Discrete elements within the SV40 enhancer region display different cell-specific enhancer activities

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The SV40 enhancer contains three genetically defined elements, called A, B and C, that can functionally compensate for one another. By using short, synthetic DNA oligonucleotides, we show that each of these elements can act autonomously as an enhancer when present as multiple tandem copies. Analysis of a progressive series of B element oligomers shows a single element is ineffective as an enhancer and that the activity of two or more elements increases with copy number. Assay in five different cell lines of two separate enhancers containing six tandem copies of either the B or C element shows that these elements possess different cell-specific activities. Parallel oligomer enhancer constructs containing closely spaced double point mutations display no enhancer activity in any of the cell lines tested, indicating that these elements represent single units of enhancer function. These elements contain either a 'core' or 'octamer' consensus sequence but these consensus sequences alone are not sufficient for enhancer activity. The different cell-specific activities of the B and C elements are consistent with functional interactions with different *trans*-acting factors. We discuss how tandem duplication of such dissimilar elements, as in the wild-type SV40 72-bp repeats, can serve to expand the conditions under which an enhancer can function.

Key words: 'core' and 'octamer' elements/enhancer mutations/oligonucleotides/promoter elements

Introduction

Eukaryotic promoters appear to be composed of many *cis*-acting control elements that function cooperatively to activate transcription. Some elements such as the TATA box are shared by most promoters, but a larger class consists of elements that confer promoter specificity. This latter set includes elements that activate transcription when located close to the transcriptional start site, including the CAATT box and Sp1 binding sites, and enhancer elements which can activate transcription over large distances from a position either upstream or downstream of the initiation site (see Gluzman, 1985; McKnight and Tjian, 1986, for reviews). The great positional flexibility of enhancer elements was first described in studies of the SV40 72-bp repeat (Banerji *et al.*, 1981; Moreau *et al.*, 1981), which is an element of the SV40 early promoter (Benoist and Chambon, 1981; Gruss *et al.*, 1981; Fromm and Berg, 1982). Subsequently, enhancers have been shown to be associated with numerous other viral and cellular promoters (see Gluzman and Shenk, 1983; Gluzman, 1985).

Enhancers display both wide and narrow ranges of cell specificity. Many viral enhancers, such as those found in SV40,

polyoma, and Moloney sarcoma virus, activate transcription in a variety of cells but the relative activities vary with the cell line used (de Villiers *et al.*, 1982; Laimins *et al.*, 1982). In contrast, the activity of some enhancers is restricted to particular cell types. For example, heavy chain immunoglobulin gene enhancer function is largely restricted to cells of lymphoid origin (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Neuberger, 1983).

Nucleotide sequence comparisons between various enhancers have identified a number of consensus sequences that are shared among different sets of enhancers. Two such consensus sequences are the 'core' element GTGG^A/T^A/T^A/T^AG (Laimins *et al.*, 1982; Weiher *et al.*, 1983) and the 'octamer' sequence ATGCAAT (see Falkner *et al.*, 1986). The octamer sequence is found associated with a number of eukaryotic genes including the immunoglobulin upstream promoter and enhancer regions (Parslow *et al.*, 1984; Falkner and Zachau, 1984; Mason *et al.*, 1985). A sequence closely related to the octamer consensus is found within the SV40 72-bp repeat. The SV40 enhancer region also contains two sequences homologous to the core consensus: one contained within the 72-bp repeat and referred to as the SV40 core (Weiher *et al.*, 1983) and a second lying just outside of the repeat. Recent mutational studies of the SV40 enhancer indicate that SV40 enhancer function involves multiple elements; many of these elements are associated with these or other consensus sequences (Herr and Gluzman, 1985; Zenke *et al.*, 1986; Herr and Clark, 1986).

In one series of experiments (Herr and Gluzman, 1985; Herr and Clarke, 1986), point mutations that impaired both viral growth and enhancer activity were engineered into three distinct regions of an SV40 virus containing a single 72-bp element. Numerous viral revertants of these mutants were subsequently isolated. These were shown to harbor characteristic tandem duplications within the enhancer region which were responsible for restored activity. These duplications identified three separate elements called A, B and C, which are 21, 22 and 15 bp long, respectively. Point mutations in one or two of these elements resulted in revertants with duplications of the remaining wild-type element(s). For example, revertants of the mutant *dpm12*, in which the A and B elements are mutated, all carried tandem duplications that contained the C element (Herr and Gluzman, 1985).

These experiments indicated that the SV40 enhancer contains multiple elements that can functionally compensate for one another. This analysis was accomplished in the context of the entire SV40 early promoter and did not address the intrinsic activity of each element. To investigate the nature of the A, B and C elements, we have employed a strategy analogous to that used by Veldman *et al.* (1985) to study a polyoma enhancer element. DNA fragments corresponding to wild-type and mutant copies of the three elements were synthesized, ligated into multiple tandem copies, and each assayed for enhancer activity in the absence of any other SV40-derived sequences. We find that each of the three elements indeed possesses intrinsic enhancer function. By assaying the function of two of these elements, B and

C, in different cell lines, we show that each displays unique cell-specific enhancer activities.

Results

Construction of oligomerized enhancer elements and test plasmids

Figure 1 shows the location within the SV40 early promoter and the nucleotide sequence of the three elements A, B and C. The 22-bp B element and the 15-bp C element lie within the 72-bp sequence that is duplicated in wild-type SV40 strain 776 (but shown as only a single copy in this figure), while the 21-bp A element lies just upstream of this sequence (Figure 1A). The precise boundaries of the 21-bp A element are ambiguous because there exists a 3-bp terminal redundancy that is shown as gtg in Figure 1B (see Herr and Clarke, 1986). The boxed sequences in Figure 1B identify the 8-bp core (in elements A and C) and octamer (in element B) consensus sequences. SV40 enhancer function is impaired by each of the double point mutations, *dpm1*, *dpm2* and *dpm6*, that lie within the A, B and C elements, respectively, and whose sequence is shown in Figure 1B (Herr and Gluzman, 1985; Herr and Clarke, 1986).

