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The sequence motifs that are involved in SV40 enhancer function also control SV40 late promoter activity

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ABSTRACT

The simian virus 40 (SV40) enhancer element is constituted of two domains which contain sequences important for late transcription (M. Ernoult-Lange, F. Omilli, D. O'Reilly and E. May, J. Virol. 61, 167-176, 1987). By analysing a series of clustered point mutations generated throughout the enhancer region we mapped domain I from nt 232 to 272 and domain II from nt 184 to 216. These two domains which are required for late promoter activity both in the presence and in the absence of T antigen correspond closely to the domains B and A respectively, identified for enhancer function (M. Zenke, T. Grundström, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman and P. Chambon, EMBO J., 5, 387-397, 1986). Similarly to the enhancer function the late promoter elements defined by these two domains contain multiple sequence motifs. Moreover there is a striking overlap between the sequence motifs within domain A, active for early enhancer function and those within domain II involved in efficient late transcription.

INTRODUCTION

The two overlapping early and late promoters of the SV40 genome located within the segment of ca. 400 base pairs (bp) extending from nucleotide (nt) 346 (Hpa II site) to nt 5170 (Hind III site) direct the early and late gene transcription in an opposite orientation with regard to one another. The SV40 early promoter is constituted by a set of at least three promoter elements. The first element, shared with most eucaryotic genes transcribed by RNA polymerase II, is a Golberg-Hogness box (TATA box) located 21 to 26 nt upstream from the "early-early" cap sites (1, 2). The second element is a guanine cytosine rich region containing six copies (G-C motifs) of a characteristic hexanucleotide 5'-GGGCGG-3'. Each of the G-C motifs can bind to the cellular transcription factor Spl (3, 4). The third element commonly referred to as enhancer, corresponds to the 72-bp direct repeats and extends 25 bp further upstream (5). The enhancer stimulates the efficiency of initiation of transcription from linked promoters irrespective of its orientation and to some extent of the distance separating this element from the start sites (6-9).

The SV40 late promoter is formed by at least two interrelated promoter elements. The first element, which we call the +7 to -53 element (10) is located from nt 332 to nt 273 and contains the major late initiation site (MLIS) at nt 325 (position +1) as well as some minor late initiation sites. It also includes sequences which specify the position of the majority of initiation sites located in this element (10, 11). Indeed a surrogate TATA box (nt 294 to nt 304) which directs the efficiency of in vitro transcription from the MLIS has been characterized in these sequences (12, 13). The second element corresponds to the enhancer element defined above for the early promoter (14-16). This element appears to be the major control element of the late promoter acting before DNA replication, in the absence of T antigen (10, 14) as well as after viral DNA replication (17). Moreover it has been proposed that T antigen induces cellular factor(s) responsible for the activation of late gene expression following viral DNA replication. Sequences required for T-antigen-mediated trans-activation have been characterized within the enhancer element (18-20).

In vivo expression of mutants deleted in the enhancer and in vitro late promoter transcription analysis suggest that the G-C motifs belong also to the late promoter (8, 21-24). However in the absence of T antigen, the deletion of the G-C motifs results in a 3- to 4-fold increase in late transcription (17). Moreover the position of the G-C motifs between the enhancer and the early initiation sites favors the early transcription before viral DNA replication (10).

By analysing a series of deletion mutants, Ernoult-Lange et al. (17) identified two domains within the enhancer element which contain sequences important for efficient late transcription. The domain I which is located at the late proximal end of each 72-bp repeat, functions before replication and in the absence of T antigen. The domain II located at the late distal part of each 72-bp repeat corresponds to the sequences identified by Keller and Alwine (19) which appeared to be responsible for T-antigen-mediated trans-activation. The contribution of this domain is much more significant after replication.

To determine more precisely the sequence requirements for late promoter activity before and after replication, we analysed a series of clustered point mutations generated throughout the enhancer region.

