antigen is able to overcome *in vivo* must be obviated under our *in vitro* conditions because we do not need to include T antigen in our assay system to be able to observe transcription from the late promoter. Under such conditions, templates with intact enhancers are transcribed more efficiently than those with mutated enhancers implying that SV40 late transcription is subject to activation by the viral enhancer *in vitro* in a manner independent of T antigen.

As far as late transcription *in vivo* is concerned, a consensus with regard to the control elements involved has still to be reached. The origin of replication, large T antigen, trans-acting factors, chromatin structure, enhancer sequences and sequences from either side of the enhancer have all been reported as being necessary for late transcription (Hartzell *et al.*, 1984; Brady *et al.*, 1984; Keller and Alwine, 1984; Tack and Beard, 1985; Fromm and Berg, 1983; Everett *et al.*, 1983; Rio and Tjian, 1984; Piatak *et al.*, 1983). However, it has also been reported that the 72 bp repeats (Gheysen *et al.*, 1983) and the SV40 origin sequences (Ernoult-Lange *et al.*, 1984) are dispensable for late transcription.

In HeLa cells we observe only a very low level of late transcription in the absence of T antigen. However, when T antigen is provided in trans (p78), the mutations in the SV40 enhancer that impair late transcription *in vitro* have the same effect *in vivo* and high levels of T antigen in the cells fail to compensate for the mutations.

In cos cells, templates that contain a functional SV40 origin are able to replicate (Gluzman, 1981). The replicating plasmids p280, p281 and p282 all give rise to similarly high levels of late transcripts in cos cells and no effect of the enhancer mutations can be detected. However, their non-replicating derivatives, pNIS80, pNIS81 and pNIS82, although they produce less RNA, clearly demonstrate that mutations in the enhancer sequences are detrimental to late transcription in these cells.

We are confident, for several reasons, that the point mutations in the enhancer mutants used in this study are specific for enhancer sequences and do not alter late promoter sequences. The mutants are two from the series employed to identify the enhancer core sequence 5′ GTGGAAAG 3′ and the relative efficiency of these enhancer mutants, as compared to wild type, has been well documented (Weiher *et al.*, 1983). The relative stimulation of late transcription by these mutants is in accordance with the values given. Furthermore, sequences containing the enhancer have been excised from the SV40 genome and reinserted at a number of different positions in either orientation without deleterious effect on late transcription (Fromm and Berg, 1983). All the point mutations in p282 except one lie within the segment that was translocated. Infectious SV40 virions have also been obtained after substituting the enhancer sequences with other viral enhancers (Weber *et al.*, 1984). Finally, all the point mutations lie within the region that has been shown to bind enhancer-specific factors *in vitro* (Wildeman *et al.*, in preparation).

The SV40 enhancer has thus been shown to activate transcription from the late promoter in three different situations, *in vitro*, in HeLa cells, which do not permit the SV40 origin to replicate, and in cos cells on replication defective templates. However, the enhancer does not stimulate late transcription from replicating templates even under conditions that allow optimal enhancement of transcription from a non-replicating rabbit β-globin template. It has been reported that in cos cells, replication of a plasmid carrying the human β-globin gene does not lead to elevated levels of β-globin RNA but that *cis*-linkage to the SV40 enhancer allows efficient β-globin transcription from replicating and non-replicating templates alike (Treisman *et al.*, 1983). This disparate behaviour of the human β-globin promoter may be due to its orientation or location relative to the viral control region in the constructions used, because these parameters differ markedly from those of the viral promoter. On the other hand, it may simply reflect qualitative differences between the two promoters studied. This inconsistency can only be resolved by further experiments.

We have demonstrated that replicating templates do not compete out enhancer-binding factors in cos cells but it is not clear from these experiments if this is due to excess factors being present in the cells or to a low affinity of replicating DNA for such factors. The successful use of competition experiments to demonstrate that specific factors bind to enhancer sequences does imply, however, that such factors are present in limiting amounts in monkey cells (Schöler and Gruss, 1984). This, in turn, argues in favour of templates undergoing replication having a reduced affinity for enhancer-binding factors. Two possible explanations can be envisaged for such an apparent reduction in affinity. One possibility is that factors are prevented from binding by the physical state of persistently replicating DNA. However the results of Treisman *et al.* (1983) are not consistent with such a model. Alternatively, other factors may bind with higher affinity to the same or overlapping sites thus competing away enhancer-binding factors or modulating their association with the DNA. In the latter case, it is necessary to explain why such factors are not able to do this early in infection of permissive cells. Again, two explanations seem feasible, both involving T antigen. Either the factors are not present in the uninfected cell and their expression is induced by T antigen or they are present but their high affinity for the binding sites relies on cooperative binding with T antigen. Whichever the case, replication of the DNA would be necessary to displace enhancer-binding factors making the binding sites available to other factors. Under conditions where replication is inhibited, enhancer-binding factors would not be displaced and the enhancer would continue to function. Evidence in favour of different factors being able to bind sites within enhancer sequences may be provided by footprinting experiments with a variety of cell extracts and in the presence and absence of T antigen.

