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Short and long range activation by the SV40 enhancer

Bohdan Wasylyk, Christine Wasylyk and Pierre Chambon*

Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'INSERM, Institut de Chimie Biologique, Faculté de Médecine, 11, rue Humann, 67085 Strasbourg-Cédex, France

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ABSTRACT

Activation of transcription by the SV40 enhancer decreases in an apparently biphasic manner when DNA sequences are interposed between the SV40 enhancer and either the homologous SV40 early or the heterologous conalbumin promoter elements. With increasing lengths of short DNA fragments (up to about 150 bp) activation of transcription decreases to less than 10% of the maximum. This short range effect is observed for both the SV40 early and conalbumin promoter elements and for either orientation of the SV40 enhancer. With the conalbumin promoter, increasing the length of the interposing DNA to 275 bp decreases activation to approximately 4 %. Larger inserts, of 650 or 3737 bp, lead to an activation of 0.5 %. However, in these recombinants, transcription is still activated at least 10 fold compared to an enhancerless recombinant. The implication of these results is discussed.

INTRODUCTION

The promoter region, which is required for efficient initiation of transcription by RNA polymerase B, can be subdivided into at least three elements, the RNA startsite, the TATA box, and one or several upstream elements generally located within approximately 110 nucleotides upstream from the RNA startsite (for refs. see 1-5). Additional elements, called enhancers, have been identified by their ability to stimulate transcription from homologous and heterologous promoters over considerable distance (several thousand base pairs) and irrespective of their orientation (for ref. see 6-9). Enhancers have been found in both viral and cellular genes, and can be located either upstream or downstream, and sometimes several thousand base-pairs, from the stimulated promoter elements. The SV40 enhancer, containing the 72 bp repeat, has been extensively studied (for refs. see 10-18). In particular, we have observed a decrease in activation of a given promoter element as it is separated from the SV40 enhancer with increasing lengths of interposed sequences (the "effect of distance", 13, 17, 18). Furthermore, enhancer-proximal promoter elements are stimulated in preference to more distant promoters. To account for these observations we have suggested that a

component of the transcription machinery may "enter" the DNA at the enhancer and then "slide" along it in such a way that proximal promoter elements are preferentially activated (13, 17, 18). We show here that there is an apparent biphasic pattern of the "effect of distance" on activation of transcription from homologous and heterologous promoter elements by the SV40 enhancer, such that interposing relatively short (up to about 150 bp) fragments of DNA leads to a drastic decrease in activation. Further insertion leads to a less dramatic decrease in enhancement, such that the enhancer stimulates transcription to a similar extent when located at 650 or 3,700 bp from the activated promoter elements. The short range, "critical" "distance"-dependence, which cannot be simply accounted for by the presence of interposed promoter elements, may reflect the existence of two functionally distinct elements within the SV40 enhancer or may be an intrinsic feature which is common to all enhancers.

MATERIALS AND METHODS

Standard cloning techniques were used (19). pTCT, pTCT (tet^+), pTCTBI3, pTCTBI37 are described in 13. pBW1 (see Fig. 1A) was constructed by ligating the small BamHI fragment of pTCT (tet^+) (from +62 of the conalbumin promoter region to 375 in pBR322) in the BamHI site at the -102 (conalbumin) -375 (pBR322) junction of pTCT (BS) (pTCT with the BamHI site at the +62 (conalbumin) -5227 (SV40) junction, destroyed by cutting with BamHI, repair with DNA polymerase I and blunt end ligation). pBW3 (see Fig. 1A) was constructed by ligating the small SalI (651 in pBR322)-AvaI (1425 in pBR322) 72 bp repeat containing-fragment from pTCTBI3 and the large SalI (651 in pBR322)-AvaI (1425 in pBR322) conalbumin promoter-containing fragment from pBW1. pBW4 (see Fig. 1A) was made by exchanging the small PstI (3204 in SV40)-PstI (3609 in pBR322) fragment from pBW1 with the equivalent 72 bp repeat containing fragment from pTCTBI37. pBW2 (see Fig. 1A) was derived from pBW3 by excision of the ClaI (23)-SalI (651) fragment, followed by repair with DNA polymerase I and ligation. pBW5 contains a XhoI linker in the DNA polymerase I repaired SalI site of pTCTBI3. pBW5.1-9 (Fig. 2A) were derived from pBW5 by Bal31 digestion from the XhoI site, ligation with XhoI linkers, digestion with AvaI (1425 in pBR322) and religation with the intact AvaI-XhoI fragment of pBW5. The deletion junctions of pBW5.1-9 were sequenced by the method of Maxam and Gilbert (19). pBW6 contains the large XhoI (-42 in conalbumin promoter, repaired with DNA polymerase I) to AvaI (1425 in pBR322) conalbumin promoter-containing fragment from pBW5.9 ligated to the small BamHI (375 in pBR322, repaired with DNA polymerase I) to AvaI (1425 in pBR322) 72 bp repeat containing-fragment from pTCTBI3. pBW7-11 and their derivatives were constructed using pMT5 (from M. Zenke and T. Grundström), which contains successively (in the 3' to 5' direction relative to the globin transcription unit) the PvuII (+1650 to -9) rabbit β-globin sequence, an polylinker from M13mp12 containing restriction site for HindIII, BamHI, XhoI, SstI, XbaI, SmaI and EcoRI, and the EcoRI (1)-PvuII (2066) fragment from pBR322 containing the ampicillin resistance gene. pBW11 (Fig. 3) was derived from pMT5 by cloning an SV40 HindIII (5171)-PvuII (272) fragment between the HindIII and SmaI sites of pMT5. This SV40 fragment is modified from wild type SV40 by a precise deletion of one 72 bp sequence and a 5'-TAGTCC-3' (SV40 coordinates 106 to 101) to 5'-GGATCC-3' mutation which creates a BamHI site at the 72 bp-21 bp repeat region junction (SV40 coordinate 179) (M. Zenke, T. Grundström,

