Stereoalignment requirements for activation of transcription by the Simian virus 40 enhancer

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ABSTRACT

We investigated the contribution to the enhancer activity of protein-protein interactions between specific trans-acting factors bound to neighbouring sequence elements of the SV40 early enhancer-promoter region. To this end, we altered the distance between the SV40 enhancer-promoter elements by inserting systematically increasing lengths of spacer DNA. We show here that the level of transcription from the SV40 early promoter decreases with the spacing between enhancer and promoter regions. In the case of insertions shorter than 125 bp, the promoter activity exhibits a strong dependence on insertion lengths of multiples of about 10 bp. This periodic effect is no longer observed for larger insertions, reflecting the torsional flexibility of DNA. These data provide evidence that, between promoter and/or enhancer elements, periodic interactions of transcriptional nucleoprotein complexes exist and stereospecific alignments are necessary to obtain an efficient initiation of transcription from the SV40 early promoter.

INTRODUCTION

The regulation of genes is controlled generally by DNA binding proteins which directly interact with other regulatory protein, as well as, with RNA polymerase to stimulate or inhibit transcription. One possible mechanism for understanding of the action at a distance, already observed with several prokaryotic and eukaryotic regulatory sequences, is DNA looping which permit contact between separated regulatory proteins (1,2,3,4). A periodic phenomenon of certain promoter activity dependent on spacing between the binding sites of its regulatory proteins suggests that efficient transcription requires the stereospecific alignment between bound proteins (5, 6, 7). Such rotational alignment has been shown previously for the simian virus 40 (SV40) early promoter (7, 8, 9). The period observed is approximately one turn of the B DNA helix that is 10,5 base pairs (bp). In the case of heat shock element (HSE), the periodic effect is seen for short insertions but not for longer ones beyond 300 bp (10).

The SV40 early promoter contains cis-acting promoter and enhancer elements. Enhancers are regulatory elements that stimulate transcription from the promoter, independent of their distance or position relative to it (11, 12). The SV40 enhancer is composed of multiple sequence motifs which appear to bind in vitro trans-acting protein factors and these sequences cooperate synergistically to generate in vivo cell-specific enhancer activity (13, 14,15, 16, 17, 18, 19, 20). The sequence motifs of SV40 enhancer are organized in at least two functionally distinct domains A and B whose multimerizations generate an enhancer effect (13).

To further investigate the protein-protein interactions of specific transacting factors bound to multiple cis-acting sequences, we altered the distance between the SV40 enhancer-promoter elements by inserting systematically increasing lengths of spacer DNA. We show here that the level of transcription from SV40 early promoter decreases slowly with the spacing between enhancer and promoter regions. In addition, we report on a strong periodic dependence of the transcription for short insertions within a natural or rearranged enhancer. Moreover an attenuation of periodic effect is observed for longer insertion lengths (125 bp), reflecting the torsional flexibility of DNA. These data provide evidence that, between promoter and/or enhancer elements, periodic interactions of transcriptional nucleoprotein complexes exist and stereospecific alignments are necessary to obtain an efficient initiation of transcription from the SV40 early promoter.

MATERIALS AND METHODS

All mutants of the pA series are derived from the recombinant pA0 as shown in Figure 1A. They were constructed by shot-gun ligation (21, 22) using synthetic linkers taking advantage of restriction sites and deletions engineered in the SV40 enhancer region (13).

All transfections have been carried out with the calcium phosphate coprecipitation technique and HeLa cells were grown as previously described (13). Specific transcripts initiated at the SV40 early-early start sites (EES) or at the start site of the internal reference β -globin gene (GLOB) were quantitated by S1 nuclease mapping, using an excess of a single-stranded ^{32}P 5'-end-labeled DNA probe that yields fragments 130–137 and 60 nucleotides

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Figure 1. General organization of transfected recombinants.