Results presented in this paper show that both domain I and II function before and after replication. Sequence motifs in these domains

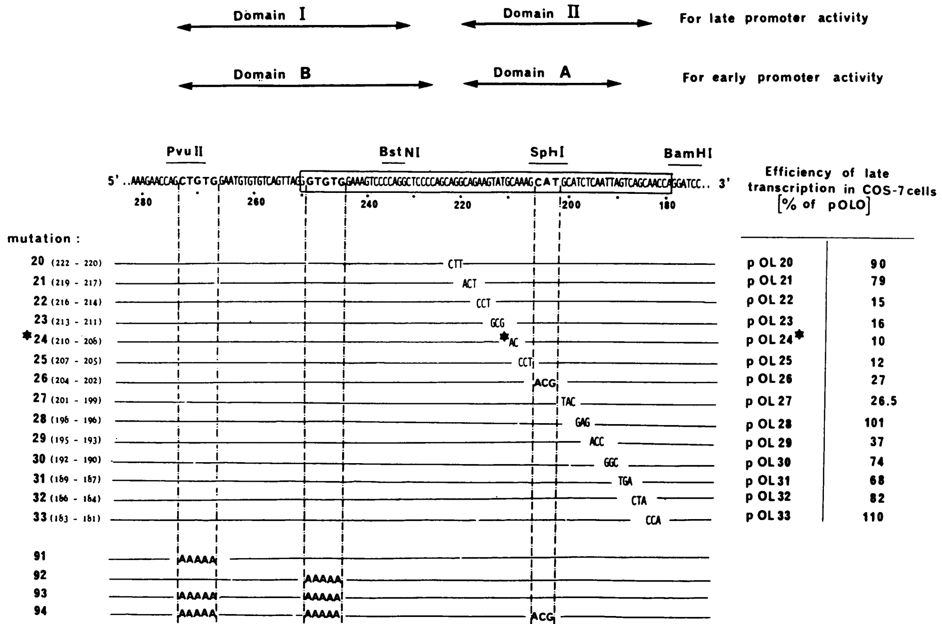

Fig. 1 - Effect of point mutations within the enhancer element, on late transcription after replication. The sequence of SV40 enhancer element present either in pAO, pOLO or pLO recombinants is indicated. The late template strand sequence is noted and some restriction sites are shown. The enhancer element of pAO recombinant have been modified relative to the wild type sequence by deleting exactly one of the two 72-bp repeats (from nt 107 to 178) and by generating a BamHI site at the junction between the 72-bp sequences and the G-C motif region (5). The boxed sequence corresponds to the unique 72-bp sequence present in the enhancer element of the pA, pOL and pL series recombinants. In the upper part of the figure, the first line shows the location of domain I and II involved in late promoter activity (see the text) and the second line the location of domains A and B required for efficient early transcription (5). Below the enhancer sequence are listed mutant derivatives with the mutated sequence. The mutations are identical to those introduced in the enhancer sequence of pA-type plasmids by Zenke et al. (5) except for the pOL24* plasmid. During the construction of pOL24, the pA24 mutation has been modified. The thymidine corresponding to the first mutated nucleotide have been deleted ; a star marks this modification. The transcriptional activity of the mutant plasmids as measured by quantitative S1 nuclease analysis after transient expression in COS-7 cells is indicated as a percentage relative to pOLO.

important for late promoter activity correspond to the sequence motifs important for enhancer function. However, the relative importance of the sequences differ for the early and late transcriptions, respectively.

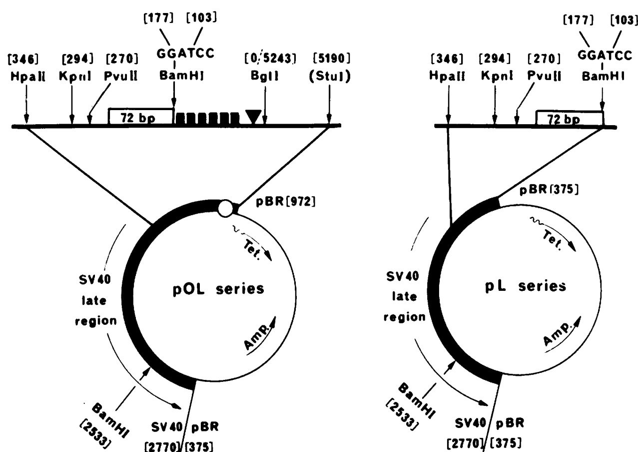

Fig. 2 - Promoter organization and structure of pOL and pL plasmid series. The position of SV40 early TATA box is indicated by a filled triangle . The six block boxes represent the six G-C motifs. The pOL and pL series recombinants contain a single 72-bp sequence represented by an open box. Some restriction sites, natural and engineered are indicated with their position. StuI site in parentheses was destroyed during construction of recombinant. Concerning the schematic representation of plasmids, the thick line represent SV40 sequences. The arrow shows the direction of late transcription. The SV40 origin of DNA replication is indicated by a small open circle (O). pBR322 sequences are indicated by the thin line.