To test the enhancer activity of oligonucleotides representing the three elements, tandemly repeated oligonucleotides were cloned as multimers into a site downstream of the β -globin gene in the plasmid $p\beta e^-$ (labeled enhancer in Figure 1C). This plasmid contains the human β -globin gene from the plasmid π SVHP $\beta\Delta$ 128 (Treisman *et al.*, 1983) cloned into the vector pUC119. This construction places the enhancer insertion site (the pUC119 *SphI* site) 2.2 kb downstream or 3.3 kb upstream of the β -globin transcriptional start site (Figure 1C). Given these distances, activation of β -globin RNA expression in the oligonucleotide-containing derivatives of $p\beta e^-$ is a strict test of enhancer function. In the experiments described below, the nomenclature used to identify each synthetic enhancer shows, in order, the number of repeats (e.g. 6 $\times$), the element involved (preceded by the letter M if mutant), the number of base pairs per repeat, and the orientation ('+' indicates the sequence shown in Figure 1B reads clockwise in $p\beta e^-$ as drawn in Figure 1C).

In the initial experiment, the activity of a 17-bp fragment (C17) corresponding to the C element was tested. This fragment was designed to create a *SmaI* site (CCCGGG) at the junction of multimerized C17 oligonucleotides. To achieve this the three wild-type SV40 Gs just upstream of the 15-bp C element (shown as ggg in Figure 1B) were included in C17 and the 3' terminal A residue of the C element was sacrificed (see Figure 1B). When preliminary experiments showed that multiple copies of C17 could function as an enhancer, we then constructed oligomers of either the A or B element sequences. We chose to test first the activity of 17-bp fragments corresponding to portions of the A and B elements that we thought were good candidates for displaying enhancer function (A17 and B17, Figure 1B). In this manner the repeat size remains constant in each construct and any difference in activity between these enhancers must therefore be sequence specific.

The sequences included in A17 (see Figure 1B) were chosen because mutagenesis experiments indicate that the A element core consensus sequence is important for SV40 enhancer function (Zenke *et al.*, 1986). When it became evident that this oligomer could not enhance transcription (see below) oligonucleotides (A21, Figure 1B) representing the entire genetically defined A element (Herr and Clarke, 1986) were tested. The design of the B17 oligonucleotides was directed by the asymmetric location of the *dpm2* mutations which inactivate the B element. When

Fig. 1. SV40 early promoter region, A, B and C element oligonucleotides, and $p\beta e^-$ experimental plasmid. (A) Diagram of the SV40 early promoter showing, from right to left, early transcriptional start sites, origin of replication, AT-rich TATA-like element, one imperfect (dashed box) and two perfect 21-bp repeats complete with Sp1 binding sites (black boxes), and a single copy of the 72-bp element. The A, B and C enhancer elements (stippled boxes) are shown below their corresponding position within this region. (B) The complete nucleotide sequence of each of the three elements A, B and C is shown in upper case letters. Any wild-type sequences flanking the element boundaries are shown as lower case letters: the 3-bp gtg terminal redundancy of the A element and the 5' ggg included in C17. The 8-bp consensus sequences, the core in A and C and the octamer in B, are boxed. The A, B and C enhancer debilitating double point mutations, *dpm1*, *dpm2* and *dpm6*, respectively, are shown above the wild-type sequences. The sequences included in the oligonucleotides used to create synthetic enhancers are shown below the wild-type sequence by a black arrow; the name of each oligonucleotide is shown to the left. The broken arrows with the Xs indicate the corresponding *dpm1*, *dpm2* or *dpm6* mutant oligonucleotides. (C) The experimental plasmid $p\beta e^-$ is depicted with the pUC119 sequences as a solid line interrupted with open boxes representing the M13 replication origin, β -lactamase gene and plasmid origin of replication. The polylinker (hatch marks) is split by insertion of the human β -globin gene sequences (stippled box). The β -globin start site is identified by a wavy arrow, with introns shown as bent lines linked to exons shown as hatched (untranslated) and solid (translated) boxes. The downstream enhancer cloning site (*SphI*) is labeled 'enhancer' and its distance from the β -globin start site is shown on the inner circle.

placed in tandem these various oligonucleotides generate sequences that share 18, 24, 18 and 17 bp (A17, A21, B17 and C17, respectively) with the wild-type SV40 sequence. For each oligomerized element a parallel oligomer construct was also created with oligonucleotides containing the *dpm1* (MA17,

Fig. 2. Multiple copies of the A, B and C elements can each independently enhance transcription in CV-1 cells. The levels of α - and β -globin probe RNAs protected from RNase digestion, by cytoplasmic RNA isolated from transfected CV-1 cells (see Materials and methods), are shown. The correctly initiated reference α -globin (132 nucleotides) and experimental β -globin (350 nucleotides) protected fragments are labeled α and β , respectively. The incorrect β -globin transcripts labeled it1 and it2 probably result from improper splicing of aberrant transcripts (see deVilliers and Schaffner, 1981). (A) Each lane represents transfection of the following p β plasmid: p β enh⁻ (lane 1), 6 $\times$ A17⁻ (2), 5 $\times$ MA17⁻ (3), 6 $\times$ B17⁻ (4), 6 $\times$ B17⁺ (5), 8 $\times$ MB17⁻ (6), 6 $\times$ C17⁻ (7), 6 $\times$ C17⁺ (8), 6 $\times$ MC17⁻ (9), 1 $\times$ 72⁻ (10) and 2 $\times$ 72⁻ (11). Lane 12 is the result of using only pUC119 DNA in the transfection. (B) Protected RNAs resulting from transfection of the A element constructions 6 $\times$ A21⁻ (lane 2), 6 $\times$ MA21⁺ (lane 3) are shown flanked by samples from enh⁻ (lane 1) and 1 $\times$ 72⁻ (lane 4) transfections. Separate experiments showed that A21 constructs in either orientation could enhance β -globin RNA levels.

MA21), *dpm2* (MB17) and *dpm6* (MC17) point mutations (Figure 1B).

Individual A, B and C elements have intrinsic enhancer activity

Initially enhancer function of each construct was assayed as the potential to stimulate expression of the human β -globin gene during transient expression in the African Green Monkey kidney cell line CV-1. Along with the experimental p β e⁻-derived plasmids, a human α -globin-containing plasmid was co-transfected to serve as a reference for transfection efficiency (see Materials and methods). The relative levels of α - and β -globin RNA were assayed by protection of internally labeled single-stranded α - and β -globin RNA probes from RNase digestion after hybridization to total cytoplasmic RNA from the transfected cells. This assay ensured that the analysis of β -globin expression would be limited to correctly initiated RNA.

