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Stimulation of *in vitro* transcription from the SV40 early promoter by the enhancer involves a specific *trans*-acting factor

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A nuclear extract prepared from HeLa cells has been used to study *in vitro* the transcription of the SV40 early promoter. The deletion of the enhancer results in a strong decrease of transcription, with spermidine and $MgCl_2$ being critical variables in the transcription reactions. Furthermore a competition assay indicates that the stimulation by the enhancer is due to a specific *trans*-acting factor(s) which acts on it. This factor appears not to interact with SV40 or adenovirus-2 major late upstream (distal) promoter sequences, and its ability to bind to the enhancer is diminished by mutations known to decrease enhancer function *in vivo* and *in vitro*.

Key words: enhancer/factor/*in vitro* transcription/RNA polymerase B (II)/SV40

Introduction

Enhancer elements increase dramatically the transcription from those genes in which they occur. First identified in the simian virus 40 (SV40) (72-bp repeat; Benoist and Chambon, 1981; Gruss *et al.*, 1981; Moreau *et al.*, 1981; Banerji *et al.*, 1981), they have been found in many viral genomes (Yaniv, 1983; Khoury and Gruss, 1983, and references therein) and more recently in some cellular genes (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Queen and Baltimore, 1983). Enhancers are *cis*-acting elements that can act in either orientation and over distances of several kilobase pairs, and potentiate either their natural promoter or heterologous promoters (Wasylyk *et al.*, 1983, and references therein). They may also play a role in cell or tissue specificity of gene expression (Yaniv, 1983; Khoury and Gruss, 1983; Banerji *et al.*, 1983; Gillies *et al.*, 1983; Queen and Baltimore, 1983; Chandler *et al.*, 1983; Picard and Schaffner, 1984, and references therein) and there has been some suggestion that specific transcription factors may recognize these sequences (Schöler and Gruss, 1984). *In vitro* transcription systems have demonstrated that for a number of genes, specific cellular factors interact with the TATA box region (Parker and Topol, 1984a; Davidson *et al.*, 1983) and upstream sequence elements (Dynan and Tjian, 1983; Parker and Topol, 1984b). We have shown that in HeLa whole cell extracts (WCE) the SV40 enhancer can stimulate transcription from heterologous promoters which are lacking a functional upstream sequence element (Sassone-Corsi *et al.*, 1984). We show here that in nuclear extracts the homologous SV40 early promoter is efficiently transcribed, and that under optimal ionic conditions and DNA concentrations this transcription is decreased 10- to 15-fold by deletion of the enhancer. This effect is observed even when the enhancer and the upstream sequence element (the 21-bp repeat) are not in close apposition. Furthermore, a competition assay reveals that the enhancement is due to the presence of a specific *trans*-acting factor(s).

Results

Enhancer-dependent transcription from the SV40 early promoter in vitro is obtained under optimal spermidine and $MgCl_2$ concentrations

Analysis of rabbit β-globin RNA synthesized from plasmids pA0 and pA56 [recombinants containing an SV40 early promoter region with an enhancer containing a single 72-bp sequence or with no enhancer, respectively (see Figure 1)] after calcium phosphate transfection into HeLa cells has revealed that the presence of the enhancer results in a dramatic stimulation of transcription from the SV40 early mRNA start sites (our unpublished results). Using HeLa whole cell (Manley *et al.*, 1980) or S100 (Weil *et al.*, 1979) extracts, the *in vivo* effect of the enhancer is reproduced only slightly, with a 2- to 4-fold stimulation of transcription normally being observed (Vigneron *et al.*, 1984; A. Wildeman, unpublished observations). Furthermore, using such extracts, the transcription products must often be analysed by quantitative S1 nuclease mapping because of the high background on run-off transcription gels. Nuclear extracts, prepared and used with the modifications discussed here, not only enable a clean run-off analysis of this promoter, but also give a greater stimulation by the enhancer.