MATERIALS AND METHODS

Construction of recombinant plasmids

The recombinants plasmids pA0, pA20 to pA33 and pA91 to pA94 have been previously described (5). In recombinant pA0, rabbit ß-globin genomic DNA is under the control of SV40 early promoter. The wild type sequence of the early promoter region (from nt 5171 to nt 346) was modified by deleting exactly one of the two 72-bp repeated sequences (from nt 107 to nt 178, see Fig. 1) and by generating a BamHI site at the junction (nt 101) between the 72-bp sequences and the G-C motifs. pA-type recombinants derive from pA0 by introducing a series of clustered point mutations throughout the enhancer region (see Fig. 1 and ref. 5).

Plasmids pL113 (containing the entire late region from nt 113 to nt 2770), pL113-7 (derived from pL113 by inserting a XhoI linker into the SV40 EcoRI site (nt 1782) and pSVL7 (containing the entire SV40 regulatory region and late genes modified by inserting a XhoI linker into the SV40 EcoRI site), have been described previously (10, 17).

pOL series. pOL0, pOL20 to 33 and pOL91 to 94 contain the regulatory region of the corresponding pA-type recombinant with the late genes in their authentic position relative to the late promoter. The ß-globin gene has been deleted. A schematic representation of this series is presented in Fig. 2. They were constructed as follows. A 6247-bp pL113 fragment extending from the KpnI site (nt 294 in SV40) to the NruI site (nt 972 in pBR322) was ligated to a 274-bp SV40 fragment (nt 294 to 5190) derived by KpnI - StuI cleavage of each one of the pA-type recombinants.

The presence of the expected cluster of point mutations in each of the pOL series recombinants was checked by nucleotide sequence analysis. This analysis revealed a change for pOL24, the first thymidine of the three mutated nucleotides T-A-C present in plasmid pA24 being deleted (see fig. 1). The mutant which harbours this modification as compared to the parental mutation is thereafter called pOL24*.

pL series. When compared with the pOL series the pL series is deleted for the origin sequences and the G-C motifs (Fig. 2). These recombinants were constructed by ligation of a 4605-bp pL113 fragment extending from the pBR322 BamHI site (nt 375) to the SV40 BamHI site (nt 2533), to a 2354-bp SV40 fragment extending from nt 179 to nt 2533 and derived by BamHI cleavage of each one of pOL series recombinants. The orientation of the inserted SV40 sequences was confirmed by restriction enzyme analysis.

Other techniques including cell growth, DNA transfection, RNA isolation and quantitative S1 analysis were as previously described (10, 17).

RESULTS

We have determined at the nucleotide level, sequences within the enhancer element required for SV40 late gene expression. Our major interest was to investigate whether the same sequence dependences were observed for late gene expression as previously characterized for the enhancer element involved in initiation of early genes. This comparison was made possible by studying an extensive set of point mutations in the enhancer element which were previously characterized for early promoter activity by Zenke et al. (5). The late promoter mutated within the enhancer element was placed upstream of the SV40 late gene in its natural position. The effect of various mutations within the enhancer element was measured by quantitative S1 analysis of late mRNA synthesized during transient expression i) after viral DNA replication by transfecting COS-7 cells which constitutively produce T antigen or ii) before DNA replication, in absence of T antigen by transfecting Hela cells. Experiments published elsewhere (17) indicated that the late promoter functions in a similar manner in both semi-permissive Hela cells and permissive CV-1P cells.

Quantitative S1 nuclease analysis involves the cotransfection of an internal marker (pSVL7 or pL113-7) and the test plasmid into the relevant cell type. The internal markers which contain a modified VP1 gene obtained by inserting a XhoI linker within the late genes at the EcoRI site (nt 1782) allow the results to be corrected for variations in the efficiency of transfection (10, 17, see also legend to Fig. 3). Cotransfection was performed with 20 μg (or 10 μg) of the test plasmid and 10 μg of the internal marker. It has been previously reported that these levels are within the linear range of response of the system (17).