The results of such an assay using a matched set of A, B and C element enhancer constructs are shown in Figure 2A. Each construct contains six tandem copies of the A17, B17 or C17 oligonucleotides or 5–8 copies of the corresponding mutant oligonucleotide (MA17, MB17 or MC17). As expected, the enhancerless plasmid p β enh⁻ alone yields a very low level of β -globin RNA (lane 1) and levels can be stimulated from 50- to >100-fold by the inclusion of a copy of the SV40 enhancer containing a single 72-bp element (1 $\times$ 72; lane 10) or two 72-bp elements (2 $\times$ 72, lane 11). Lanes 2–9 show the relative activity of the different oligomerized enhancer elements. The relative

Table I. Activity of synthetic A, B and C enhancers in CV-1 cells

Enhancer	Relative levels of β -globin RNA
1 $\times$ 72	1.0 (8)
2 $\times$ 72	2.5 $\pm$ 0.3 (4)
6 $\times$ A17	0.03 $\pm$ <0.01 (2)
5 $\times$ MA17	0.03 $\pm$ 0.01 (2)
6 $\times$ A21	0.25 $\pm$ <0.01 (2)
6 $\times$ MA21 ⁺	0.03 $\pm$ <0.01 (2)
6 $\times$ B17	0.74 $\pm$ 0.09 (7)
6 $\times$ B17 ⁺	0.65 (1)
8 $\times$ MB17	0.02 $\pm$ <0.01 (4)
6 $\times$ C17	0.24 $\pm$ 0.04 (8)
6 $\times$ C17 ⁺	0.21 (1)
6 $\times$ MC17	0.02 $\pm$ <0.01 (2)
enh ⁻	0.02 $\pm$ 0.01 (4)

Values are shown relative to the level of β -globin RNA produced by the 1 $\times$ 72 construct. The samples were quantitated as described in Materials and methods and are shown with the standard deviation. The number of determinations is shown in parentheses. All samples are in the ‘-’ orientation except where indicated.

levels of β -globin RNA were determined by measuring the level of radioactivity in each band, followed by normalization to the level of radioactivity in the α -globin reference band. The relative

Fig. 3. Progressive increase in B element oligonucleotides results in increased enhancer activity. Plasmids were transfected into CV-1 cells and RNAs probed as described in Materials and methods. The bands corresponding to the reference α -globin and experimental β -globin RNAs are labeled α and β , respectively. (Incorrect transcripts it1 and it2 are described in the legend to Figure 2.) **Lane 1**, the enhancerless control $p\beta enh^-$; **lane 2**, $1 \times B22^+$; **lanes 3–7**, two to six tandem copies of the B17 oligonucleotide; **lanes 8–10**, eight, ten and fourteen copies of B17, respectively; **lane 11**, $8 \times MB17^+$; and **lanes 12 and 13**, $1 \times 72^+$ and $2 \times 72^-$ wild-type controls. The B17 enhancer constructs in this figure are all in the (+) orientation.

normalized levels of correctly initiated β -globin RNA are shown in Table I.

Both the wild-type and mutant A17 constructs ($6 \times A17^-$ and $5 \times MA17^-$) failed to enhance β -globin expression in CV-1 cells (lanes 2 and 3). The lack of any detectable $6 \times A17^-$ enhancer function suggested that this 17-bp fragment does not represent a functional A element. To investigate this possibility further we tested the ability of A21 (Figure 1B) to enhance transcription. In contrast to the A17 oligomer, six copies of A21 ($6 \times A21^-$) do enhance β -globin RNA levels in CV-1 cells (Figure 2B, lane 2; Table I). This activity is specific to the A element because the activity is abolished by the presence of the *dpm1* point mutations in the $6 \times MA21^+$ construct (lane 3). These results indicate that the A element core consensus sequence is not sufficient for enhancer function.

In contrast to the A17 oligonucleotides, six copies of the B17 (lanes 4 and 5) or C17 (lanes 7 and 8) oligonucleotides can enhance β -globin expression in CV-1 cells. The $6 \times B17^-$ construction yields nearly equal levels of β -globin RNA as the 1×72 control while the $6 \times C17^-$ plasmid generates 24% the level of 1×72 (see Table I). These results demonstrate that the B and C elements alone are authentic enhancers when present as multiple copies. The $6 \times B17^-$ construct is more active than the $6 \times C17^-$ enhancer in CV-1 cells, but this does not necessarily mean that

the B element is an intrinsically stronger enhancer because the B17 and C17 oligonucleotides do not necessarily reflect the entire B and C elements.

The parallel enhancer constructs that contain the *dpm2* ($8 \times MB17^-$) and *dpm6* ($6 \times MC17^-$) point mutations are not capable of enhancing β -globin expression in our assays (lanes 6 and 9). (It was not considered necessary to obtain a matched $6 \times MB$ construct because $8 \times MB17$ itself does not display any function.) These results indicate the activity of the B17 and C17 constructs are a true reflection of SV40 enhancer activities. The *dpm* mutations in the oligomerized constructs have a greater effect than the same mutations have when they are in the context of the SV40 enhancer (compare with Herr and Clarke, 1986). This is probably because in the $8 \times MB17^-$ and $6 \times MC17^-$ constructs there are no other compensatory elements. The inactivity of the MA21, MB17 and MC17 constructs indicates that the *dpm1*, *dpm2* and *dpm6* mutations completely inactivate the A, B and C elements in the wild-type enhancer. Furthermore, the lack of enhancer activity of $8 \times MB17$ indicates that the B element octamer consensus sequence is not sufficient for enhancer function because the *dpm2* mutations lie outside of this sequence (see Figure 1B).

Individual enhancer elements function bidirectionally

One of the hallmarks of enhancers is that they can activate transcription independently of orientation. To test the orientation dependence of the B and C elements, we measured the relative activity of the $6 \times B17$ and $6 \times C17$ enhancers in both orientations. After transfection into CV-1 cells, the pair of $6 \times B17$ enhancers (Figure 2A, lanes 4 and 5) and the pair of $6 \times C17$ enhancers (lanes 7 and 8) each yield nearly identical levels of β -globin RNA (see Table I). These results suggest that the bidirectionality of the SV40 enhancer region is due to the accumulation of a number of individual bidirectional enhancers and is not due to the grouping of a number of unidirectional elements oriented in opposite directions.