Transcription *in vitro*, using whole cell or nuclear extracts, of several eukaryotic promoters [SV40, adenovirus-2 major late (Ad2ML), human β-globin, ovalbumin] is stimulated by the addition of polyamines such as spermidine or spermine to the transcription reaction (A. Wildeman, unpublished observations). While most *in vitro* transcription systems include $MgCl_2$ at a concentration of 4–12 mM, the addition of polyamines requires that less magnesium be used. Using *Acc*I digests of the constructions shown in Figure 1, the effects of $MgCl_2$ and spermidine were analyzed systematically. The run-off transcripts shown here correspond to RNA initiated at the early-early start sites (EES) (see Figure 1, and Vigneron *et al.*, 1984 and references therein). Long exposures of the gels reveal the late-early start sites (LES), and S1 nuclease mapping verified that the cap sites used *in vitro* correspond to those used *in vivo* (not shown). Furthermore, these S1 mapping experiments showed that the stimulatory effect of the enhancer was similar when linear and circular templates were used (not shown). Figure 2A shows that in the presence of 3 mM spermidine, increasing the $MgCl_2$ concentration above 1.4 mM is inhibitory for RNA synthesis, but optimizes the stimulatory effect of the enhancer sequence (pA0). Under these ionic conditions, both an enhancer containing a 72-bp repeat (pAW2 and pAW22, Figure 1) and one containing a single 72-bp element (pA0 and pA71, Figure 1) stimulate

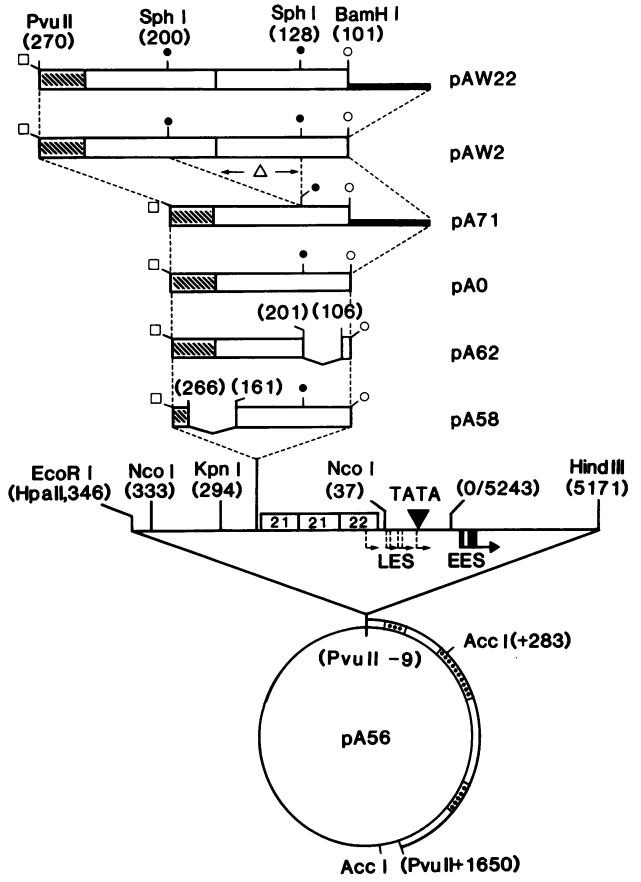

Fig. 1. Structure of recombinants used for studying the effect of the 72-bp repeat on transcription from the SV40 early promoter. pA56 contains the SV40 origin region from *Hpa*II (346) to *Hind*III (5171) fused to a rabbit β-globin coding sequence from −9 to +1650 (Van Ooyen *et al.*, 1979) (double line), and pBR322 sequences (single line) from *Pvu*II (2066) to *Eco*RI (4361). Within the SV40 sequences a deletion extending from a *Bam*HI site (101) generated by *in vitro* site-directed mutagenesis of nucleotides TAGTCC (106−101) to GGATCC (T. Grundström, M. Zenke, M. Winzerith, H. Matthew, A. Staub and P. Chambon, personal communication) to the *Pvu*II site (270) created an enhancerless promoter. pAW2 and pAW22 contain a wild-type 72-bp repeat region, with pAW22 having a pBR322 fragment (*Bam*HI to *Nar*I, coordinates 375−413, with a *Bgl*II linker ligated to the *Nar*I site) inserted at the *Bam*HI site (solid line). pA0 and pA71 have a single 72-bp sequence [generated by a deletion between *Sph*I (128) and *Sph*I (200)] with pA71 carrying the same pBR322 insertion as pAW22. pA62 and pA58 were derived from pA0. pA62 contains a deletion from 106 to 201 generated by a cutting at *Bam*HI and *Sph*I sites, followed by blunt-ending with Klenow enzyme and ligation. The deletion present in pA58 has been described previously (mutant TB101, Moreau *et al.*, 1981), and was cloned into the pA series by a *Kpn*I (294) - *Sph*I (128) transfer. Early-early (EES) and late-early (LES) start sites and TATA box and 21-bp repeat regions are indicated (Vigneron *et al.*, 1984). Hatched areas are sequences between the 5′ end of the 72-bp repeat (coordinate 251) and the *Pvu*II site (270). Nucleotide coordinates for SV40 follow the BBB system (Tooze, 1982).