(A) structure of the basal recombinant pA0 which contains the SV40 early promoter region with only one copy of the 72 bp sequence (position 179–250) from EcoRI (346) to HindIII (5171) sites cloned in front of the coding sequence of rabbit beta-globin gene in the pBR322 plasmid between EcoRI (4361) and PvuII (2066) sites. The restriction sites, the 72-bp sequence, the 21-bp repeat region, the TATA box and the early-early start site (EES) and late-early start site (LES) are indicated. For more details see (13). (B) Organization of pA500–pA600 insertion mutant series. For pA500 construction a 65 bp length sequence containing three new created restriction sites SstI, XbaI and XhoI has been inserted at the BamHI site (101) of the recombinant pA0. Between XbaI and XhoI sites, the added 42 bp sequence is that of the pA249 recombinant which has no enhancer activity (13). DNA fragments between the SstI and XbaI sites from 10 to 110 bp (10 to 10) have been inserted corresponding to pA510–pA610 mutant series, respectively, as shown in Table I.

Figure 2. Organization and sequences of insertion mutants between enhancer and 21-bp repeat region and between domains A and B of the SV40 promoter. The enhancer region (position 277/179), 21-bp repeat region and below the proximal sequence of 72-bp repeat (noncoding early strand) are shown. The sequences inserted (underlined) in pA constructions and the amount of RNA initiated at the early-early start site (EES) expressed relative transcription to pA0, taken as 100%, are indicated. The values given correspond to the average of the relative transcription levels measured. The inserted sequences are represented, on the left-hand side, for the insertion mutants between positions 221–220 in pA0 (pA751–760 series), on the right-hand side, for the insertion mutants between enhancer and 21-bp region (pA701–730 series) and, below, for two 'double' insertion mutants between domains A and B and between enhancer and 21-bp repeat region (pA765–pA770).

long for hybrids corresponding to RNA initiated at the EES and the GLOB start sites, respectively (Fig. 1). The results of these analyses are expressed as the amount of RNA initiated at the EES sites on each recombinant, relative to pA0 (or pA550 in figure 4B) taken as 100%, and represent the average of different transfection experiments using at least two different DNA preparations, after correction for transcription from the

cotransfected reference recombinant pG1B, indicated by GLOB in the figures (13, 17).

RESULTS

Insertions between the enhancer and 21-bp repeat region

As expected from previous study (8), introduction of progressive insertions from 1 to 30 bp DNA fragments (pA701–pA730)

Figure 3. Quantitative S1 nuclease analysis of RNA synthesized by various insertion mutants. Cytoplasmic RNA molecules from co-transfected HeLa cells by pA recombinants (as indicated) and reference recombinant have been analysed by quantitative nuclease S1 mapping as described elsewhere (13). EES and LES represent single-strand DNA probe fragments of 130–137 and 164–178 nucleotides in length, respectively, hybridized to initiated RNA at the SV40 EES and LES, respectively. GLOB corresponds to RNA transcribed from the reference recombinant pG1B (17). (A) Effect of the insertions between enhancer and 21-bp repeat region (see Fig. 2). (B) Effect of inserting length between enhancer domains A and B (see Fig. 2). (C) Effect of the insertions between domains A and B at a distance in recombinant pA550 and, in pA655 and pA660 'double' insertion mutants (see Table I).

between the enhancer and 21-bp repeat region led to a cyclic variation of RNA synthesis at the early-early start site (EES) of the SV40 early promoter. Increasing spacing by 1 to 5 bp reduced transcription at maximum for 5 bp to 6 fold, about 15% to wild-type level of pA0, whereas 6, 7 and 10 bp reaugmented transcription to 68% for 10 bp inserted (Fig. 2, 4). Increasing spacing by an odd number of half a DNA turn (5, 15, 25 bp) caused a decrease of transcription activity by a factor 6–4 fold, whereas the insertions by an even number (10, 20, 21, 30 bp) reduced the level of transcription by 1.5 fold (Fig. 2, 4). The length of the cycle is close to 10.5 bp, one turn of the B DNA form helix. An autoradiogram corresponding to such quantitative S1 nuclease analyses of these spacing mutants is shown in Figure 3A.