Comparative activities of single and multiple B elements

To study the activity of both a single enhancer element and the effect of progressive tandem addition of oligonucleotide units, the activity of a series of B element enhancers was assayed. The results are shown in Figure 3. When assaying the activity of one or a few oligomers it is necessary to take into account the exact junction sequences of each construct. As described above, the tandem ligation of B17 oligonucleotides creates an 18-bp sequence of the wild-type SV40 B element. Therefore, a single B17 element is an under-representation of the B element sequence found in the B17 repetitions. Furthermore the double-stranded B17 DNA fragments contain a single 3' nucleotide overhang which would be lost before ligation into $p\beta e^-$ (see Materials and methods). To guarantee the best possibility of a single element displaying enhancer activity the plasmid construct $p\beta 1 \times B22$, which contains the entire 22-bp B element sequence (see Figure 1B), was used. In separate experiments, three tandem copies of this element ($3 \times B22$) displayed excellent enhancer function (about equivalent to $6 \times B17$, data not shown). The single B22 element does not exhibit any evident enhancer function (compare lanes 1 and 2, Figure 3), nor does $1 \times B22$ in the opposite orientation (data not shown). These results suggest that solitary enhancer elements possess little if any enhancer activity.

In contrast to the results with $1 \times B22$, two tandem copies of the B17 fragments (Figure 3, lane 3) exhibit a detectable level of enhanced β -globin RNA relative to the enhancerless control

Fig. 4. The B and C enhancer constructions display different cell specificities. A set of nine samples were transfected into NIH/3T3 (A), NS-1 (B), MDCK (C) and HeLa cells (D), corresponding to the following lanes: 1, enh^- ; 2 and 3, $6 \times A17^-$ and $5 \times MA17^-$; 4 and 5, $6 \times B17^-$ and $8 \times MB17^-$; 6 and 7, $6 \times C17^-$ and $6 \times MC17^-$; 8, $1 \times 72^-$; and 9, pUC DNA alone. RNAs were isolated from cells and probed as described in Materials and methods. The protected α - and β -globin RNAs are shown labeled α and β , respectively. In **panel D**, lanes 10 and 11 show the result of transfection of $6 \times B17^+$ and $6 \times C17^+$, respectively. The samples in lane 5 and 6 of **panel A** are over-represented (see the α -globin bands and the quantitation in Table II).

Table II. Relative activities of $6 \times B17$ and $6 \times C17$ in five different cell lines

Cell line	Enhancer				Ratio $6 \times B17/6 \times C17$
	1×72	$6 \times B17$	$6 \times C17$	enh^-	
CV-1	1.0 ± 0.06	0.74 ± 0.9	0.24 ± 0.04	0.02 ± 0.01	3.2
NIH/3T3	0.9 ± 0.22	0.10 ± 0.05	1.30 ± 0.30	0.34 ± 0.23	0.1
NS-1	1.0 ± 0.10	0.95 ± 0.07	2.00 ± 0.74	0.25 ± 0.06	0.5
MDCK	1.0 ± 0.10	0.41 ± 0.05	0.23 ± 0.05	0.07 ± 0.03	1.8
HeLa	1.0 ± 0.15	0.08 ± 0.02	0.13 ± 0.04	$0.02 \pm <0.01$	0.6

Levels of β -globin RNA relative to the 1×72 sample were determined as described in Materials and methods. The enhancer constructs are the same as those assayed in Figure 4. Determination of activities in CV-1 cells was as described in Table I. For NIH/3T3, NS-1 and MDCK cells two separate transfections were performed with duplicate samples for $1 \times 72^-$, $6 \times B17^-$ and $6 \times C17^-$ (four determinations) and a single sample of enh^- DNA per transfection (two determinations). In one transfection of NIH/3T3 cells the samples were normalized to twice the $1/2 \times 1 \times 72$ control because of unusually low 1×72 β -globin levels. For HeLa cells three separate transfections were performed as above (i.e. six determinations).

(lane 1). (Fortuitously, in the orientation used for this experiment the vector sequences recreate an 18-bp B repeat. Therefore $2 \times B17$ contains two complete 18-bp repeats.) These results suggest that two, but not one, enhancer elements can enhance transcription. Lanes 4–10 show that progressive addition of B17 oligonucleotides (from 3 to 14 copies) results in a linear increase in enhancer activity (quantitation not shown). As expected, eight copies of the mutant B element in the same orientation ($8 \times MB17^+$, lane 11) do not enhance transcription. These results indicate that beyond two elements there is no obvious synergism between elements and that, in this assay, the cumulative enhancer activity is rate limiting for transcription. Such a limitation is consistent with the results of Zenke *et al.* (1986) in which a similar progression in the number of 72-bp

elements could generate a linear increase in activity. The inactivity of a single B element and activity of two elements are consistent with the results of Veldman *et al.* (1985) in which two copies, but not one copy, of a synthetic 26-bp fragment of the polyoma enhancer could support enhancer-dependent polyoma virus DNA replication.

The B and C elements display cell-specific enhancer activities

To investigate whether the activity of the A, B and C elements is dependent on the cell line used, we have examined the enhancer activity of the various oligomer constructs in four other cell lines besides CV-1: NIH/3T3, a mouse embryo fibroblast cell line; NS-1, a non-secreting mouse myeloma cell line; MDCK, a canine kidney epithelial-like cell line; and HeLa, a human epitheloid

cervical carcinoma cell line. The results of one set of assays are shown in Figure 4. Table II gives the averaged results of four determinations. Consistent with the results in CV-1 cells the 6×A17 enhancer does not function in any of the four cell lines (lane 2, Figure 4A–D), supporting the conclusion above that the A element core homology is not sufficient to confer enhancer function. A functional A element enhancer (6×A21) has not yet been assayed in these cell lines. To directly compare an A element construct with the 6×B17 and 6×C17 it will be important to generate a new 17-bp A oligomer that can enhance transcription. The 6×B17 enhancer shows clear activity in three of the cell lines (NS-1, MDCK and HeLa; lane 4 in Figure 4B–D), while the 6×C17 enhancer is active in all four cell lines tested (lane 6 in Figure 4A–D). These results demonstrate that the B and C elements, originally defined genetically by SV40 revertant analysis in CV-1 cells, can actually function as enhancers in a broad spectrum of cell lines.