transcription, with the former being slightly more efficient (Figure 2A, right panel), particularly when moved 43 bp away from the 21-bp repeat element (compare pAW22 and pA71). Different ratios of spermidine/$MgCl_2$ concentrations were also examined, using plasmids pA0, pA62, pA58, pA56 and pA71 (Figure 2B). pA62 and pA58 contain internal deletions in the single 72-bp sequence (see Figure 1) which reduce its efficiency *in vivo* by 15- and 50-fold, respectively (M. Zenke, T. Grundström, H. Matthes, M. Wintzerith and P. Chambon, personal communication). As seen in Figure 2B (lanes 1−6), very low concentrations of spermidine

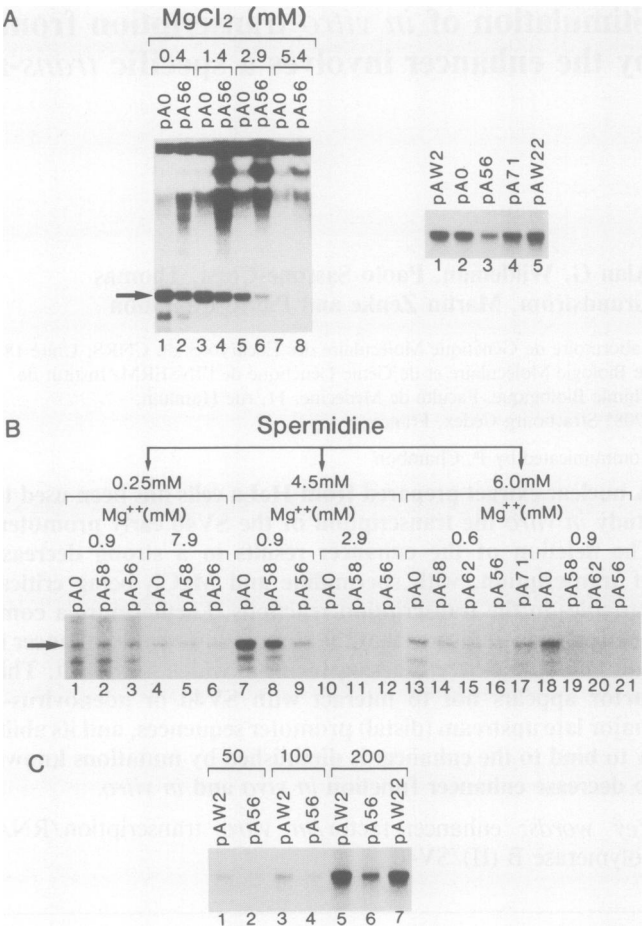

Fig. 2. Effect of spermidine and $MgCl_2$ on transcription from the SV40 early promoter with nuclear extracts. Run-off transcriptions were carried out as described in Materials and methods using total *Acc*I digests (see Figure 1) of the recombinants as indicated. KCl was added to 50 mM. **(A)** 200 ng of DNA were transcribed in the presence of 3 mM spermidine with either variable (left, as indicated) or constant (right, 2.9 mM) $MgCl_2$ concentrations. **(B)** 100 ng of DNA were transcribed in reactions in which both spermidine and $MgCl_2$ concentrations were varied as indicated. **(C)** 50, 100 or 200 ng of DNA were transcribed in the presence of 4.5 mM spermidine and 3.0 mM $MgCl_2$.