H. Matthes and M. Wintzerith, in preparation). To construct pBW7 and pBW8 (Fig. 3) first a HindIII linker was ligated to the DNA polymerase I repaired ClaI site at position +62 of the conalbumin (+62 to -102) promoter fragment in pTCT (BS). The HindIII-BamHI conalbumin promoter fragment was then ligated between the HindIII and BamHI sites of pMT5. The SV40 BamHI (179)-PvuII (272) fragment containing one 72 bp sequence (179 is the first nucleotide of the 72 bp sequence next to the in vitro generated BamHI site, see above) was repaired with DNA polymerase I, ligated with XhoI linkers, digested with XhoI and ligated into the XhoI site of the polylinker to give pBW7 and 8. DNA sequence analysis confirmed the presence of one linker sequence at each extremity. For construction of pBW7.3-6 and pBW8.3-6, the conalbumin HindIII (+62)-BamHI (-102) fragment (see above) was ligated to HindIII-XhoI-digested pMT5 together with the corresponding fragment of pBR322 bounded by BamHI and XhoI sites from pBW5.3-6 (Fig. 2A), respectively. The enhancer fragment (179-272), bounded by XhoI sites (see above), was then ligated into the XhoI site. pBW9.3-6, pBW10.3-6, pBW7.8 and pBW8.8 were constructed similarly, except that either the SV40 HindIII (5171)-BamHI (102) (102 is the coordinate of the last nucleotide of the SV40 early promoter sequence) or the conalbumin +62 to -42 fragment (modified at the +62 BamHI site by DNA polymerase I repair and addition of a HindIII linker) were used instead of the conalbumin HindIII (+62)-BamHI (-102) fragment. Other techniques, including quantitative S1 nuclease mapping, RNA isolation and HeLa cell short term transfection by calcium phosphate precipitation were as described previously (18).

RESULTS

A. The SV40 enhancer preferentially activates a proximal promoter element only when the latter is in close vicinity.

Our previous results have shown that the 72 bp repeat preferentially potentiates transcription from proximal promoter elements. In particular, in a recombinant containing two identical conalbumin (+62 to -102) promoter fragments in tandem, and the 72 bp repeat directly upstream, a tenfold greater initiation of transcription was observed on the enhancer-proximal conalbumin startsite than on the enhancer-distal startsite (see recombinant pTCTCTB in 18). To test whether the enhancer-proximal promoter of a tandem pair is always preferentially transcribed, even when the 72 bp repeat is not in close apposition to the promoters, recombinants pBW2, pBW3 and pBW4 were constructed (Fig. 1A). They consist of the SV40 early T-antigen coding region (5227 to 2553), two conalbumin (+62 to -102) promoter fragments in tandem and the 72 bp repeat region (113 to 272 from pHS102, see 13) in an inverted orientation (relative to SV40 early coding sequences). The SV40 enhancer and the conalbumin sequences are separated by 36 bp (pBW2), and approximately 650 bp (pBW3) and 3700 bp (pBW4) of pBR322 DNA. pBW1 contains no enhancer element.

The recombinants were transfected by calcium phosphate precipitation into HeLa cells together with a reference plasmid pβ(244+)β [which contains the rabbit β globin gene and the polyoma enhancer (20)]. After 48 h, total RNA was extracted and analyzed by quantitative S1 nuclease mapping.

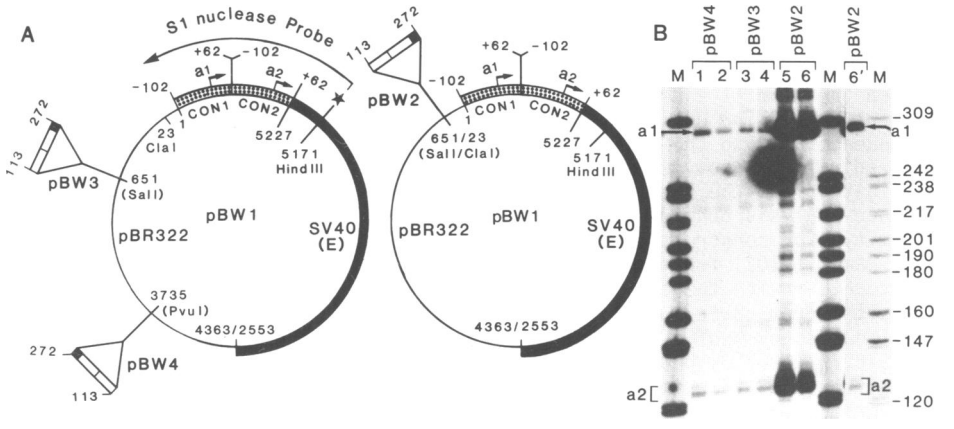

Fig. 1 : **Structure of pBW1-4 recombinants and S1 nuclease quantitation of their RNA after short-term transfections in HeLa cells.** (A) Recombinants pBW1-4 contain tandemly repeated conalbumin (+62 to -102) promoter fragments (CON1 and CON2, dotted boxes), the SV40 early T antigen coding sequences [SV40 (E)] from 5227 to 2533 (filled line) and pBR322 sequences (thin line). The SV40 enhancer fragment is represented by two open boxes (the 72 bp repeated sequences) and one solid box. a1 and a2 are the conalbumin +1 startsites on CON1 and CON2 respectively. SV40 nucleotides are numbered according to the BBB system (48). Restriction sites lost during cloning are indicated in parentheses. SV40 and pBR322 coordinates are indicated inside the circle, conalbumin and SV40 enhancer coordinates outside the circle. (B) Total RNA from transfections with pBW4 (lanes 1 and 2), pBW3 (lanes 3 and 4) and pBW2 (lanes 5, 6 and 6') was analyzed by S1 nuclease mapping using a [^{32}P] 5' end-labeled HindIII (5171) -ClaI (23) probe from pBW1 (see Fig. 1A) (5% polyacrylamide/8.3M urea gel.) a1, a2 = S1 nuclease resistant fragments expected for RNA starting at sites a1 and a2. M = [^{32}P] 5' end-labeled MspI digest of pBR322. Lane 6' is a shorter exposition of lane 6. Lanes 1-6 correspond to separate transfection experiments with different recombinant DNA preparations.

Different exposition of autoradiograms were scanned, and the results were corrected for variations in RNA synthesis from the reference plasmid. In agreement with our previous report, when the enhancer is close to the tandem promoters (pBW2) transcription from the distal promoter (CON2) is about 10% of that from the proximal promoter (CON1) (compare a1 and a2 in Fig. 1B, lanes 5, 6 and 6' and see table 1). When the enhancer is further away, in both pBW3 and pBW4, the amount of transcription from both CON1 and CON2 is very similar, and is approximately 0.5% of the amount of transcription from site a1 of pBW2 (lanes 1-4 of Fig. 1B, and Table 1). When the enhancer was absent (pBW1), transcription from the conalbumin promoters was undetectable (TABLE 1, a signal five times lower than from pBW3 or pBW4 would have been

Table 1

RELATIVE AMOUNT OF RNA INITIATED FROM THE TANDEM CONALBUMIN PROMOTER ELEMENTS WITH THE SV40 ENHANCER (WITH TWO 72 bp SEQUENCES) LOCATED AT VARIOUS DISTANCES.