Although the B and C element enhancers are active in multiple cell lines, their level of activity differs in separate lines. For example, in HeLa and MDCK cells the synthetic enhancers are not as active as the wild-type 1×72 enhancer (compare lanes 4 and 6 with lane 8 in Figure 4C and D) but in NS-1 cells the 6×B17 and 6×C17 enhancers are either as active (Figure 4B, lane 4) or more active (lane 6) than the 1×72 enhancer (lane 8). Most striking is that the individual elements display different activities relative to each other in the various cell types: the 6×B17 enhancer is more active than 6×C17 in CV-1 cells (Figure 2; compare lanes 4 and 7) and MDCK cells (Figure 4C; lanes 4 and 6), whereas in NIH/3T3, NS-1 and HeLa cells the 6×C17 enhancer is the more active (Figure 4A, B and D; compare lanes 4 and 6; Table II). In HeLa cells, these relative activities are independent of orientation (panel D, lanes 10 and 11). These results indicate that the SV40 enhancer B and C elements have different cell-specific enhancer activities.

The closely spaced *dpm* mutations within the B and C elements (MB17 and MC17; Figure 4, lanes 5 and 7) abolish enhancer function in all cell lines tested. Of particular interest is that the mutant B17 construct, in which the octamer sequence is unaffected, does not function in a lymphocyte cell line (NS-1); therefore, the SV40 octamer sequence itself is not likely to be a lymphoid-specific enhancer element. Taken together, the activity of the wild-type oligomers and inactivity of the mutant oligomers indicate that the B and C elements represent single units of enhancer function.

Discussion

We have shown that each of the three SV40 enhancer elements, A, B and C, which were originally defined by revertant analysis of SV40 enhancer mutants in CV-1 cells, can enhance transcription when present as multiple copies. Thus the SV40 enhancer can be characterized as a region composed of at least three discrete enhancer elements. Unlike the SV40 enhancer, the A, B and C enhancer elements probably represent single units of enhancer function because: (i) they were not subdivided by the revertant duplications that originally defined the elements (Herr and Gluzman, 1985; Herr and Clarke, 1986) and (ii) the *dpm* mutations in the B and C elements inactivate these elements in a variety of different cell lines (see Figure 4). Thus the SV40 revertant analyses were able to identify three independent enhancer elements, at least two of which display a relaxed cellular specificity. The revertant analysis was limited to CV-1 cells and therefore may not have detected additional elements that cannot compensate for loss of SV40 enhancer function in these cells.

Fig. 5. Putative interactions and relative activities of the 1×72 and 2×72 SV40 enhancers in the presence of different sets of *trans*-acting factors. The position of the A, B and C elements is shown by the labeled rectangular boxes and the regions located within the 72-bp element are shown by the black arrows. Putative element-specific *trans*-acting factors are shown as circles with the bottom portion shaded differently to emphasize the postulated different DNA binding specificities of these factors. (A) shows the 1×72 enhancer and (B) the 2×72 enhancer in three states: in the presence of (i) all three A, B and C element-specific factors, (ii) the C element-specific factor alone, and (iii) the B element-specific factor alone. The expected relative activities of each enhancer in these three states is shown as very high activity (heavy arrow), high activity (light upward arrow), and low activity (downward arrow).

In contrast to the drastic effect of the *dpm* mutations on the activity of the oligonucleotide constructs, the effects of these point mutations vary when placed in the context of the SV40 enhancer region. In CV-1 cells, the *dpm6* mutations in the C element have the largest effect on enhancer function, while the *dpm1* mutations in the A element have the least effect (Herr and Clarke, 1986; unpublished results). These results suggest that the relative contribution of these three elements to SV40 enhancer function in CV-1 cells is $A < B < C$. The experiments described here do not address whether these relative activities are due to the intrinsic strength of the elements, their neighboring sequences, or their relative position with respect to other promoter elements.

Each of the enhancer elements, A, B and C, contain sequence motifs that have been implicated in SV40 enhancer function. The core (Laimins *et al.*, 1982; Weiher *et al.*, 1983) and related consensus sequences (Weiher and Botchan, 1984; Zenke *et al.*, 1986) either lie entirely within or overlap the A and C elements. Point mutations within these sequences, including *dpm6* (see Figure 1B), affect SV40 enhancer function (Zenke *et al.*, 1986; Herr and Clarke, 1986) suggesting that they are important constituents of the SV40 enhancer. Nevertheless, the core element does not appear to be sufficient for enhancer function because the A17 oligonucleotide, which contains an entire core element, does not

enhance transcription and the *dpm1* mutations in MA21, which lie outside of the A core element, inactivate the A element. These results are consistent with the hypothesis that the core sequences within the A and C elements represent sub-elements, which themselves do not possess intrinsic enhancer function but which cooperate to create an autonomous enhancer element (see Herr and Clarke, 1986).

As with the A element core sequence, the B element octamer consensus sequence is not sufficient to confer enhancer activity because *dpm2* lies outside of the octamer but inactivates the B element in all cell lines tested. This conclusion is consistent with the result that a set of three point mutations within the octamer sequence does not have much effect on SV40 enhancer function in HeLa cells (mutant 24; Zenke *et al.*, 1986). That the octamer also does not work in the NS-1 lymphocyte cell line (see Figure 4B, lane 5) contrasts with the results of Davidson *et al.* (1986) who suggest that the octamer alone does function as an enhancer in the lymphocyte cell line MPC 11. These apparently conflicting results may reflect a difference in the two assays. In the experiments described here enhancer function was assayed by activation of transcription over a large distance (> 2 kb) whereas Davidson *et al.* (1986) assayed the octamer element in its natural context, ~ 140 bp upstream of the SV40 early transcriptional start sites. Thus the octamer homology could belong to a class of upstream activation elements that can only act over a limited distance.