(0.25 mM) result in levels of transcription which are virtually unchanged by the deletion of the enhancer (compare pA0 with pA56). Background transcription can be lowered by increasing the $MgCl_2$ concentration (lanes 4−6), but again no enhancer effect is observed. The addition of spermidine to 4.5 mM (lanes 7−12) causes a specific enhancer-dependent stimulation of transcription at low $MgCl_2$ concentrations, lanes 7−9); the mutation in pA58 decreases this stimulation. Addition of $MgCl_2$ to 2.9 mM (lanes 10−12), a concentration which in the presence of 3.0 mM spermidine reveals a strong effect of the enhancer (Figure 2A, lanes 5 and 6, left panel) now inhibits transcription completely. As more spermidine is added (lanes 13−21) transcription is supressed further, with mutations present in pA58 and pA62 reducing the efficiency of the enhancer. Under these conditions the 72-bp sequence can still exert its influence when moved 43 bp away from the 21-bp repeat (pA71, lane 17). Lanes 18−21 show that when slightly more $MgCl_2$ is present (0.9 mM), transcription of pA0 is at an optimum for this spermidine concentration, while an enhancerless promoter is very inefficient (lane 21, pA56). Mutations which impair enhancer function *in vivo*

(pA58 and pA62, lanes 19 and 20) also do so *in vitro*. The addition of more $MgCl_2$, in the presence of 6 mM spermidine, inhibits all transcription (not shown).

In summary, transcription of the SV40 early promoter in nuclear extracts can be made sensitive to the presence of the enhancer by the inclusion of polyamines in the transcription reaction. Although less effective, spermine has an effect similar to that of spermidine (not shown). To compensate for the addition of spermidine to the transcription reactions the protocol for preparation of nuclear extracts was modified to include final dialysis with a low concentration of $MgCl_2$. The inhibitory effects of super-optimal concentrations of spermidine and $MgCl_2$ can be overcome by using more DNA template (Figure 2C). Since 100–300 ng of DNA were present in all subsequent experiments, incubations were routinely carried out in the presence of 3 mM $MgCl_2$ and 4.5 mM spermidine. For each preparation of extract the DNA concentrations must be optimized.

It is interesting to observe in Figure 2A (left panel) that the presence of the enhancer results in a reduction in the amount of background transcription visible at the top of the gel, suggesting that the 72-bp sequence is acting in the *in vitro* system to focus transcription on the SV40 promoter.

Stimulation of in vitro transcription by the SV40 enhancer involves a trans-acting factor(s)

A competition assay was used to investigate whether the stimulation of transcription by the 72-bp repeat could be due to a specific *trans*-acting factor(s). *In vitro* transcriptions of *Acc*I-digested pA0 and pA56 were carried out in the presence of various purified promoter elements to monitor the competition for transcription factors. The initial experiments were designed to see if transcription from the SV40 early promoter could be selectively competed for by a DNA fragment containing both the 21-bp repeat region and the enhancer. A purified *Kpn*I-*Nco*I fragment (position 294 to 37, see Figure 1) spanning this region was found to compete efficiently for transcription of pA0 (Figure 3A, lanes 2–4), whereas a fragment containing the upstream element of the Ad2ML promoter (Ad2MLP) (Hen *et al.*, 1982) did not (lanes 5–7). This result is in agreement with the previous observation made by Miyamoto *et al.* (1984) showing that the upstream element of the Ad2MLP did not compete out the effect of the 21-bp repeat region. When increasing amounts of a fragment containing the enhancer (*Kpn*I-*Bam*HI segment of pA0, see Figure 1) were used as a competitor, transcription of pA0 template was reduced to the level of that seen from a pA56 template (Figure 3B). (The decrease in transcription seen in lane 4 with 50 ng of competitor DNA was not observed in duplicate experiments; see also panel C). Such a result would be expected only if this fragment were trapping a factor(s) which acts on the enhancer present in the template DNA.

In Figure 3A and B it can be seen that the level of transcription of pA0 and of pA56 rises slightly as more Ad2MLP or pA0 fragments, respectively, are added. The magnitude of this phenomenon, which was also seen when non-specific DNA, such as pBR322, was added to the *in vitro* transcription system (e.g., Dignam *et al.*, 1983) varied with different extracts. To ensure that the competition effects observed were indeed enhancer specific, and not due to changes in DNA concentrations, various competitors were tested simultaneously using the same extract and DNA template preparations. Figure 3C shows that a pBR322-derived fragment (lanes 3 and 4) did not compete for transcription from either