RNA SYNTHESIS (%)		
Recombinants (a)	Initiation site	
	a1	a2
pBW1	< 0.1	< 0.1
pBW2	100	11, 10
pBW3	0.6, 0.5	0.5, 0.7
pBW4	0.5, 0.4	0.4, 0.5

(a) see Fig. 1A.
The values, from independent experiments, using different DNA preparations, are expressed as a percentage of RNA initiated from pBW2 site a1, and are corrected for variations in transcription from the internal control pβ(244+)β.

detected). These results show that the proximal promoter element of a tandem repeat is preferentially transcribed only when the enhancer is close by. This suggests that intervening promoter elements may have no effect on transcription of a downstream located promoter. Rather, it appears that there could be a critical distance requirement for maximum stimulation, such that a conalbumin promoter element that is adjacent to the enhancer (pTCTCTB in 18) or separated from an enhancer by a short distance (CON1, 36 bp in pBW2) is stimulated much more than a promoter element located 164 bp further away (CON2 in pBW2). Tandem promoter elements located over 200 bp from the enhancer element (the distance of CON2 in pBW2) are much less stimulated, since there is very little or no difference between promoters located 650 or 3,700 bp from the enhancer, and in both cases CON1 and CON2 promoters are equally stimulated. To refine the analysis of the "distance"-dependence of stimulation we constructed a series of deletions in the recombinant pBW5 (Fig. 2A).

B. Stimulation of transcription from the conalbumin promoter depends critically on its distance from the SV40 enhancer.

pBW5 contains the SV40 early coding region, a single conalbumin +62 to -102 promoter fragment and an "inverted" (with respect to SV40 early transcription) enhancer element separated from the conalbumin sequences by 275 bp of pBR322 (Fig. 2A). pBW5 resembles pTCTBI3 (18), except for a XhoI linker at the 72 bp repeat-pBR322 junction. Deletions were made from the XhoI site in the pBR322 sequences towards the conalbumin sequences, so that the 72 bp repeat was progressively brought closer to the conalbumin startsite a, in approximately 50 bp steps. The sequences deleted in recombinants pBW5.1-9,

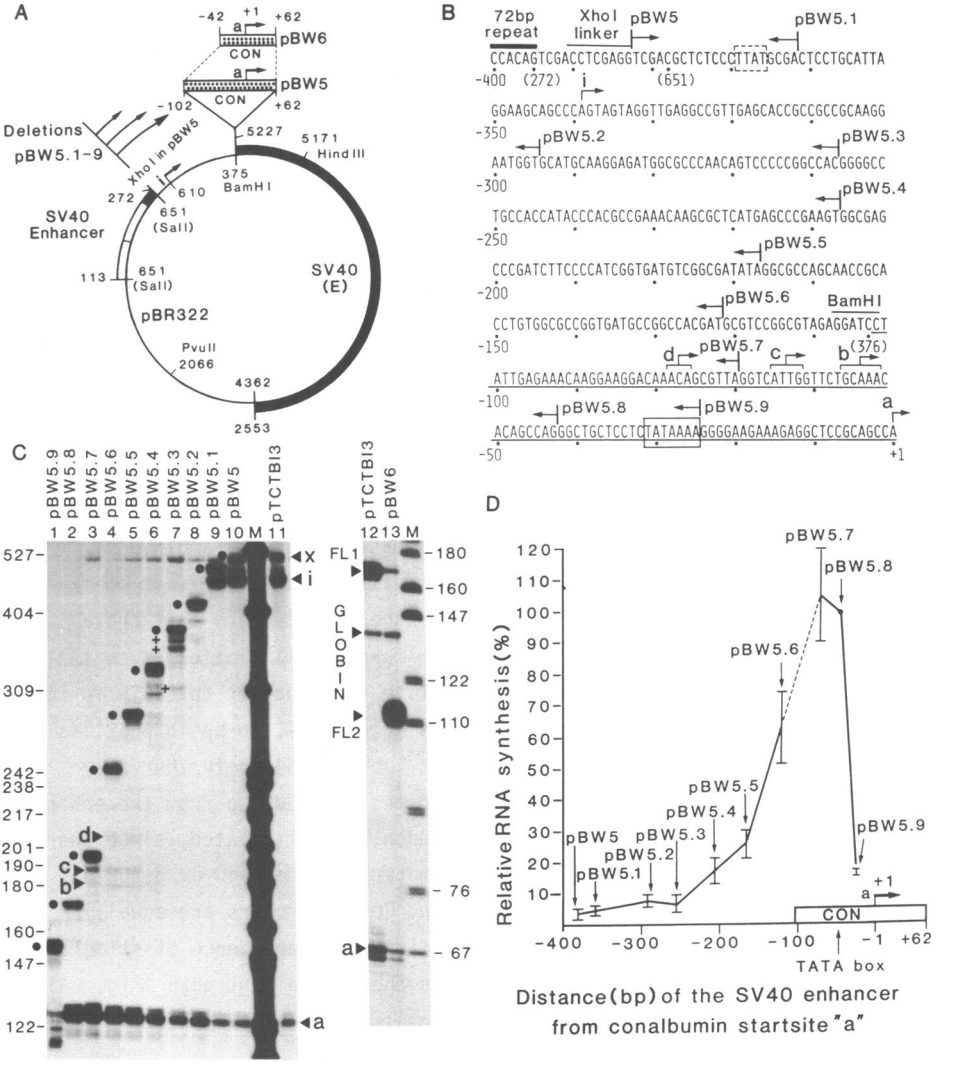

Fig. 2 : **Structure of recombinants pBW5, pBW5.1-9 and pBW6 and S1 nuclease quantitation of RNA after short term transfections in HeLa cells.** (A) Recombinants pBW5, pBW5.1-9 (see under B) and pBW6 contain SV40 T antigen coding sequences [filled line labeled SV40 (E)], a conalbumin promoter element (CON, dotted box) extending from +62 to either -42 (pBW6) or -102 (pBW5 series), pBR322 (thin line) and the 72 bp repeat region from 113 to 272. pBW 5.1-9 contain deletions from the XhoI site towards conalbumin sequences (see below). a = RNA startsite at +1 of the conalbumin promoter. i= SV40 enhancer-induced prominent RNA startsite in pBR322 identified previously (18). SV40 and pBR322 coordinates are outside and inside the circle, respectively. (B) Sequence of pBW5 between the SV40 enhancer sequence