Similar to the structure of the SV40 enhancer region, the enhancer of polyoma virus contains multiple redundant enhancer elements of which one, but not another, was shown to function autonomously as an enhancer (Veldman *et al.*, 1985). The polyoma enhancer region can be strategically divided into two separate restriction fragments; both of these fragments can enhance transcription and both probably contain multiple enhancer elements (Herbomel *et al.*, 1984; Veldman *et al.*, 1985). As with the small SV40 B and C enhancer elements, these two relatively large (109 and 137 bp) polyoma enhancer fragments exhibit different cell-specific enhancer activities (Herbomel *et al.*, 1984). Consistent with these different cell specificities, duplications of specific regions within these polyoma enhancer fragments are responsible for altered cell specificities of polyoma enhancer function (see Amati, 1985). It is not yet known how many individual units of enhancer function are responsible for the polyoma enhancer cell-specific activities.

It is significant that the relative activities of the B and C elements can actually invert with respect to one another. In the experiments described here the B element construct (6×B17⁻) enhanced β -globin expression more effectively in CV-1 and MDCK cells, whereas the C element construct (6×C17⁻) was more effective in HeLa, NS-1 and NIH/3T3 cells (see Table II). This strongly suggests that these two elements interact differently with the transcriptional apparatus and may well reflect functional interactions with different *trans*-acting factors. The activities of the B and C elements are quite broad — enhancer function went undetected only with the 6×B17 construct in NIH/3T3 cells — indicating that they interact with *trans*-acting factors that are common to a large number of different cell types. The different relative activities of the B and C elements may reflect different patterns of expression of these putative B and C element-specific transcription factors. As an example, the activity of these transcription factors could fluctuate according to the cell cycle. The different activities we observe could thus represent the assay of enhancer function in cell lines with different cell-cycle patterns.

Why a 72-bp repeat?

The prototype SV40 enhancer is derived from the SV40 strain 776 and contains a 72-bp tandem repeat. Numerous other SV40 wild-type strains contain enhancer duplications of different sizes ranging from 64 to 93 bp in length (Reddy *et al.*, 1978; Ghosh *et al.*, 1981; Benoist and Chambon, 1981; Been *et al.*, 1984). Although the 72-bp repeat contains only elements B and C (see Figure 1) the larger SV40 enhancer duplications include all three elements. SV40 can grow effectively without a duplicated enhancer (van Heuverswyn and Fiers, 1979) but the 72-bp repeat generates both a more active enhancer and robust virus (see Herr and Gluzman, 1985). We describe below how such tandem duplications, that span multiple dissimilar elements, could serve to expand the range of activities of an enhancer.

The analysis of single and multiple synthetic B element oligomers indicates that a single element (1×B22) is ineffective as an enhancer but that two or more elements are clearly capable of enhancing transcription (see Figure 3). The revertants of SV40 enhancer mutants indicate that duplicated B or C elements can also function autonomously as enhancers within the context of the SV40 enhancer. Revertants of the *dpm12* SV40 enhancer mutant can effectively enhance transcription and these revertants contain duplications of the wild-type C element in the presence of mutated A and B elements (Herr and Gluzman, 1985). Likewise revertants of the mutant *dpm16* show that duplications of the B domain can function when the A and C elements are mutated (J. Clarke and W. Herr, unpublished results). It is also clear that single elements in conjunction with other elements (as in 1×72) are effective enhancers (see Figures 1 and 4). These results suggest that these elements may either cooperate with themselves or with each other to create a functional enhancer.

In vivo and *in vitro* experiments involving competition between *cis*-acting elements suggest that the SV40 enhancer interacts with cellular *trans*-acting factors to effect activation of transcription (Wildeman *et al.*, 1984; Scholer and Gruss, 1984, 1985; Mercola *et al.*, 1985; Sassone-Corsi *et al.*, 1985). DNase I and dimethylsulfate protection of the SV40 enhancer by nuclear extracts indicate that a large number of factors interact with specific sequences within this region (Wildeman *et al.*, 1986; Davidson *et al.*, 1986). Point mutations known to adversely affect SV40 enhancer function *in vivo* also inhibit the interaction of nuclear factors with the mutated sequences (Wildeman *et al.*, 1986; Zenke *et al.*, 1986). Taken together these results suggest that the deleterious effect of the *dpm1*, *dpm2* and *dmp6* mutations is functionally equivalent to the absence of *trans*-acting factors specific for these elements. Thus the low activity of the *dpm12* enhancer, in which A and B are mutated, may be analogous to the activity of the wild-type 1×72 enhancer in the absence of A and B element-specific factors; similarly, the relatively high activity of the *dpm12* revertants containing two copies of the C element may reflect the activity of a duplicated wild-type SV40 enhancer under the same conditions.

Figure 5 shows a schematic representation of the 1×72 and 2×72 enhancers in three such different states: in the presence of putative factors interacting with all three elements (i), or in the presence of just the C element-specific (ii), or B element-specific (iii), transcription factors. Also shown are the deduced activities of these two enhancers under the three different conditions by analogy to the activities of the various mutants and revertants. Therefore, the 1×72 enhancer is shown to be active in the presence of all three factors (state i) but relatively inactive when only one specific factor is present (states ii and iii); this

is analogous to the activities of the wild-type 1×72 enhancer, and the mutant *dpm12* and *dpm16* enhancers, respectively (Herr and Gluzman, 1985; J. Clarke and W. Herr, unpublished results). The duplicated enhancer, however, is functional in all cases because states (ii) and (iii) are analogous to the functional *dpm12* and *dpm16* revertants, respectively.

These interpretations suggest that by carrying single copies of two elements that recognize different *trans*-acting factors, the function of an enhancer would be restricted to the presence of both *trans*-acting factors. By tandem duplication of these elements expression would be expanded to the presence of either factor. Thus appropriate manipulation of enhancer elements could either restrict or expand the conditions under which a promoter can operate.