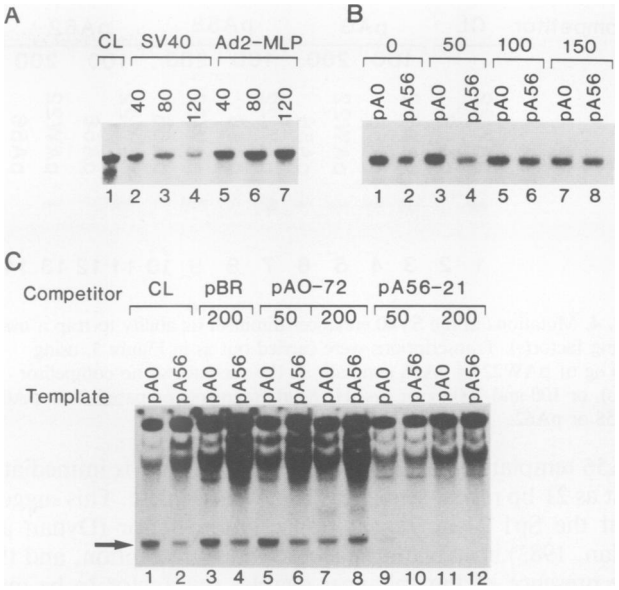

Fig. 3. Transcription from the SV40 early promoter is stimulated by an enhancer-specific *trans*-acting factor(s). 100 ng of either pA0 or pA56 template were transcribed using $MgCl_2$, spermidine and KCl concentrations of 4.5 mM, 3.0 mM and 50 mM, respectively, in the presence of various purified competitor fragments. **(A)** Transcription of pA0 in the presence of no competitor (CL), and 40, 80 or 120 ng of an *Nco*I-*Nco*I fragment (SV40 coordinates 37–333) of pA0 containing the SV40 21-bp and 72-bp repeat promoter elements (SV40), or a *Xho*I-*Xho*I fragment from the −260 to −29 positions of the Ad2MLP (Hen *et al.*, 1982). The *Xho*I site at −29 was created *in vitro* by site-directed mutagenesis (a gift of R. Hen and M. Wintzerith). **(B)** Transcription of pA0 and pA56 in the presence of 0, 50, 100 or 150 ng of a *Bam*HI to *Kpn*I fragment (SV40 coordinates 101 and 294, respectively) from pA0. **(C)** Transcription of pA0 and pA56 in the presence of no competitor (CL), 200 ng of an *Eco*RI to *Bam*HI fragment of pBR322 (position 185–375) (pBR), 50 and 200 ng of the *Bam*HI-*Kpn*I fragment used in **(B)** (pA0-72), or 50 and 200 ng of a *Nco*I to *Kpn*I fragment (coordinates 37 and 294) of pA56 containing the 21-bp repeat (pA56-21). Transcription of pA0 and pA56 in the presence of no competitor (CL) or 50, 125 and 200 ng of the Ad2MLP *Xho*I-*Xho*I competitor fragment (Ad2MLP), the 21-bp repeat fragment (pA56-21), or the 72-bp fragment (pA0-72).

pA0 or pA56. The fragment containing an enhancer sequence (lanes 5–8) competed only for the transcription from a promoter with an enhancer (pA0), but not for that from an enhancer-less template (pA56). At the same time, however, a fragment containing only the upstream element (the 21-bp repeat, lanes 9–12) competed for the transcription from either template, indicating that the enhancer factor is not acting on the upstream element, whereas the Ad2MLP upstream sequence again was unable to compete for transcription from either SV40 template (not shown).

In all of the competition experiments it is found that the amount of 72-bp repeat competitor required to totally compete the effect of the enhancer (~150–200 ng) represents a 20- to 40-fold molar excess. With respect to the template DNA, this excess is ~5 times greater than that required to compete for 21-bp repeat factor(s). Whether these molar values reflect a greater quantity of available enhancer-binding factor(s) than upstream sequence binding factor(s) in the extract, or a difference in the stabilities of the complexes between the competitor fragments and their cognate factors, should become more apparent as the nuclear extract is further purified. It is interesting to note that, as shown in Figure 3C, the difference in background transcription between pA0 and