To compare the effect of various mutations on early and late

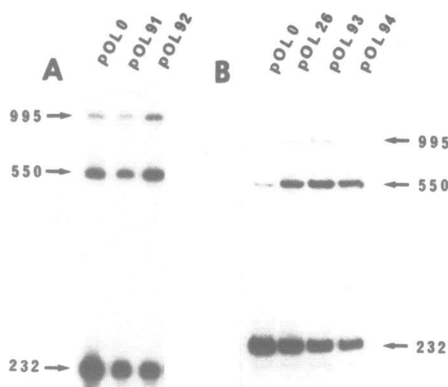

Fig. 3 - Quantitative S1 nuclease analysis of late transcription directed by a set of pOL series recombinants after replication. Total RNA was extracted from COS-7 cells cotransfected with 20 μg of one of the test plasmid and 10 μg of internal marker pSVL7. The internal marker pSVL7 contains an XhoI linker within the coding sequence of late genes at nt 1782. 2.5 μg of total RNA were hybridized with an excess of the single stranded DNA (corresponding to the late template strand) of AvaII fragment of pSVL7 (nt 2013 to 1018) ^{32}-P labelled at the 5' end (nt 2013). Hybridization was performed for 15 h. at 42°C in the presence of 50 % formamide, 0.4 M NaCl and 40 mM Pipes, pH 6.4. S1 nuclease digestion was performed by 9-fold dilution of the hybridization mixture in 0.23 M NaCl, 40 mM $ZnCl_2$, 30 mM Na acetate (pH 4.5) and 250 U. of S1 nuclease (BRL), followed by 2 h. incubation at 25°C. Ethanol precipited S1 digestion products were taken up in 10 ul of 80 % formamide solution (1 mM EDTA, 0.05 % bromophenol blue, 0.1 % xylene cyanol), heated for 2 min. at 95°C, and electrophoresed on 5 % polyacrylamide-7 M urea gels in 0.5 x TBE buffer. The dried gels were exposed to Kodak-X-Omat AR film for 2 days without intensifying screen. When the probe DNA was hybridized with 16 S mRNA transcribed from the test plasmid, the sequence homology, stopped at the XhoI linker at nt 1782 and a 232-nt fragment was protected. However, when the probe DNA was hybridized to 16 S mRNA transcribed from the marker plasmid, DNA sequence homology continued up to the acceptor splice junction at nt 1463 and a 550-nt hybrid fragment was protected from S1 digestion. The 995-nt fragment corresponds to the entire probe which can be protected by 19 S mRNA transcribed from the internal marker. Panels A and B correspond to two different gels.

transcription we often refer to the results obtained by Zenke et al. (5) with pA series plasmids.

Effect of point mutations within the enhancer element on late gene transcription after the onset of viral DNA replication.

- Analysis of recombinants mutated in three motifs of the enhancer element critical for early transcription.

Using a site directed mismatched - primer mutagenesis to

Table I - Relative amount of late mRNA initiated from late promoter in presence (COS-7 cells) or in absence (HeLa cells) of viral DNA replication.

	Relative efficiency of transcription (%)[b]		
	COS-7 cells	HeLa cells	
	pOL series	pOL series	pL series
Wild type	100	100	100
Mutation[a] 91	49	ND[c]	ND
92	36	ND	ND
93	25	65	35
26	26	67	69
94	6.5	52	16

[a] Position of point mutation clusters for the different mutants are given in figure 1.
[b] The values from five independent experiments with different DNA preparations are expressed as a percentage of RNA initiated from wild type late promoter (pOLO for pOL series and pLO for pL series) and are normalized relative to the level of transcription from the internal control (pSVL7 for pOL series and pL113-7 for pL series).
[c] ND : not determined.

introduce point mutation clusters, Zenke et al. (5) define precisely the nucleotides within the enhancer element that are involved for efficient early gene expression. This analysis revealed three regions of the enhancer that tolerated poorly the introduction of mutations. These regions included approximately nucleotides 190 - 216, 239 - 250 and 262 - 273. Individual clusters of point mutations in each of these regions reduced only partially the efficiency of transcription from the early promoter. However the recombinant pA94 which has three nucleotide clusters mutated at key positions (nt 202 to 204 ; nt 245 to 249 and nt 268 to 272) (Fig. 1) is almost totally inactive for transcription from the early promoter (5).

We then analysed the effect on late gene expression, of the mutation clusters present in mutant pA94 either individually or in combination. The pOL series plasmids which contain an intact viral origin of replication were transfected into COS-7 cells which constitutively express functional T antigen. Total RNA was extracted and the amount of late specific RNA initiated from various mutants was determined by quantitative S1 nuclease analysis. The results of such an experiment are illustrated in Fig. 3. The 232 nucleotides protected fragment corresponds to 16S and 19S mRNA

transcribed from test plasmids. While 16S mRNA transcribed from the internal marker protects a fragment of 550 nt, the 19S mRNA synthesized by the internal marker protects the entire probe and yields a band at 995 nt. The efficiency of late transcription directed by each plasmid was estimated by densitometric scanning of suitably exposed autoradiograms. The average value of at least five independent transfection experiments using at least three different plasmid preparations are given table I. The results corrected for the level of transcription from the cotransfected reference recombinant (pSVL7) are expressed relative to total transcription from wild type recombinant pOL0.