Then, we analysed the effect of longer insertions to know whether the enhancer activity is biphasic at a distance as described previously (23), and whether the stereospecificity alignment changes with the distance. As shown in Table I and Figure 4, the activating transcription of recombinants bearing various added lengths of relatively long DNA fragments, from 65 to 175 bp between enhancer and promoter regions, decreased slowly from 40 to 15%, respectively. Nevertheless, a most pronounced diminution of transcription appear for longer spacing, beyond 115–125 bp inserted, reflecting a less influence of proteins-proteins interactions. However at this distance, the periodic effect due to the rotational alignment is still found, merely attenuated, by inserting systematically 1 to 1 bp between the enhancer and 21-bp repeat region, the insertion mutant series pA550–pA560 (Table I; Fig. 4B). A similar cyclic variation of RNA synthesis is also observed for smaller spacing by comparing the recombinants pA500, 502, 505, 506 and 510 or pA540, 546 and 550 (Table I, Fig. 4A).

Taken together, these results of the spacing effect between the enhancer and 21-bp repeat region indicate that: (i) natural arrangement of promoter and enhancer elements seems optimal for initiation of transcription from SV40 early promoter; (ii) a spacing by a half helical turn of DNA at short distances strongly

Table I. The effect, at a distance, on transient expression of the pA500–pA600 mutant series in HeLa cells. The insertion lengths between enhancer and 21-bp repeat region (Enh/Prom) and between domains A and B (A/B) are indicated in bp. The values given correspond to the average of the relative transcription levels measured.

RECOMBINANT	INSERTION (bp)		TRANSCRIPTION RELATIVE to pA0 (EES) percent
	Enh/Prom	A/B	
pA500	65		40
pA510	75		30
pA520	85		30
pA530	95		27
pA540	105		30
pA550	115		36
pA560	125		32
pA570	135		27
pA580	145		20
pA581	146		16
pA590	155		16
pA600	165		20
pA610	175		15
pA655	115	5	24
pA660	115	10	43

RECOMBINANT	INSERTION (bp)		TRANSCRIPTION RELATIVE to pA550 (EES) percent
	Enh/Prom		
pA550	115		100
pA551	116		89
pA552	117		96
pA553	118		85
pA554	119		63
pA555	120		65
pA556	121		67
pA557	122		80
pA558	123		92
pA560	125		94

alters activity, an effect which is lessened at greater distances. Thus, a similar effect of cyclic variation but attenuated with the distance.

Since the enhancer functions bidirectionally, we tested whether a similar influence of the distance positioning between the enhancer and 21-bp repeat region can also be seen when the enhancer (domain A and B) is inverted, the pA320–pA327 mutants series, as described in Figure 5A. A periodic variation of transcriptional expression is effectively found with these insertion mutants, as represented in Figure 6A.

Can the spacing between enhancer domains alter transcriptional activity?

In a previous study (13), we observed that the synergy between the domains A and B does not appear to depend critically on the exact distance between them, up to 58 bp. To further investigate, we introduced systematically from 1 to 10 bp between the two domains A and B (Fig. 2). The insertions of this mutant series, pA751–pA760, caused a cyclic variation in RNA initiated from EES (Fig. 2, 6B). The sequences inserted of 6 or 7 bp reduced, at the most, transcription to about 35% of the wild-type level (Fig. 2, 3B, 6B). The synergic activity between the domains A and B is so moderately altered, however enhancer is still functional or conserved, compared to a construction bearing only one domain A, pA233 = 2.3% (13).