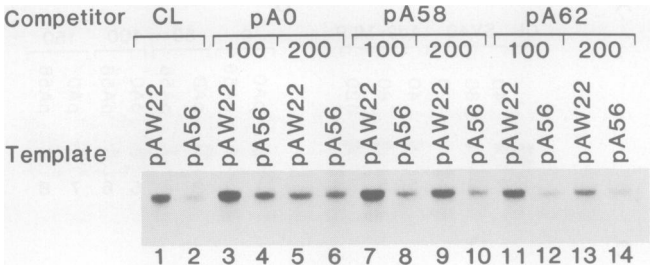

Fig. 4. Mutations in the SV40 enhancer diminish its ability to trap a *trans*-acting factor(s). Transcriptions were carried out as in Figure 3, using 200 ng of pAW22 or pA56 template, in the presence of no competitor (CL), or 100 and 200 ng of *Bam*HI-*Kpn*I fragments prepared from pA0, pA58 or pA62.

pA56 templates, visible at the top of the gel, is immediately lost as 21-bp repeat sequences begin to compete. This suggests that the Sp1 21-bp repeat transcription factor (Dynan and Tjian, 1983) is activating background transcription, and that the presence of the enhancer enables this factor to be more selective.

Mutated SV40 enhancers do not compete for the enhancer factor(s)

Competitor fragments (*Kpn*I-*Bam*HI segments) were prepared from pA58 and pA62 and tested for their ability to compete enhancer-dependent transcription using pAW22 and pA56 as templates. As shown in Figure 4, a competitor fragment derived from pA0 and containing an intact enhancer (lanes 3 – 6) competes only for transcription from the pAW22 template. When fragments derived from pA58 (lanes 7 – 10) and pA62 (lanes 11 – 14) were used, the efficiency of competition was strongly reduced compred with that of the fragment from pA0. Some weak competition against the pAW22 template was observed with 200 ng of these competitors lacking either the 5′ or the 3′ domain of the enhancer (lanes 9 and 13). These results show that a single 72-bp sequence can compete with an enhancer containing a 72-bp repeat, and that the competition is unaffected when the enhancer and 21-bp elements are separated.

Discussion

The enhancer can stimulate transcription from heterologous promoter elements *in vitro* (Sassone-Corsi *et al.*, 1984). The present study shows that using nuclear extracts a similar stimulation can be observed with the homologous promoter elements present. Transcription conditions which were not necessarily the most efficient were sought, to supress transcription coming from promoter elements in the absence of an enhancer. The role of various parameters was investigated, including Mg^{2+}, K^+ and polyamine concentrations, as well as temperature and time of transcription reactions. Interestingly, polyamines were found to impart much lower $MgCl_2$ requirements for transcription and to allow a more efficient transcription from a promoter containing an enhancer. The effect of altering KCl concentration was much less dramatic, and 50 mM was chosen as the working concentration. Unlike the initial protocol for transcription with nuclear extracts (Dignam *et al.*, 1983), the temperature optimum in the present study (35°C) was near the *in vivo* temperature; full-length run-off transcripts appeared after 10 min of incubation. Transcripts from a promoter with an enhancer were not synthesized noticeably earlier in the reaction than those from a promoter without an enhancer (not shown). The intracellular concentrations of polyamines can be in millimolar ranges (e.g., Cohen, 1971), and it is possible that the addition of spermidine to the nuclear extract and the use of 35°C for the transcriptions results in a more physiological condition. Polyamines are known to stimulate the transcription activity of purified RNA polymerase B (II) (Mandel and Chambon, 1974). It is also noteworthy that spermidine can have an optimizing effect, and lower the Mg^{2+} requirement, in *in vitro* translation systems (Konecki *et al.*, 1975).

The competition experiments indicate that transcription of the SV40 early promoter *in vitro* is stimulated by a specific *trans*-acting factor(s) which acts on the enhancer element. This factor appears to be unique from those which act on upstream promoter elements, and mutations which both *in vivo* and *in vitro* diminish the ability of the enhancer to stimulate transcription also diminish its ability to compete for the factor in a transcription reaction. It seems clear, therefore, that the mechanism of enhancer function involves, at least in part, the specific recognition of the enhancer element by a cellular factor(s). At the present time it is not possible to say if other properties of enhancers contribute to their function. The SV40 enhancer is capable of creating an 'open' chromatin structure *in vivo* (Jongstra *et al.*, 1984, and references therein), but whether this is due to specific proteins which are bound to the enhancer or because of some intrinsic property of the sequence (Wasylyk *et al.*, 1979) is not known. If it is due to specific proteins, it is possible that the factor revealed in the present *in vitro* study is also involved in the generation of the open chromatin structure. The availability of an *in vitro* system will now enable both a further purification of the factor, and a closer analysis of its interaction with the DNA template. The fact that an enhancer fragment of only 120 nucleotides is able to trap a factor suggests that chromatin structure is not necessary for recognition of the enhancer by the factor, and studies to examine the structure of the competitor fragment in the transcription reaction are currently in progress.