(overlined with a thick line, coordinate 272 in parentheses) and the +1 to -102 conalbumin promoter region (underlined with a thin line). The bars with an arrow indicate the extents of pBR322 deletions in pBW5.1-pBW5.9 (pBR322 coordinates 376 and 651 are indicated in parentheses). RNA startsites a, b, c, d and i (see text) are indicated on the sequences. Nucleotides are numbered negatively from the conalbumin startsite a. The conalbumin TATA box is boxed, and a short TTAT sequence 30 nucleotides upstream from site i is indicated by a broken-line box. XhoI linker and BamHI indicate restriction enzyme sites used for cloning (see A). (C) RNA from transfections with pBW5, pBW5.1-9, pBW6 and pTCTBI3 was analysed by S1 nuclease mapping using either a [^{32}P] 5' end-labeled HindIII (5171 in SV40)-PvuII (2066 in pBR322) double stranded DNA probe (lanes 1-11) or a mixture of [^{32}P] 5' end-labeled coding strand probes of the conalbumin +62 to -102 and rabbit β globin +139 to -84 DNA fragments (lanes 12, 13). (The internal control recombinant pβ(244+)β was cotransfected with all recombinants). Gels were 3.5.% acrylamide/8.3M urea (lanes 1-11) and 8% acrylamide/8.3M urea (lanes 12, 13). a, b, c, i and globin indicate previously mapped RNA startsites (see text). FL1 correspond to the full length conalbumin probe; FL2 in lane 13 and the bands labeled with a dot in lanes 1-11 correspond in size to the length of homology between the input recombinant DNA and the probe. The crosses (corresponding to bands in lane 7), d and x indicate bands discussed in the text. M = [^{32}P] 5'end-labeled MspI digest of pBR322. (D) The relative amounts of RNA initiated at the conalbumin startsite a with pBW5 and pBW5.1-9 is plotted as a function of the distance of the enhancer fragment (coordinate 272) from the conalbumin promoter startsite a (see under B). The values, expressed as a percentage of site a for pBW5.8, were obtained by scanning autoradiograms from two independent transfection experiments, using different DNA preparations, and were corrected for transcription from the internal control recombinant pβ(244+)β. The values for relative RNA synthesis for pBW5.6 and pBW5.7 are joined by a dotted line because mutants with deletions between these two recombinants were not analyzed, whilst it is likely, from the results in Table 2, that recombinants with the enhancer at -119 (pBW7) and -55 (pBW7.8) relative to the conalbumin capsite are transcribed to very similar extents.

derived from pBW5, are shown in Fig. 2B. pBW5 and pBW5.1-9 were transfected into HeLa cells, and RNA was analyzed by quantitative S1 nuclease mapping, using as a probe a [^{32}P] 5'-end labelled HindIII (5171 in SV40) to PvuII (2066 in pBR322) fragment prepared from pTCTBI3. For recombinant pTCTBI3, three major S1 nuclease resistant bands were observed, labelled a, i and x in Fig. 2C, lane 11. Bands a and i result from RNA initiating at the conalbumin startsite and a previously mapped (18) prominent pBR322 startsite, respectively (see Fig. 2B). X probably results from RNA initiating at a minor startsite located upstream from site i. Similar bands were obtained for pBW5 (lane 10). The band indicated by a dot is similar in size to x and may correspond to RNA initiating at a similar startsite in pBR322. Alternatively it may result from S1 nuclease cutting of a hybrid between the probe and either RNA initiating from upstream (i.e. beyond the sequence discontinuity due to the XhoI linker between the probe and the transfected pBW5 recombinant) or the

input pBW5 DNA which is present as a minor contamination in the RNA preparation. Similarly, in lanes 1-9 a band is observed (indicated by a dot), which corresponds to the length of the homology between the probe and the transfected recombinants pBW5.1-9 and may result from either RNA initiated from upstream of the break point or hybridization to contaminating transfected DNA present in the RNA preparation. Other bands, of smaller size, are observed, the length of several of which correspond to previously identified startsites (18). These include RNA initiated at the natural conalbumin capsite (band a in all lanes) and RNA synthesised from substitute promoter elements in both conalbumin promoter upstream sequences (sites b and c for pBW5.4-7, lanes 3-6), and pBR322 (site i in pBW5.1, lane 9).

Transcription from the natural startsite a (lanes 2-10) increases as the enhancer is brought closer to it. In Fig. 2D, the amount of RNA initiated at site a is expressed as a percent of that for pBW5.8, and is plotted as a function of the distance of the enhancer from the conalbumin capsite. A slight stimulation in RNA synthesis is observed as the enhancer is moved from about 400 bp to 250 bp from the startsite (from approximately 4% to 7% with pBW5 and pBW5.1-3, Fig. 2D). Decreasing the enhancer-capsite distance to 70 bp results in a dramatic increase in RNA synthesis (from 7% to 100% with pBW5.3-7) ; a further decrease to 42 bp has no effect (pBW5.8), whereas a drastic decrease in RNA synthesis is observed when the enhancer is brought to within 24 nucleotides from the startsite (approximately 17% with pBW5.9). The latter decrease is most probably due to the deletion of the TATA box, which influences the efficiency of RNA synthesis from the conalbumin startsite (18). A dramatic increase in RNA synthesis from startsites b and c is also observed as the enhancer is brought closer to these substitute promoter startsites (lanes 3-10); similar increases are noticed for other potential startsites such as the conalbumin promoter site d in lanes 4-10, and those located in pBR322 sequences and labelled + in lanes 7-10 (confirmation that the latter are genuine startsites will require additional primer-extension mappings). It is important to stress that all of the potential startsites located in the pBR322 sequences of pBW5.3-6 are much weaker than site i.