Point mutants pOL91 and pOL92 in which the sequence 5'-G/CTGTG-3' (nt 272 to 268 and nt 249 to 245) is replaced by a cluster of five A residues (Fig. 1) diminish the efficiency of late transcription by a factor of 2 and 3 respectively. A combination of the mutations present in pOL91 and pOL92 (pOL93, Fig. 1) decreases the late transcriptional activity by a 4-fold factor.

A clearcut result is obtained with mutant pOL26. The only change introduced within the wild type regulatory region is the substitution of the three nucleotides C-A-T at positions 204-202 by the nucleotides A-C-G. This cluster of point mutations brings about a 4-fold reduction in late promoter strength. Maximum inhibition of late promoter activity (activity reduced to ca 7 % of wild type late promoter activity) is obtained with mutant pA94 having the cluster of point mutations present in pA91, pA92 and pA26 (Fig. 1).

Comparison of our results with those of Zenke et al. (5) shows that for most of the mutants, a mutation that decreases the enhancer activity also reduces the late promoter activity although to a different extent. While mutations at the late proximal part of the 72-bp repeat are less deleterious for late than for early transcription (ca. 3- and 6-fold for pOL92 and pA92 respectively), the contrary is obtained by mutating the late distal part of the 72-bp repeat (ca. 4- and 2.5-fold reduction for pOL26 and pA26 respectively). Moreover combinations of mutations appear to be less detrimental for late than for early transcription (ca. 15- and 200-fold for pOL94 and pA94 respectively).

- Analysis of a series of point mutations scanning the late distal moiety of the 72-bp repeat.

In the next series of experiments we delimitated at the nucleotide level, those sequences within the late distal part of the enhancer

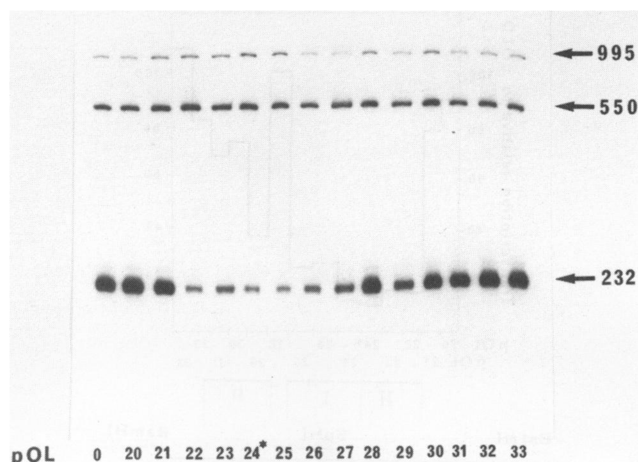

Fig. 4 - Quantitative S1 nuclease analysis of late transcription directed by pOL0 and pOL20 to pOL33 recombinants after replication. Total RNA was extracted from COS-7 cells cotransfected with 10 µg of one of the test plasmid and 10 µg of internal marker pSVL7. This was analyzed as described in legend to Fig. 3. The dried gel was exposed to Kodak X-omat AR film for 1 day without intensifying screen. Fragments 232 and 550 nt long correspond to 16 S mRNA transcribed from the test and marker plasmids respectively.

element that were critical for late gene expression following DNA replication. By analysing a series of clustered point mutations, Zenke et al. (5) defined within this region three sequence motifs of importance for early transcription. These motifs called by the authors Sph I and II motifs (nt 199 to 207 and nt 208 to 216) and P motif (nt 186 to 196) are defined by mutants pA22-pA27 and pA29-pA30 respectively. We then determined if similarly to enhancer activity, late promoter activity of this region is due to the presence of several specific sequence motifs. The recombinants pOL20 to pOL33 were constructed by inserting the regulatory sequences of the corresponding pA recombinants upstream of the late gene in their wild type position (Fig. 2).