The present work has identified at least one transcription factor acting on the SV40 enhancer. Whether there is more than one protein involved is not yet clear. The activity of the enhancer is lowered by mutations in its 5′ or 3′ domains, implying either a relatively large protein contact site, the binding of more than one protein, or a coiled enhancer sequence. Preliminary experiments to compete with a mixture of the two fragments which each carry a deletion (i.e., prepared from pA58 and pA62) have been unsuccessful, suggesting that the two deletions may not represent binding sites for two different proteins, or that if they do, these proteins must interact with each other to activate the promoter.

The TATA box and the upstream sequence elements of eukaryotic class B (II) promoters have already been shown to interact with specific transcription factors (Parker and Topol, 1984a, 1984b; Davison *et al.*, 1983; Dynan and Tjian, 1983). The binding of an enhancer-specific factor, demonstrated in the present study, to its cognate element appears to have a stability *in vitro* similar to that of other factors (our unpublished results). Considering that the upstream element (the 21-bp repeat region, Vigneron *et al.*, 1984; Baty *et al.*, 1983) and the enhancer (Moreau *et al.*, 1981; Sassone-Corsi *et al.*, 1984, and our unpublished results) of the SV40 early promoter can still function when their position and/or orienta-

tion (with respect to the cap site and the TATA box element) are modified, it is interesting to speculate how the factors which bind to these three promoter elements can cooperate to promote efficient transcription. There must be either bending of the DNA template or movement of the factors from their recognition site to bring them into the necessary proximity. Alternatively, binding of the factors to their recognition sites may result in transmission of some DNA perturbation along the template by an as yet unknown mechanism. Considering that the SV40 enhancer factor operates *in vitro* either when the enhancer is in its natural position or moved away from the 21-bp repeat region (see pAW22, Figures 2 and 4), it should be possible to further explore the mechanism involved. Because the effect of the enhancer is seen *in vitro* using linear templates, it seems probable that DNA supercoiling is not required for the positional flexibility of promoter elements.

Materials and methods

In vitro transcriptions were carried out using HeLa cell nuclear extracts (NE) prepared as previously described (Dignam *et al.*, 1983), with the following modifications. The nuclear pellet was resuspended with the aid of a Dounce homogenizer (type B pestle) in 3 ml per 10^9 cells of 20 mM Hepes (pH 7.9), 25% glycerol (v/v), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 0.5 mM DTT. After gentle stirring for 30 min at 0°C, the lysate was centrifuged 20 min at 25 000 *g*. The supernatant was collected and precipitated by the addition of ammonium sulphate (0.33 g/ml), again with gentle stirring on ice. The precipitate was collected by centrifugation for 20 min at 25 000 *g*. The pellet was resuspended in 1/12 the volume of this supernatant in 20 mM Hepes (pH 7.9), 20 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, 17% glycerol, and dialyzed 10–12 h at 4°C against two changes (100 volumes each) of the same buffer. Aliquots were frozen in liquid nitrogen and stored at −80°C.

Transcription reactions were done in a final volume of 10 μl, using 4 μl of extract. Unlabeled ATP, UTP and GTP were added to give a final concentration of 0.5 mM each and CTP (α-^{32}P, 10 Ci/mmol) was added to 10 μM. $MgCl_2$, KCl, spermidine trihydrochloride, DNA template and DNA competitor fragments were added as indicated in figure legends. After mixing all of the components on ice, the reactions were carried out for 30 min at 35°C. Samples were SDS-phenol-$CHCl_3$ extracted and the transcription products analyzed on 5% polyacrylamide gels containing 8.3 M urea (Maxam and Gilbert, 1980). DNA templates were obtained by *Acc*I digestion of the pA series of plasmids shown in Figure 1. Templates were phenol-$CHCl_3$ extracted following *Acc*I digestion, and suspended in H_2O at an appropriate concentration. Fragments to be used as competitors in the *in vitro* transcription reactions were obtained by digestion of the recombinants with restriction enzymes as indicated in legends to figures, and purification on 6% polyacrylamide gels.

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