When the enhancer is brought to within about 20 bp of a startsite, transcription from a particular startsite may decrease as shown for site a (pBW5.9 in lane 1, Fig. 2C) or site i (pBW5 in lane 9). In pBW5.9 deletion of the TATA box leads to a strong decrease in RNA initiated at startsite a and the appearance of multiple new startsites (lane 1, Fig. 2C). This result is in accord with the known function of the TATA box (i.e. directing initiation

of transcription approximately 30 bp downstream) and suggests that sequences located in the startsite region also participate in the mechanism which ensure accurate initiation of transcription at the natural capsite. In pBW5.1, RNA synthesis decreases from site i (lane 9), and increases from the site corresponding to the base A located three nucleotides downstream (Fig. 2B and results not shown). It is possible that this decrease is due to deletion of the TATA box-like sequence TTAT which is located 30 nucleotides upstream from the startsite i (boxed with hatched line in Fig. 2B). In contrast to startsites a and i, the deletion in pBW5.7, which deletes conalbumin promoter sequences to within about 4 base-pairs of the startsite c, leads to a further increase in transcription from this startsite. Apparently a TATA box-like sequence located 30 bp upstream is absent.

The deletions in pBW5.7 and pBW5.8 remove some of the conalbumin promoter sequences located upstream from the TATA box, yet transcription from site a increases relative to pBW5.6, which contain all conalbumin promoter sequences up to -102. These results suggest that these conalbumin promoter upstream sequences have little effect in HeLa cells on transcription initiated from the normal capsite, or that the 72 bp repeat can compensate for them when they have been deleted. To test this possibility we constructed pBW6, which is similar to pTCTBI3 except that the conalbumin promoter sequences extend from +62 to -42 (Fig. 2A). In both recombinants the enhancer is separated from conalbumin sequences by approximately 275 bp of pBR322. The enhancer is 60 bp closer to the conalbumin capsite in pTCTBI3 than in pBW6, but this difference should have little effect on stimulation by the 72 bp repeat (see Fig. 2D). pTCTBI3 and pBW6 were transfected into HeLa cells, and total RNA was analyzed by S1 nuclease-mapping (Fig. 2C, lanes 12 and 13). Approximately four times less RNA is initiated from startsite a when the conalbumin -102 to -42 sequences are deleted. These results show that the conalbumin promoter upstream sequences located between -42 and -102 have an effect on the efficiency of transcription initiated from the natural conalbumin startsite in HeLa cells, and that the SV40 enhancer, when substituted for these sequences, can compensate for the effect of this deletion (see also pBW7.6, pBW7 and pBW7.8, and pBW8.6, pBW8 and pBW8.8, Table 2 and Fig. 3).

From these results we conclude that stimulation of transcription from the conalbumin startsite a depends critically on the location of the enhancer. Moving the 72 bp repeat from approximately 260 (pBW5.3) to 120 bp (pBW5.6) upstream from the startsite causes a more than ten-fold increase in transcription. This stimulation is not related to the deletion of potential subs-

titute promoter elements located between the enhancer and the conalbumin promoter. Indeed, the deletion in pBW5.2, which removes startsite i, does not lead to the appearance of detectable new startsites between the enhancer and the conalbumin capsite, nor to an increase in transcription from startsite a when compared to pBW5 (see Fig. 2B). Further, consecutive deletions, which bring the enhancer and the startsite closer together, do not lead to the appearance or dissappearance of major startsites, yet there is a dramatic increase in transcription. In addition, the effect of interposed sequences of similar lengths is very similar (in that they reduce transcription from the distal startsite about ten-fold) whether they contain no major startsites (e.g. pBW5.2) or a strong startsite (a1 in CON1 of pBW2). Since the 72 bp repeat is known to enhance transcription in both orientations, it was interesting to investigate whether a similar "distance" phenomenon would be observed for the other orientation of the enhancer, and if the same "distance"-dependence holds for the homologous SV40 early promoter.

C. The critical "distance"-dependence of activation by the SV40 enhancer is observed with both orientations of the enhancer, and also with the SV40 early promoter region.

pBW7 (Fig. 3) contains the rabbit β globin sequences from +1650 to -9, the conalbumin promoter fragment from +62 to -102, a polylinker, and the pBR322 sequence from 1 to 2066. The enhancer, containing a single 72 bp sequence and extending from nucleotide 179 to 272, was cloned with XhoI linkers into a XhoI site located immediately upstream from the conalbumin promoter. We have previously shown that deletion of one 72 bp repeated sequence in its natural location has no noticeable effect on SV40 early transcription (13, 21). The enhancer was isolated as a BamHI-PvuII fragment from an *in vitro* generated mutant which has only one 72 bp repeated sequence and a BamHI site at coordinate 102 between the 21 bp and 72 bp repeat (M. Zenke et al., in preparation, see pBW11). pBW8 contains the enhancer with a single 72 bp sequence in the opposite orientation. pBW7.3-6 and pBW8.3-6 (Fig. 3) were derived from pBW7 and pBW8 by inserting the pBR322 sequences from pBW5.3-6 (which are bordered by BamHI and XhoI sites, Fig. 2A) between the enhancer and the conalbumin promoter sequences. The recombinants pBW7.8 and pBW8.8, contain a shorter conalbumin promoter fragment (+62 to -42) and the enhancer directly upstream (Fig. 3). These recombinants were transfected into HeLa cells and total RNA was analyzed by quantitative S1 nuclease mapping. The results are given in table 2, where the amount of RNA initiated from startsite a is expressed relative to recombinants pBW7.8 or pBW8.8. The distance