Materials and methods

Oligonucleotide enhancer constructions

Synthetic A, B and C enhancers were constructed in the plasmid p β e⁻. This plasmid contains the β -globin gene on a *Xba*I–*Pst*I fragment, from π SVHP β Δ128 (Treisman *et al.*, 1983), inserted into the pUC119 polylinker region between the *Xba*I and *Pst*I sites. The p β e⁻ vector was prepared by *Sph*I digestion followed by end-repair with DNA polymerase I and calf intestinal alkaline phosphatase treatment. Religation of this DNA produced p β enh⁻ which was used as the enhancerless control. The A, B and C element enhancers were constructed using complementary synthetic oligonucleotides. The oligonucleotides were phosphorylated by polynucleotide kinase, annealed, ligated into tandem unidirectional repeats by virtue of non-palindromic overlapping nucleotides, and end-repaired by DNA polymerase I. This population of multiple repeats was fractionated by size on a native 5% polyacrylamide gel and selected populations of differently sized fragments were blunt-end ligated to the p β e⁻ vector. DNAs were transfected into the *Escherichia coli* strain MV1193. Potential clones were screened by isolating single-stranded DNA (making use of the M13 origin of replication in pUC119) and dideoxynucleotide sequencing (Sanger *et al.*, 1977) using a 15-nucleotide-long primer specific to the adjacent β -globin sequences and a single chain terminator (T-tracking). Clones selected for analysis were sequenced in entirety.

In certain cases (6×A21⁻, 8×MB17⁺ and 6×C17⁻) the orientations were obtained by inverting the oppositely oriented enhancer fragment by digestion at the adjacent *Pst*I and *Hind*III sites, end-repair using DNA polymerase, and religation into the *Sph*I-cut p β e⁻ DNA. The 1×B22⁺ construction contains the entire 22-bp B element, as shown in Figure 1B, and was made by filling in the 3-bp 5' overhang of the annealed B19 oligonucleotides followed by ligation into the p β e⁻ vector. The wild-type 1×72 enhancer was cloned from the plasmid pAO (Zenke *et al.*, 1986) as a blunt-end *Bam*HI–*Pvu*II fragment into the *Sph*I site of p β e⁻ to create p β 1×72. To create p β 2×72, the 72-bp SV40 *Sph*I fragment was cloned into the *Sph*I site of p β 1×72.

The A, B and C oligonucleotides used for cloning are listed 5' to 3' as shown in Figure 1B. The underlined nucleotides represent the single-stranded overhangs that are formed after being annealed to their complementary oligonucleotide:

A17, GTGGAATGTGTGTCAGT;
 MA17, GTGGAATGAGAGTCAGT;
 A21, GTGGAATGTGTGTCAGTTAGG;
 MA21, GTGGAATGAGAGTCAGTTAGG;
 B17, AAGTATGCAAAGCATGC;
 MB17, AGGAAGTATGCAAAGCA;
 B19, GCAGAAGTATGCAAAGCAT;
 C17, CGGGTGTGGAAAGTCCC;
 MC17, CGGGTGTCCAAAGTCCC.

Some of the constructs contain small changes from the expected structure. These are: in 6×C17 the CGG 5' overhang is deleted in the first repeat, 8×MB17 contains a G to A transition in the sixth repeat and a C insertion between the sixth and seventh repeats, and 6×A21 contains a G insertion between the first and second repeats (the repeats are ordered with respect to the sequencing primer).

Cell lines

CV-1 cells, originally obtained from the ATCC (#CCL70), and HeLa cells were the same lines as used previously (Herr and Gluzman, 1985; Herr and Clarke, 1986). NIH/3T3, MDCK and NS-1 cells were gifts from L. Rodgers, M. Quinlan and E. Harlow, respectively, all at Cold Spring Harbor Laboratory. HeLa, MDCK, NS-1 and NIH/3T3 cells were maintained in 10% fetal bovine serum in Dulbecco's

modified Eagle's minimal medium with 100 U/ml penicillin and 100 μ g/ml streptomycin. CV-1 cells were maintained in the same medium but with 5% fetal bovine serum.

DNA transfections

DNAs for cell transfections included the 5.5-kb p β test plasmid, the 2.7-kb α -globin containing π SVHP α 2 plasmid (see Treisman *et al.*, 1983), and either pUC13 or pUC119 carrier DNA where appropriate. Mock transfections contained pUC DNA alone. To determine whether the assay was in a linear range of response each series of transfections included a sample which contained one-half the concentration of either p β 1×72 or p β 2×72 DNA. Plasmid DNAs were prepared by alkaline lysis, purified by CsCl gradient centrifugation, and further purified by polyethylene glycol precipitation.

The CV-1, HeLa, NIH/3T3 and MDCK cells were transfected by calcium phosphate co-precipitation. Cells were seeded 1 day prior to transfection as follows: CV-1 cells were seeded to produce 50–70% confluency the day of transfection; HeLa cells were transfected at 30–50% confluency; MDCK cells were seeded at $\sim 1.5 \times 10^6$ cells/100-mm plates (30–40% confluency); and NIH/3T3 cells were seeded at $\sim 5 \times 10^6$ cells/plate (30–50% confluency). Calcium phosphate co-precipitates were prepared as described previously (Herr and Clarke, 1986) and left on the cells for 12–16 h. Cytoplasmic RNA was prepared by NP40 lysis as described (Herr and Gluzman, 1985) 60 h post initiation of the transfection except for MDCK and NIH/3T3 cells. For these latter transfections the precipitate was left for 20 h and RNAs were collected 48 h (MDCK) or 50–60 h (NIH/3T3) after initiation of the transfection. A total of 20 μ g of plasmid DNA was used per 100-mm plate: CV-1 and HeLa cells were transfected with either 9 or 15 μ g of p β DNA and 3 or 5 μ g π SVHP α 2 DNA; MDCK and NIH/3T3 cells were transfected with 8 μ g p β and 2 μ g π SVHP α 2 DNA.

To prepare the lymphocyte cell line NS-1 for transfection the cells were seeded at 6×10^6 cells/100-mm plate 24 h prior to transfection. Cells were transfected with 4 μ g p β plasmid DNA and 1 μ g π SVHP α 2 DNA without carrier DNA by treatment with DEAE–dextran followed by chloroquine exposure essentially as described by Grosschedl and Baltimore (1985). Cells were split 1 to 2 12 h post-transfection and cytoplasmic RNAs were prepared 24 h post-transfection.