The periodic effect of insertions between the enhancer and 21-bp repeat region and the two domains A and B could reflect a requirement for unique rotational alignment of three bindings regions, as well as between each enhancer domain and upstream promoter region. As shown in Figure 2, a 'double' insertion mutant (5 bp between domains A and B and between enhancer and 21-bp repeat region), recombinant pA765, is transcribed less efficiently than pA705. By contrast, a 10 bp inserted fragment between the domains A and B in another 'double' insertion mutant (pA770) has an higher activity than pA765. In addition, two 'double' insertion mutants at a distance, pA655 and pA660, behave in a similar fashion as the recombinants pA755 and pA760 (Table I). Therefore the interactions between protein factors bound to this three regions require a unique rotational alignment.

These results are likewise confirmed by the spacing between the two domains A and B, the mutant series pA341–pA346, when the enhancer is inverted as shown in Figure 5A. Thus, even in reversed orientation, a stereospecific alignment between domains A and B appears to be required for maximal enhancer activity (Fig. 6A).

Spacing effect with rearranged enhancer domain construction

The fact that the enhancer can be divided into domains and the dimerization of each domain can restore an enhancer effect (13, 14), we assayed the spacing requirement between two domains A in a dimer insertion mutant series, pA270–pA276, as shown in Figure 5B. A strong dependence of stereospecific alignment reveals a cyclic variation in transcription initiated at the EES (Fig. 5B, 6B). Indeed, the lower activity (2%), obtained with the recombinant pA273, is about at the same level as that of a monomer of domain A construct (pA233 = 2.3%), this former does not contain the XbaI-XhoI region or domain B (13), suggesting that only one domain A in pA273 is functional.

In a construction series bearing only one domain B, pA282–pA288 (Fig. 5C), a similar requirement is also observed between enhancer domain B and 21bp repeat region for optimal transcriptional activation. The half of a periodic cycle is close to 5 bp (Fig. 6D).

Figure 4. Diagrams of the results of transcriptional expression from SV40 early promoter obtained with pA mutant series containing insertion between enhancer and 21-bp repeat region. (A) Three series of recombinants have been constructed: insertions from 1 to 7 bp, from 10 to 30 bp in 5 to 5 bp and from 65 to 175 bp in 10 to 10 bp (see fig. 2, 1B). The length of the insertions are represented on the abscissa. The relative efficiency of transcription relative to pA0 (=100%), is given on the ordinate. (B) Transcription results obtained with insertions from 1 to 8 bp between the XhoI and BamHI sites, between the enhancer and the 21-bp repeat region in the pA550 (see fig. 1B). The insertion mutant series pA550–pA560 are represented. The length of the insertions are represented on the abscissa and the relative efficiency of transcription relative to pA550 (=100%), is given on the ordinate. The values given correspond to the average of the relative transcription levels measured (number of experiments are given in parenthesis). The bars represent the highest and lowest values obtained.

DISCUSSION

Within the SV40 early promoter are a number of separate cis-acting enhancer-promoter elements that can activate transcription in a variety of different contexts (13, 17). In most cases stereospecific alignments between these elements are required for optimal initiation from promoter (8, 9, 10).

The results presented in this manuscript, clearly demonstrate and generalize that transcriptional activation, in various

Figure 5. Organization and sequences of various insertion mutants with SV40 rearranged enhancer domains. The rearranged enhancer region, the restriction sites and the orientation of domains (arrows) are represented. The sequences inserted (underlined) in pA constructions and the amount of RNA initiated at the EES expressed relative transcription to pA0, taken as 100%, are indicated. (A) Insertions from 0 to 7 bp (pA320–pA327) between the inverted enhancer and 21-bp repeat region and below 'double' insertions from 1 to 6 bp (pA341–pA346) between the two inverted domains A and B in the recombinant pA327; (B) Insertions from 0 to 6 bp between two domains A (pA270–pA276) (C) Insertions from 2 to 8 bp between the domain B and 21-bp repeat region (pA282–pA288). The values given correspond to the average of the relative transcription levels measured. Recombinant pA234 has been described (13)

arrangements of SV40 transcriptional elements, is a cyclic function of physical spacing between the enhancer-promoter elements. The cycle varies as a period approximately one turn of B DNA helix. Stereospecific alignments between these elements is necessary to obtain an optimal initiation of transcription from the SV40 early promoter. These observations could be explained by flexibility of nucleoprotein complexes, reflecting that protein-protein interactions play a key role in the

generation of an active transcriptional initiation complex at the SV40 promoter.