The efficiency of late transcription of each of these mutants was measured following transfection into COS-7 cells. 48 hours post transfection, late viral RNA initiated by various mutants was determined by quantitative S1 analysis as described above. An autoradiogram corresponding to such an experiment is shown in Fig. 4. Results are summarized in Fig. 1 where late promoter activity of pOL series mutants is given relative to the wild type pOL0 recombinant. Each value corresponds to six independent

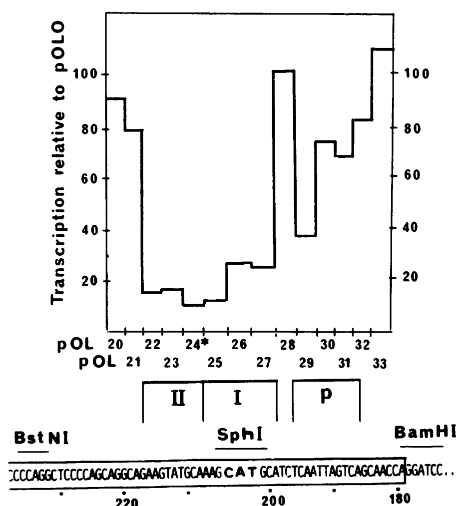

Fig. 5 - Profile of the effect of point mutations on transcriptional efficiency of the late promoter. The amount of late RNA was measured by quantitative S1 nuclease analysis after transient expression in COS-7 cells. Values relative to pOLO were obtained by densitometric scanning of autoradiograms similar to the one shown in Fig. 4. These values which represent the average of six independent transfection experiments with three different plasmid DNA preparations are reported Fig. 1. The lower part shows the late distal moiety of the 72-bp sequence. This sequence is lined up with the position of the nucleotide substitutions present in the tested mutants. Above the sequence are indicated the position of the Sph- motifs I and II (noted I and II) and the P-motif (noted P) defined for enhancer function by Zenke et al. (5).

transfection experiments using three different plasmid DNA preparations. In Fig. 5 these values are lined up with the positions of mutations. Results reveal a sequence motif crucial for late transcription, defined by mutants pOL22-pOL27. Any clustered point mutations throughout this region brings a large reduction in promoter strength (residual activity of 10 to 25 % relative to pOLO). This sequence motif (extending from nt 216 to 199) overlaps exactly both the Sph I - Sph II motifs. It is interesting to note that mutant pOL24* which differs from the parental mutant by the deletion of the first nucleotide of the mutated three nucleotide cluster (see Fig. 1) shows a lower efficiency of transcription (10 % relative to pOLO). On the contrary the parental mutation has a minor effect on the efficiency of early transcription (5). The differential effect observed between the two mutants is possibly due to the nucleotide deletion. Mutations present in plasmids pOL29, pOL30, pOL31 and pOL32 are also detrimental to late transcription.

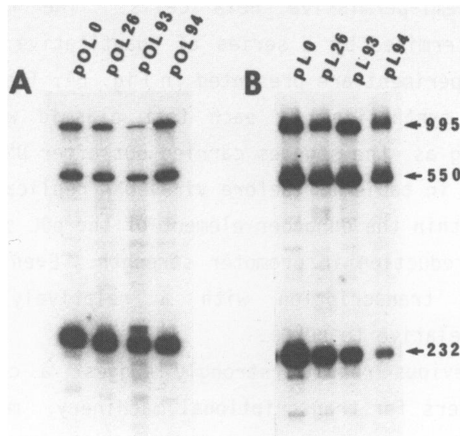

Fig. 6 - Quantitative S1 nuclease analysis of late transcription directed by pOL and pL recombinants before replication. Total RNA was extracted from Hela cells cotransfected with 20 µg of one of the pOL (panel A) or pL (panel B) recombinants and 10 ug of internal marker pSVL7 or pL113-7 respectively. This was analyzed as described in the legend to Fig. 3 except that 20 µg of total RNA was hybridized to the 5'-end labelled probe. The dried gels were exposed to Kodak X-Omat AR film for 6 days without intensifying screen. Protected fragments 232 and 550 nt long correspond to 16 S mRNA transcribed from the test and marker plasmid respectively.

These mutants defined the P motif previously identified for the enhancer function.

As compared to result already published for early transcription (5) it is noticed that although mutations in this region are generally more detrimental for late than for early transcription there is a striking overlap between sequences important for late transcription after replication (called domain II by Ernoult-Lange et al. (17)) and sequences essential for enhancer activity (called domain A by Zenke et al. (5)) (see Fig. 1).

Effect of point mutations within the enhancer element on late gene transcription preceding viral replication.

- Effect of point mutants on late promoter activity of the regulatory sequence in its entirety.