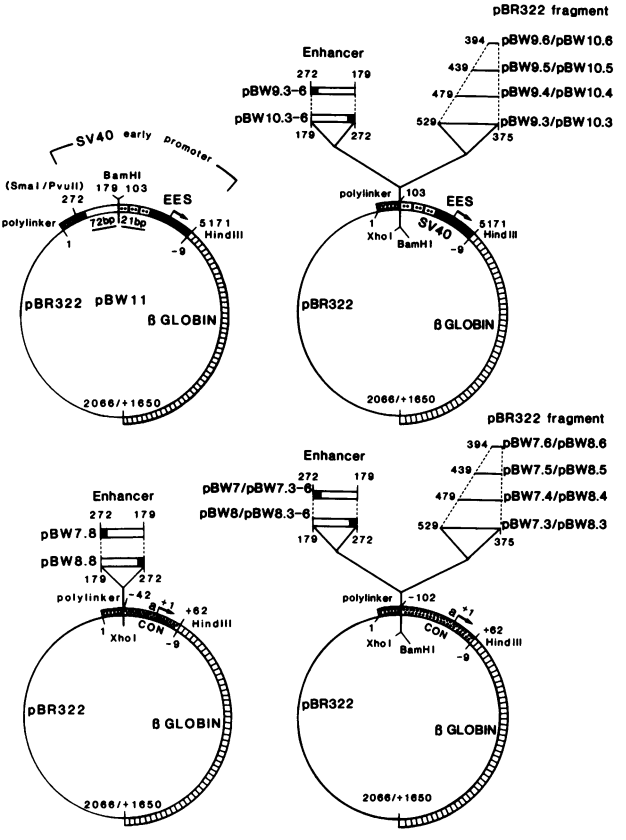

Fig. 3 : **Stucture of recombinants pBW7, pBW8, pBW11, pBW7.3-6, pBW8.3-6, pBW9.3-6 and pBW10.3-6.** pBW11 contains successively the rabbit β globin PvuII fragment from +1650 to -9 (hatched thick line), the SV40 Hind III (5171)-PvuII (272) fragment with a precise deletion of one 72 bp sequence and a TAGTCC (nucleotides 106-101) to GGATCC in vitro mutation (a gift of M. Zenke, T. Grundström, H. Matthes and M. Wintzerith) which creates a BamHI site (coordinate 103) at the junction between the SV40 21 bp region upstream element (indicated by 21 bp) and the enhancer (indicated by 72 bp), and pBR322 sequences from coordinates 1 to 2066. pBW9.3-6 and pBW10.3-6 are similar to pBW11, except that they contain upstream (relative to the direction of SV40 early transcription) from the BamHI site, BamHI-XhoI pBR322 fragments from pBW5.3-6 (Figs. 2A and B), the DNA polymerase I repaired BamHI-PvuII "enhancer" fragment (identical to that in pBW11 with XhoI linkers at its extremities), and the XhoI-EcoRI portion of the polylinker from M13mp12. pBW 7.3-6 and pBW8.3-6 are similar to pBW9.3-6 and pBW10.3-6, except that they contain the conalbumin +62 to -102 fragment in the place of the SV40 5171 (HindIII) -103 (BamHI) early promoter fragment. pBW7 and pBW8 contain no pBR322 sequences between the conalbumin and "enhancer" sequences. pBW7.8 and pBW8.8 are similar to pBW7 and pBW8 respectively, except that they contain the +62 to -42 conalbumin promoter fragment. Nucleotide 179 in the enhancer fragment corresponds to the first nucleotide of the 72 bp sequence which is present.

Table 2

RELATIVE AMOUNT OF RNA INITIATED FROM THE CONALBUMIN AND SV40 EARLY-EARLY PROMOTER REGIONS WITH THE SV40 ENHANCER (WITH ONE 72 bp SEQUENCE) LOCATED AT VARIOUS DISTANCES FROM THE PROMOTER.

PROMOTER REGION					
CONALBUMIN			SV40 EARLY		
Recombinant	RNA synthesis from site "a" (%)		Recombinant	RNA synthesis from the early-early Startsites (EES) (%)	
pBW7.3 (-269)	18	10	pBW 9.3 (-274)	4	6
pBW7.4 (-219)	21	25	pBW 9.4 (-224)	6	8
pBW7.5 (-179)	59	24	pBW 9.5 (-184)	31	32
pBW7.6 (-134)	92	81	pBW 9.6 (-139)	55	36
pBW7 (-119)	77	110			
pBW7.8 (- 55)	100	100	pBW10.3 (-269)	4	6
			pBW10.4 (-219)	9	7
pBW8.3 (-264)	5	6	pBW10.5 (-179)	23	27
pBW8.4 (-214)	26	10	pBW10.6 (-134)	47	31
pBW8.5 (-174)	58	59			
pBW8.6 (-124)	74	56			
pBW8 (-114)	68	83			
pBW8.8 (- 50)	100	100	pBW 11 (-112)	100	100

The amount of RNA initiated at either the conalbumin startsite "a" or the SV40 early-early startsites (EES, see 21) was quantified by scanning autoradiograms after quantitative S1 nuclease mapping of total RNA isolated from HeLa cells transfected with the indicated recombinants. For the conalbumin promoter-containing recombinants (see Fig. 3) values are expressed for pBW7 and pBW7.3-6 relative to pBW7.8, and relative to pBW8.8 for pBW8 and pBW8.3-6. For the SV40 early promoter region recombinants (see Fig. 3), values are expressed relative to pBW11. The values given correspond to two independent transfection experiments using different DNA preparations, and are corrected for transcription from the internal control recombinant, pβ(244+)β. Numbers in parentheses are the distances from the respective RNA startsites to the beginning of the enhancer.

of the enhancer from the conalbumin capsite is given in parentheses. For recombinants derived from pBW8, moving the enhancer element from -264 (pBW8.3) to -114 bp upstream from the conalbumin promoter startsite (pBW8) increases transcription from startsite a over 10-fold. Deleting the conalbumin promoter upstream sequences (pBW8.8) has little further effect. These results are similar to those for the pBW5 recombinants, and show that there is a critical "distance"-dependence of activation whether the enhancer fragment contains two or one 72 bp sequence. When the enhancer is present in the opposite orientation, a similar pattern of stimulation of transcription is observed. Deletions which bring the enhancer from -269 (pBW7.3) to -119

(pBW7) bp upstream from the conalbumin promoter startsites result in an approximate seven-fold increase in transcription. Further deletion, in the conalbumin promoter upstream sequences (pBW7.8) has no effect. It is clear from the above results that stimulation of transcription from the conalbumin promoter by the SV40 enhancer is most efficient when the latter is located less than 150 nucleotides from the conalbumin capsite.