It has been shown previously (8) by inserting either odd or even multiples of half turns of the DNA helix between the enhancer and 21-bp repeat region and between 21-bp region and TATA box that there are marked differences in the in vivo effects of these two types of insertions on initiation of transcription from SV40 early promoter. We also observed that an odd number of

Figure 6. Diagrammatic representation of the transcriptional results obtained with the various insertion mutants. The length of the sequences inserted between enhancer and/or promoter elements is indicated on the abscissa and the relative efficiency of transcription relative to pA0 (= 100%) is given on the ordinate (see figure 5). The given values correspond to the average of the relative transcription levels measured (number of experiments are given in parenthesis). The bars represent the highest and lowest values obtained. A) Insertions between the enhancer inverted and the 21-bp repeat region (pA320–pA327). (B) Insertions between the two domains A and B of the enhancer (pA751–pA760). (C) Insertions between two domains A in a dimer of A (pA270–pA276). (D) Insertions between a domain B and the 21-bp repeat region in a monomer of B construct (pA282–pA288).

helical half turns of DNA decreases transcription about 6 fold and an even number maintains a higher transcription level. These results suggest that efficient initiation of transcription requires stereospecific alignments between the Sp1 and enhancer factors. Thus, the binding sites may be situated on the same side of the double DNA helix facilitating optimal protein(s)–protein(s) interactions with each other and/or some element of the transcriptional machinery. This hypothesis of a correct localization along the DNA helix is reinforced by the periodic effects on transcription observed by altering the spacing between (i) two A domains in a dimer of A; (ii) between a single B domain and 21-bp repeat region in a monomer of B or (iii) between the two domains A and B and between enhancer and 21-bp region. Additionally, the study of the ‘double’ insertion mutants series (pA341–pA346), at the same position as the latter mutant series, shows that an interaction between the A domain and the rest of promoter region may exist even when the enhancer is inverted. These results clearly support that stereospecific interactions between regulatory protein factors or sequences are involved in the formation of an active transcriptional complex.

Our present results of transient expression in HeLa cells with the spacing recombinant series, pA701–pA730 and pA550–pA560, indicate that the importance of a specific alignment between enhancer and 21-bp repeat region appears to decrease with the distance, as suggested (8). The study of the

transcriptional effect at greater distances when the enhancer is separated from the promoter region reveals a progressive diminution of promoter activity but there is no strong evidence for a break or a biphasic decrease of the enhancer activity beyond 150 bp; in particular at 146 bp distance which is the length of DNA contained in a nucleosome core particle, as suggested previously (23).

The importance of the position of the regulatory cellular factors along of the double DNA helix has been well documented for the bacteria, phage lambda or yeast system (1, 2, 3). This type of interaction plays a key role in the regulation of the transcription by specific protein–DNA and protein–protein interactions, involving the flexibility of regulatory proteins or DNA. Particularly, when two separated regulatory nucleoprotein complexes may contact in forming a loop of DNA, as proven by electron microscopy visualization of loops seen, for an example, with the lambda phage repressors (24).

In conclusion, DNA looping provides a conceptually simple explanation for the action at a distance phenomenon permitting a possible regulatory mechanism for gene activity. Furthermore our results clearly support that the requirement for correct rotational alignment of enhancer–promoter elements reflect multiple protein–protein interactions involved in the generation of an active transcriptional initiation complex.

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