The pOL series which contains the complete SV40 late region, retains the entire late and early promoter sequences and an intact origin of replication (Fig. 2). The SV40 early genes are deleted from these plasmids and consequently replication from the SV40 origin is blocked. The efficiency of late transcription of each of these mutants was measured following

transfection into semi-permissive Hela cells. The relative level of transcription was determined by a series of quantitative S1 analyses. The results of such an experiment are presented in Fig. 6A. The variations in the amount of late RNA synthesized by each test plasmid were quantified by densitometric scanning as the studies carried out after DNA replication. The results are presented in table I. Before viral DNA replication, introduction of point mutations within the enhancer element of the pOL series results in a relatively moderate reduction in promoter strength. Even the triple mutant pOL94 directs late transcription with a relatively high efficiency (approximately 52 % relative to pOL0).

Our previous results strongly suggest a competition between early and late promoters for transcriptional machinery, mediated by the G-C motifs (10, 17). The relatively low effect of the mutations present in recombinant pOL94 on late promoter activity measured before viral DNA replication may be a consequence of the dramatic decrease in early promoter activity observed with these mutations. To test this hypothesis we then checked the effect of point mutations within the enhancer element on the promoter activity of minimal late promoter sequences.

- Effect of point mutations on the activity of minimal sequences required to drive basal late transcription.

We showed previously that sequences required for basal late gene expression are located from nt 332 to nt 113 (10, 14). These sequences include the enhancer element but not the G-C motifs. In consequence we constructed the pL recombinants by deleting the part of regulatory sequences which extends from nt 5190 to 101 (Fig. 2). The expression of late genes is then regulated by minimal late promoter sequences. The major part of the early promoter except the enhancer sequences are deleted. In these experiments the internal marker used was pL113-7 which is deleted for the origin sequences as are pL recombinants. At 48 hours after transfection, late viral RNA initiated by the various mutants was determined by quantitative S1 nuclease analysis as described above. The results of such an experiment are illustrated on Fig. 6B. The efficiency of late transcription directed by each plasmid was estimated by densitometric scanning of suitably exposed autoradiograms. The values presented in table I are expressed relative to late transcription from pL0 (taken as 100 %) and correspond to a mean of six independent experiments. The two clusters of point mutations present in the late proximal end of the enhancer element (mutant pL93) decrease the efficiency of transcription by a factor of ca. 3-fold. The inhibition

observed after viral replication with the recombinant pOL93 is within the same range of order (see below and table 1).

On the contrary, the effect of mutations within the late distal end of the enhancer element is less deleterious to late transcription before viral DNA replication. The plasmid pL26 directs the late transcription with an efficiency approximately 70 % relative to pLO. The same mutation in plasmid pOL26 brings about a 4-fold reduction in late promoter strength measured in COS-7 cells. These results confirm our previous data obtained by deleting the late distal moiety of the single 72-bp repeat of the plasmid pOLO (17) and clearly indicates that sequences located in this region are less critical for late transcription before than after DNA replication.

A combination of both pL93 and pL26 mutations (plasmid pL94) decreases the efficiency of late transcription by a factor of ca. 6-fold. This effect is approximately as severe as expected if the effects of pL93 and pL26 mutations were additive. However both before and after DNA replication (see below), triple mutations present in plasmid pL94 are less deleterious to late than to early transcription. In the case of early transcription, triple mutations within the enhancer element reduce the transcriptional activity of the early promoter to approximately the same extent as recombinants having the entire enhancer deleted (5). This is in marked contrast to the effect of the same triple mutations on the basal late promoter activity since compared to the effect produced by deleting the enhancer element, this mutation has a relatively moderate effect on late gene expression (the transcription efficiency being 16 % that of pLO). We have previously shown that the deletion of the enhancer element completely abolishes the activity of the minimum sequences required for basal late promoter activity (10).

DISCUSSION

In this paper we define those sequences within the 72-bp enhancer element that are required for late promoter activity by analysing the efficiency of late transcription directed by mutants containing nucleotide substitutions. When compared with deletion mutations, point mutations preserve the relative position of different elements. Results presented here confirm and extend our previous results obtained with deletion mutants (17). Sequences within the enhancer element are critical for the expression of late genes measured in transient expression both in absence of T-antigen-mediated replication and after viral DNA replication.