In the SV40 genome, the 72 bp is located 112 nucleotides upstream from the early-early startsite (EES), the major startsite of the early region used for initiation of transcription shortly after SV40 infection (Wasylyk et al., 1983b). To test whether there is a similar "distance"-dependent effect of the enhancer on the SV40 early promoter, recombinants pBW11, pBW9.3-6 and pBW10.3-6 were constructed (Fig. 3). pBW11 (Fig. 3) contains the SV40 HindIII (5171)-PvuII (272) sequences required for efficient early transcription upstream from the rabbit β globin region (-9 to 1650). The SV40 sequences differ from wild-type by the deletion of one 72 bp repeated sequence and by the presence of a BamHI site between the 21 bp repeat and the enhancer. pBW9.3-6 and pBW10.3-6 have pBR322 sequences from pBW5.3-6, which are bordered by a BamHI site and a XhoI site, inserted between the BamHI site located just upstream from the 21bp repeat and the XhoI site of the poly-linker. The enhancer sequence (from 179 to 272) with XhoI linkers at its extremities is inserted in either orientations in this XhoI site (pBW9 and pBW10 series, for details of the construction see Materials and Methods). Transfection into HeLa cells, and analysis of total RNA by quantitative S1 nuclease mapping, showed that inserting fragments of pBR322 of increasing size between the enhancer and the 21 bp repeat progressively decreases transcription from the early-early startsite EES (Table 2 and Fig. 3). The insertion of as little as approximately 25 bp of DNA, between the 21 bp region upstream element and the enhancer (recombinants pBW9.6 and 10.6), leads to a noticeable decrease in RNA synthesis (approximately 45 % of pBW11, Table 2). Larger insertions, of approximately 70 and 100 bp of DNA, result in an even greater decrease in RNA synthesis (pBW9.4-5 and pBW10.4-5 in Table 2). In pBW9.3 and pBW10.3, which contain inserts of approximately 160 bp, and in which the enhancer is about 270 bp from EES, transcription is dramatically reduced to about 5% of pBW11. This decrease is independent of the orientation of the 72 bp sequence (Table 2, compare pBW9.3-6 with pBW10.3-6), and is similar to the effect observed with the conalbumin promoter.

DISCUSSION

A) The effect of "distance" on the stimulation of a given promoter by the SV40 enhancer cannot be simply accounted for by the presence of interposed promoter elements.

We have previously reported that activation of transcription by the SV40 enhancer is mediated through discrete "natural" or "substitute" promoter elements, and that the same elements are equally stimulated by both orientations of the enhancer (bidirectional effect). We have also observed that there is a drastic decrease in the activation of a given promoter element when it is separated from the enhancer with increasing lengths of interposed sequences (the effect of "distance" ; 13, 18). In addition, when the SV40 enhancer is inserted immediately upstream from a tandem repeat of the +62 to -102 conalbumin promoter element, transcription from the enhancer-proximal element is preferentially activated (18). These observations led us to propose an entry-site model for the mechanism of activation of transcription by the SV40 enhancer, in which some component(s) of the transcription machinery enters the template at the 72 bp repeat and then slides along the DNA, scanning it in either direction for potential promoter elements, in such a way that efficient activation of distal promoter elements would be prevented by the interposition of more proximal ones (18). Thus the decrease in efficiency of activation of a given promoter region with increasing distance would be related more to the presence of potential "natural" or "substitute" promoter elements in the interposed sequences than to an effect of distance per se. In accord with this model, de Villiers et al. (15) and Kadesh and Berg (16) have reported that, in constructions containing the SV40 enhancer and tandemly repeated transcription units, the presence of a strong potential promoter element in the proximal unit precludes efficient transcription from the distal one, whereas the same distal transcription unit was efficiently expressed when the promoter of the proximal unit was weakened by mutation.

Our present observations clearly indicate that the effect of "distance" on activation of transcription by the SV40 enhancer cannot be simply accounted for the presence of interposed "natural" or "substitute" potential promoter elements. First, a low, but equal, activation of transcription is observed from both conalbumin promoter elements of the tandem repeat in pBW3 and pBW4 (Fig. 1 and Table 1), eventhough the enhancer in pBW4 is located 3000 bp further away from the conalbumin tandem repeat than in pBW3. In constrast, in pBW2 the distal conalbumin promoter element CON2, which is about 200 bp downstream from the enhancer, is much better activated than

either of the two tandemly repeated conalbumin promoter elements of pBW3 or pBW4, although its activation is only 10% of that of the proximal CON1 promoter element which is in close apposition to the enhancer (Fig. 1 and Table 2). Second, a critical dependence of activation on distance is observed when, starting with the enhancer separated from a single conalbumin promoter element by 275 bp of pBR322, the enhancer is brought progressively closer to the promoter (pBW5 series, Fig. 2). The presence of 150 bp of intervening sequence is sufficient to reduce transcription from the conalbumin promoter element more than ten-fold. The same effect of "distance" is observed with an enhancer element containing a single 72 bp sequence in either orientation (series pBW7 and pBW8, Fig. 3, Table 2). This effect is obviously not related to the presence of potential "substitute" promoter elements in the interposed pBR322 sequences, since there is very little increase in RNA synthesis from the conalbumin promoter when the major pBR322 substitute startsite i is deleted (pBW5.1 and 5.2) and there is no other important "substitute" promoter element in the remaining interposed pBR322 sequences (Fig. 2C). Moreover, interposing between the enhancer and conalbumin promoter element, either another 164 bp conalbumin promoter element (CON1 in pBW2, Fig. 1, Table 1) or 153 bp of pBR322 sequence containing no evident "substitute" promoter element (pBW5.3, Fig. 2), results in a similar decrease in transcription of approximately 90%. Clearly, there is an effect of distance per se, which cannot be related to the preferential activation of more proximal potential promoter elements. Our present study shows that the striking effect of "distance" on activation by the SV40 enhancer is apparent only within approximately 260 bp upstream from the activated startsite. There is a very little effect of "distance" when the SV40 enhancer is moved from 650 bp to more than 3700 bp upstream from the potential conalbumin promoter elements (Fig. 1, Table 2). The possible significance of this apparently biphasic pattern will be discussed below. Incidently, our observations also rule out that, in the present case, the effect of "distance" could be due to transcription from an upstream promoter which would lead to "transcriptional interference" or "promoter occlusion" of a downstream promoter (16, 22, 23).

B) The effect of "distance" on the activation by the SV40 enhancer is observed with a variety of potential promoter elements and interposed sequences.

The critical distance-dependence of activation of potential promoter elements by the SV40 enhancer is clearly not peculiar to the heterologous conalbumin promoter, since exactly the same effect of "distance" is observed, when sequences of increasing length are interposed between the enhancer in

either orientation, and the homologous SV40 early promoter elements (the pBW9 and pBW10 series, Fig. 3 and Table 2). Similar effect of "distance" on activation by the SV40 enhancer have been observed in our laboratory for the Adenovirus major late promoter element (see 2, 24). Moreover, in the latter case, the interposed sequences are derived from Adenovirus-2 and not pBR322. Similarly, when the SV40 enhancer is separated from the conalbumin startsite by 400 bp of conalbumin upstream sequences, the activity of the conalbumin promoter region is reduced over ten-fold, compared with a recombinant in which the enhancer is located at 102 bp upstream from the startsite (A. Dierich, personal communication).