Assay of α - and β -globin RNA expression

RNA isolations and RNase protection of α - and β -globin anti-sense probes generated by SP6 RNA polymerase were carried out as previously described (Herr and Gluzman, 1985). Each series of hybridizations included a sample which contained a dilution (usually 4-fold) of an RNA sample derived from a highly expressing β -globin plasmid (usually 1×72) to test for probe excess during the hybridization. Digested RNA samples were electrophoresed through 6% denaturing polyacrylamide gels, dried down, and autoradiographed. In most cases the samples were visually normalized to the level of α -globin RNA and refractionated by acrylamide gel electrophoresis to generate the figures. In general, the level of α -globin RNA (i.e. transfection efficiency) varied no more than 2- to 3-fold. The samples were quantitated by cutting out the bands corresponding to correctly initiated α - and β -globin RNA and radioactivity measured by scintillation counting. To determine the relative levels of β -globin RNA the β -globin samples were first normalized to the corresponding α -globin level, and then normalized to the average of two 1×72 samples, but in a few cases only one 1×72 sample was available.

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References

- Amati, P. (1985) *Cell*, **43**, 561–562.
- Banerji, J., Rusconi, S. and Schaffner, W. (1981) *Cell*, **27**, 299–308.
- Banerji, J., Olson, L. and Schaffner, W. (1983) *Cell*, **33**, 729–740.
- Been, M. D., Burgess, R. R. and Champoux, J. J. (1984) *Nucleic Acids Res.*, **12**, 3097–3114.
- Benoist, C. and Chambon, P. (1981) *Nature*, **290**, 304–310.
- Davidson, J., Fromental, C., Augereau, P., Wildeman, A., Zenke, M. and Chambon, P. (1986) *Nature*, **323**, 544–548.
- de Villiers, J. and Schaffner, W. (1981) *Nucleic Acids Res.*, **9**, 6251–6264.

- de Villiers, J., Olson, L., Tyndall, C. and Schaffner, W. (1982) *Nucleic Acids Res.*, **10**, 7965–7976.
- Falkner, F.G. and Zachau, H.G. (1984) *Nature*, **310**, 71–74.
- Falkner, F.G., Mocikat, R. and Zachau, H.G. (1986) *Nucleic Acids Res.*, **14**, 8819–8827.
- Fromm, M. and Berg, P. (1982) *J. Mol. Appl. Genet.*, **1**, 457–481.
- Ghosh, P.K., Roy, P., Barkan, A., Mertz, J.E., Weissman, S.M. and Lebowitz, P. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1386–1390.
- Gillies, S.D., Morrison, S.L., Oi, V.T. and Tonegawa, S. (1983) *Cell*, **33**, 717–728.
- Gluzman, Y. (ed.) (1985) *Eukaryotic Transcription: the Role of cis- and trans-acting Elements in Initiation*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Gluzman, Y. and Shenk, T. (eds) (1983) *Enhancers and Eukaryotic Gene Expression*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Grosschedl, R. and Baltimore, D. (1985) *Cell*, **41**, 885–897.
- Gruss, P., Dhar, R. and Khoury, G. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 943–947.
- Herbomel, P., Bourachot, B. and Yaniv, M. (1984) *Cell*, **39**, 653–662.
- Herr, W. and Clarke, J. (1986) *Cell*, **45**, 461–470.
- Herr, W. and Gluzman, Y. (1985) *Nature*, **313**, 711–714.
- Laimins, L.A., Khoury, G., Gorman, C. Howard, B. and Gruss, P. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6453–6457.
- Mason, J.O., Williams, G.T. and Neuberger, M.S. (1985) *Cell*, **41**, 479–487.
- McKnight, S. and Tjian, R. (1986) *Cell*, **46**, 795–805.
- Mercola, M., Gorman, J., Mirell, C. and Calame, K. (1985) *Science*, **227**, 266–270.
- Moreau, P., Hen, R., Wasylyk, B., Everett, R., Gaub, M.P. and Chambon, P. (1981) *Nucleic Acids Res.*, **9**, 6047–6068.
- Neuberger, M.S. (1983) *EMBO J.*, **2**, 1373–1378.
- Parslow, T.G., Blair, D.L., Murphy, W.J. and Granner, D.K. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 2650–2654.
- Reddy, V.B., Thimmappaya, B., Dahr, R., Subramanian, K.N., Zain, B.S., Pan, J., Ghosh, P.K., Celma, M.L. and Weissmann, S.M. (1978) *Science*, **200**, 494–502.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
- Sassone-Corsi, P., Wildeman, A. and Chambon, P. (1985) *Nature*, **313**, 458–463.
- Scholer, H.R. and Gruss, P. (1984) *Cell*, **36**, 403–411.
- Scholer, H.R. and Gruss, P. (1985) *EMBO J.*, **4**, 3005–3013.
- Treisman, R., Green, M.R. and Maniatis, T. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7428–7432.
- Van Heuverswyn, H. and Fiers, W. (1979) *Eur. J. Biochem.*, **100**, 51–60.
- Veldman, G.M., Lupton, S. and Kamen, R. (1985) *Mol. Cell. Biol.*, **5**, 649–658.
- Weiher, H. and Botchan, M.R. (1984) *Nucleic Acids Res.*, **12**, 2901–2916.
- Weiher, H., König, M. and Gruss, P. (1983) *Science*, **219**, 626–631.
- Wildeman, A.G., Sassone-Corsi, P., Grundström, T., Zenke, M. and Chambon, P. (1984) *EMBO J.*, **3**, 3129–3133.
- Wildeman, A.G., Zenke, M., Schatz, C., Wintzerith, M., Grundström, T., Matthes, H., Takahashi, K. and Chambon, P. (1986) *Mol. Cell. Biol.*, **6**, 2098–2105.
- Zenke, M., Grundström, T., Matthes, H., Wintzerith, M., Schatz, C., Wildeman, A. and Chambon, P. (1986) *EMBO J.*, **5**, 387–397.

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Note added in proof

In recent experiments we have found that the A element, as defined by the 6×A21[−] construct, can also enhance transcription in HeLa cells at a level between that found with the 6×B17[−] and 6×C17[−] constructs. Shirm *et al.* (1987) have also recently reported the use of synthetic oligonucleotides to examine the cell specific activities of three similar segments of the SV40 enhancer region [Schirm, S., Jiricny, J. and Schaffner, M. (1987) *Genes and Develop.*, **1**, 65–74.]