By using deletion mutants we previously defined two domains

within the enhancer element (17). Data presented in this paper permit these two domains to be mapped more precisely. Results obtained with mutant pOL91 indicate that domain I previously mapped from nt 232 to 265 indeed extends further up to nt 272. Domain II consists in the 33-bp sequences extending from nt 184 to 216. The two domains are required for late promoter activity both before and after viral DNA replication.

Keller and Alwine (19) also defined two late promoter elements located respectively at the late proximal end of the enhancer (nt 200 to 270) and within the 33-bp sequence which spans the junction of the 72-bp repeats (nt 168 to 200). The element located at the late proximal end of the enhancer overlaps the domain I defined above from nt 232 to 272. In the experimental conditions used by these authors, the activity of these two elements was undetectable in the absence of T antigen. Moreover, the element extending from nt 200 to 270 needs an intact origin of replication to be active in addition to the presence of a functional T antigen.

Our experimental conditions allow us to detect the activity of domain I and II in the absence of T-antigen. Under these conditions, results obtained with pL recombinants indicate that in the absence of the origin of replication, sequences within domain I are even more critical for basal late promoter activity than sequences within domain II. On the contrary, sequences within domain II are more critical for late promoter activity after DNA replication. This increased importance of domain II may be related to T-antigen-mediated stimulation of late gene expression (18, 19) and/or to template modification(s) resulting from viral DNA replication.

It is interesting to note that as has been described for enhancer function of early promoter, the effect of individual point mutation are additive for late promoter activity. Nevertheless the same combination of point mutations present either in pA94, pOL94 and pL94 recombinants, decrease the efficiency of early transcription to a larger extent compared to that in late transcription. This may be because mutations present in these recombinants preserve some sequences more critical for late than for early transcription. A systematic point mutation scanning analysis of the late distal moiety of the 72-bp repeat reveals a striking identity between the map position of domain A required for efficient early transcription (5) and domain II particularly involved in the efficiency of late transcription following DNA replication (see Fig. 1 and 5). Moreover the sequence motifs Sph and P required for enhancer function within domain A are also identified when late promoter activity is monitored.

In vitro and in vivo analysis of early promoter have indicated that trans-acting factors are involved in enhancer function (for review see 25). In vitro DNase I footprinting experiments strongly suggest that these trans-acting factors are exerting their function by binding to the various enhancer motifs identified by point mutation scanning analysis (26). The close similarity between domain A and domain II raises an important question concerning the possible similarity of the factors interacting in trans with this region either in the early phase of infection, in the absence of T antigen or in the late phase of infection in the presence of T antigen.

We previously showed (27) that in the early phase of infection when late genes are expressed at a relatively low level late mRNAs are initiated mainly within the 62-bp segment extending from nt 325 (MLIS) to 264. On the contrary, RNAs transcribed after viral DNA replication are extremely heterogeneous since the initiation of late mRNAs takes place at various sites distributed over almost the entire SV40 control sequence including the 72-bp repeats (28, 29). On the other hand our previous results (10, 17) strongly suggested that late gene expression is regulated negatively by the G-C motifs in the early phase of infection but after viral DNA replication this region stimulates late promoter activity two-fold. Finally, results presented here and elsewhere (17) indicate that mutations within the 72-bp moiety located close to the G-C motifs have a moderate effet on late gene expression before DNA replication but decrease dramatically the efficiency of late transcription after viral replication. These different results strongly suggest that the sequence requirements for the regulation of late gene expression are different before and after replication.

Results presented by Takahashi et al. (30) have shown that protein-protein interactions between Spl factor which binds specially to G-C motifs and trans-acting factor(s) bound to enhancer may be involved in activation of early genes. On the other hand, results obtained by two groups indicated that T-antigen has a direct stimulatory effect on late gene expression independent of and separate from replication or gene amplification. Large T-antigen-binding sites and a functional enhancer region are required for maximal stimulation (31, 32). To account for the change in the regulation of late genes concomitant with the initiation of viral DNA synthesis it is tempting to hypothesize that the binding of T-antigen to its natural binding sites might modify directly or indirectly some interactions between the different trans-acting factors bound to promoter elements, leading to down-regulation of the early promoter and to an up-regulation of

late gene expression. However regulation of late transcription might be more complex as in SV40 abortively infected or transformed cells the presence of relatively high level of T-antigen is not sufficient to induce substantial late gene expression (27, 33). Moreover a study performed with mutants which produce a temperature-sensitive large T-antigen (tsA mutants) indicates that accumulation of late mRNA at late times after infection depends at least to some extent, on the amplification of the viral genome (34).

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