The critical distance-dependence of activation by the SV40 enhancer has gone unnoticed in several studies of the effect of "distance" on activation of heterologous and homologous potential promoter elements. For the immunoglobulin λI light chain (25) and the Drosophila hsp70 heat shock gene (26) activation was observed only when the SV40 enhancer was approximately 150 bp from the promoter elements, no transcription being detected when the enhancer was far away. In contrast, for the rabbit β-globin gene, there was no significant effect of distance when the enhancer was moved from 425 bp to 5600 bp upstream (or 2500 bp downstream) from the startsite (15). In the light of our present results, it seems reasonable to assume that the immunoglobulin λI gene in HeLa cells and the heat shock gene in non heat-induced cells have weak potential promoter elements, so that transcription could be detected in the presence of the SV40 enhancer only with maximal proximal stimulation. On the other hand, and especially if one assumes that the rabbit β globin gene has a stronger promoter element, the critical effect of distance could have been missed, since there would be very little difference in transcription activation whether the enhancer is located at 425 bp or further away from the startsite. Fromm and Berg (27) have reported, in studies using SV40 viral recombinants, that the 72 bp repeat region inserted over 2 kbp from the enhancer, and in either orientation, enhanced early and late region promoter functions. However, since the RNA was isolated late in infection, after viral replication, it is possible that a less efficient enhancement of transcription, when the enhancer is at a distant location, would have been missed.

C) Does the biphasic pattern of distance-dependence reflect the existence of two functionally distinct elements within the SV40 enhancer or is it an intrinsic feature of enhancer function ?

The apparent biphasic pattern of decreased enhancing activity as the SV40 enhancer is moved away from a promoter element raises the question

whether the 72 bp repeat contains two functionally distinct elements, one responsible for the short-range activation which decreases rapidly with increasing distances to the promoter element, and the other responsible for a long-range activation, which is almost invariable as this distance is increased. The latter would correspond to the true enhancer function. In other words, could the 72 bp repeat region contain both an element analogous to the known upstream promoter elements (see Introduction) as well as an enhancer ?

There is no clear answer to this question at the present time. The three as yet most extensively studied upstream promoter elements, those of the SV40 early promoter (the 21 bp region ; for refs., see 5, 28), the Herpes virus thymidine kinase gene (29) and the Drosophila heat shock gene (26), exhibit some properties which suggest that a similar element may be present in the 72 bp repeat region. First, these upstream elements continue to stimulate transcription when they are moved away from their corresponding TATA box. However, this stimulation decreases more dramatically as a function of increasing lengths of interposed sequences than in the case of the SV40 72 bp repeat. Little stimulation is detectable when these upstream elements are 50 bp from their original location (13, 29). Second, both the SV40 21 bp region (5, 28) and the Herpes virus thymidine kinase (30) and glycoprotein D (31) gene upstream elements exhibit some stimulatory activity when inserted in their original location, but in the opposite orientation. Whether or not this "bidirectionality" is a general property of upstream promoter elements remains to be established. It is also interesting to note that the activity of some promoters [Adenovirus major late transcription unit (2, 24); the Drosophila heatshock gene (26)] become independent of the presence of their upstream elements when the SV40 72 bp repeat is inserted in place of, or in close apposition to, these elements. However a deletion in the SV40 early promoter, which removes the 21 bp repeat upstream element, and brings the 72 bp repeat in its place, is a very strong down-mutation (pMD102 in 5, 28). A better characterization of the property of upstream elements may help in deciding whether or not the SV40 72 bp repeat contains two functionally distinct elements, an upstream element and the enhancer. Alternatively, apart from the magnitude of their effect, it is not clear at the present time whether we will ever be able to draw a clear distinction between the properties of upstream elements and enhancers. The comprehensive point mutagenesis in the 72 bp repeat region, which is in progress in our laboratory, will reveal whether or not the same sequences are responsible for both the short-range and the long-range stimulatory activities of this region.

On the other hand, a critical "distance"-dependence may be an intrinsic feature of activation of transcription by enhancers. Both Adenovirus E1A (32, 33) and mouse Moloney leukemia virus (Augereau and Wasylyk, in preparation) enhancers are much less efficient when moved away from heterologous promoter elements by a few hundred bp, suggesting that the distance-dependent biphasic pattern of enhancer activity is not a peculiarity of the SV40 enhancer. It should, however, be noted that in both the Bovine papilloma virus early gene (34-36) and immunoglobulin heavy and kappa light chain (37-42) genes, the enhancers are located several thousand bp 3' to the promoter startsites. It remains to be seen if these enhancers would also be more efficient when moved closer to their respective potentiated promoter elements.

How could the biphasic pattern of stimulatory activity be an intrinsic feature of the SV40 enhancer ? It has been recently demonstrated that the SV40 enhancer can generate, over its own sequences, an alteration of chromatin structure which is evidenced by increased DNaseI sensitivity (27, 43, 44) and the formation of a nucleosome-free region (43, 44). As discussed elsewhere (43-45), this enhancer-induced chromatin alteration may be one of the mechanisms responsible for the "entry site" function of the SV40 72 bp repeat, the transcription factor(s) entering on the template at the chromatin "open window" generated by the enhancer. One may speculate that the transcription factor(s) could then either slide along the DNA towards closely located potential promoter elements which would be preferentially activated, or move to more distant promoter elements by direct intra-molecular transfer. The latter mechanism would account for the long range effect of the SV40 enhancer much better than sliding, since it is much less dependent on the length of the sequences interposed between the potential promoter and the enhancer. Both sliding and intramolecular direct transfer have been evoked in prokaryotes as possible complementary search mechanisms to account for binding of regulatory proteins to specific DNA sequences (for refs., see 46). It is striking that moving the enhancer by approximately 150 bp upstream from either the heterologous conalbumin +62 to -102 promoter element (Fig. 2D) or the homologous SV40 early 21 bp repeat region (Table 2) result in an almost complete disappearance of the preferential short range activation. Since a nucleosome core particle contains 146 bp of DNA (for refs. see 47), it is possible that the short range activation reflects also a more ready access of transcription factor(s) to the promoter region when the promoter elements and the enhancer are close enough not to be separated by a nucleosome. Clearly, which molecular mechanisms are underlying the short and long range stimula-

tory effect of the SV40 enhancer imperatively requires the further development of *in vitro* systems in which all *in vivo* effects can be mimicked.

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*To whom reprint requests should be sent

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