In conclusion, SV40 late transcription can be shown, *in vitro* and *in vivo*, to be potentiated by the viral enhancer. *In vivo*, T antigen is needed to stimulate late transcription above a basal level but such expression, prior to replication, is still enhancer dependent. During replication, however, late transcription proceeds efficiently, independent of the viral enhancer so that poten-

tiation of late transcription by the enhancer, although manifest has no clear role in the lytic cycle of the virus.

Materials and methods

Cloned DNA templates

The cloned transcription units used for these studies are presented in Figure 2. The SV40 sequence numbering is according to Tooze (1981). p280, p281 and p282 have been described in the results section. The modified SV40 genomes were opened at their unique *TaqI* sites and inserted into the *ClaI* site of plasmid pAD190 (Weiher *et al.*, 1983). Plasmids p1-3CAT, p5CAT and p18CAT were constructed by inserting SV40 *HindIII* fragment covering the entire early and late promoter region (nucleotide 5171 – 1046 of p280, p281 and p282 respectively) into plasmid pSV-2, upstream of the CAT gene (Weiher *et al.*, 1983).

The Non-replicating Insertion Series plasmids pNIS80, pNIS81 and pNIS82 were derived from plasmids p280, p281 and p282 respectively and have the *BglI* site at the SV40 origin partially deleted and replaced by an *XhoI* octamer linker (Figure 2a). The constructs were sequenced through the enhancer to confirm the point mutations and across the mutated replication origin to ascertain the sequence. Three nucleotides were deleted at the *BglI* site in the pNIS plasmids and a complete linker octamer iserted.

Plasmid p78 contains the SV40 early gene with the complete promoter sequences (*BamHI* to *HpaII* fragment) fused into plasmid vector pA10 (Schöler and Gruss, 1984).

Plasmid p9511 carries the rabbit β-globin gene linked to the enhancer sequence contained in the SV40 DNA fragment extending from nucleotide 100 – 296, and plasmid pHX2 contains the β-globin gene without enhancer sequences (de Villiers *et al.*, 1982).

Preparation of whole-cell extracts

Whole-cell extracts were prepared from HeLa cells essentially as described by Hernandez and Keller (1983) except that the final dialysis was made against 50 mM Tris-HCl (pH 8), 17% glycerol, 0.1 mM EDTA, 0.25 mM dithiothreitol and 40 mM $(NH_4)_2SO_4$.

In vitro transcription

In vitro transcription assays contained in 50 μl: 5 mM creatine phosphate, 6 mM $MgCl_2$, 0.5 mM ATP, CTP, GTP and UTP, 25 μl of whole-cell extract and different DNA concentrations, indicated in the figure legends. After incubation at 30°C for 60 min, RNAs were isolated as described (Hernandez and Keller, 1983).

Cell culture and transfections

HeLa and cos-1 cells (Gluzman, 1981) were grown in DMEM (Gibco) supplemented with 10% (v/v) fetal calf serum. Cells were seeded at 10^6 per 100 mm Petri dish 24 h prior to infection, and the medium was changed 4 h before transfection. Transfections were performed by the addition of DNA-calcium phosphate co-precipitates as described (Weber *et al.*, 1984). 16 h after transfection, the precipitate was removed and the cells were incubated for 4 min with 3 ml of a 15% (v/v) glycerol solution. The cells were then washed and incubated in fresh medium.

RNA extraction

At 48 h after transfection, cells were rinsed twice with cold phosphate-buffered saline and lysed by the addition of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 20 mM EDTA, 2% (v/v) N-lauryl-sarcosine and 200 μg/ml proteinase K. The lysate was incubated for 10 min at 30°C and then vortexed thoroughly to reduce its viscosity. The clear lysate was mixed with CsCl (1 g/ml) and underlayed with 1.2 ml of 5.7 M CsCl (Glisin *et al.*, 1974). After 16 h at 36 000 r.p.m. in a Beckman SW60 rotor, the RNA was recovered as a pellet, free of DNA and dissolved in 100 μl of 200 mM sodium acetate (pH 5.4) and precipitated with 3 vol. ethanol.

RNA analysis

RNA from transfected cells or from *in vitro* transcription assays was resuspended in 10 μl of 10 mM Tris (pH 7.4), 300 mM NaCl, 0.2 mM EDTA, 50% formamide (v/v) and mixed with an excess of single-stranded DNA probe or primer. The probes used for S1 analyses of early and late RNA were the relevant strands of the *AvaIID* fragment (coordinates 5118 to 557) of SV40 labelled at their 5′ ends. The primers for reverse transcription were isolated from the same fragment: early primer, **AvaII-HindIII* (5118 – 5171); late primer, *HaeIII-AvaII** (363 – 557). For the analysis of rabbit β-globin RNA, the *PstI-SfaNI** fragment was used as a single-stranded primer. Asterisks denote labelled ends (see Figure 2).

After heating at 80°C for 5 min, probes were allowed to hydridize for 18 h at 30°C. following primer extension (Hernandez and Keller, 1983) or S1 digestion (Weaver and Weissmann, 1979), the complementary DNA products or the S1-resistant, radioactive DNA fragments were analysed on 6% (w/v) acrylamide/8.3 M urea sequencing gels.

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