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Stimulation of SV40 DNA replication by the human c-*myc* enhancer

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In earlier studies we had shown that a transcriptional enhancer sequence exists about 2 kb upstream of the human c-*myc* gene. The core sequence necessary for enhancer activity was defined therein as a 21 bp nucleotide element, which also showed autonomous replicating activity [EMBO J. (1988) 7, 3135–3142; EMBO J. (1989) 8, 4273–4279]. Recently, several reports have substantiated the notion that transcription and replication can be concertedly regulated in a larger number of cases than expected. In this report, we took the simian virus 40 (SV 40) *ori*/promoter as a model system. The SV40 enhancer is known to enhance transcription from its *ori*/promoter, but to reduce its replication (probably due to a negative feedback). The SV40 enhancer was replaced by the c-*myc* enhancer core in order to see its effect upon SV40 DNA replication and transcription. The results showed that besides stimulating transcription, the c-*myc* enhancer promoted SV40 DNA replication in monkey Cos1 cells. Stimulation was only observed when the c-*myc* enhancer was inserted in the 'up-to-down' orientation to the SV40 promoter. The promoting function of the c-*myc* enhancer on DNA replication correlated with the transcriptional activation function, as determined by systematic point mutations introduced within the 21 bp core sequence.

c-*myc*; Enhancer; DNA replication

1. INTRODUCTION

Precise mechanisms of DNA replication in mammalian cells are still not clarified. Studies using animal virus systems suggest that DNA replication and transcription are coordinately regulated [1,2]. Two types of coordination are considered. One is that common sequences act as regulatory elements both in DNA replication and transcription. A typical example is the polyomavirus system, where the transcriptional enhancer sequence is necessary for viral DNA replication [3,4]. In other virus systems including adenovirus, simian virus 40 (SV40), bovine papilloma virus and EB virus, transcriptional regulatory sequences are placed near or overlapping with the origin of DNA replication [1]. Another case was reported where a transcriptional silencer sequence derived from mouse teratocarcinoma cells suppressed DNA replication as well as transcription in polyomavirus [5]. Recently, it was also found that a transcriptional silencer and an origin of replication in yeast require a common element [6]. The other type of coordination is that common protein factors affect both DNA replication and transcription. At least two well-characterized transcription factors have turned out to be identical to cellular proteins required for viral DNA replication. CTF, a transcription factor responsible for specific recognition of the sequence CCAAT in eukaryotic promoters, is indistinguishable form nuclear factor I (NF-1), a cellular DNA-binding protein essential for the initiation of adenovirus DNA replication in vitro [7]. An octamer-binding transcription factor (OTF-1), which exists ubiquitously in mammalian cells, is physically and biologically identical to another cellular DNA-binding protein, nuclear factor III (NF-III), which is also required for adenovirus DNA replication [8–11]. Both CTF/NF-I and OTF-1/NF-III can serve as transcription factors for RNA polymerase II, as well as initiation factors for viral DNA replication.

We have previously identified a transcriptional enhancer sequence about 2 kb upstream from the first exon of the human c-*myc* gene [12], and determined the core sequence of 21 bp necessary for enhancer action [13]. The sequence could also function as an autonomously replicating sequence (ARS) both in cultured cells and in transgenic mice [14]. The sequence is probably involved in autoregulation of c-*myc* transcription by the c-*myc* protein itself [15].

Here, we linked the c-*myc* enhancer to the SV40 promoter/origin instead of the SV40 enhancer. The results showed that the c-*myc* transcriptional enhancer stimulated SV40 DNA replication, which substantiates the notion of concerted mechanisms for DNA replication and transcription in mammalian cells.

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Abbreviations: CAT, chloramphenicol acetyl transferase; *ori*, origin of DNA replication; SV40, simian virus 40.

2. MATERIALS AND METHODS

2.1. *Cells*

Monkey Cos 1 and mouse L cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, at 37°C and 5% CO_2.

2.2. *Plasmids and oligonucleotides*

Oligonucleotides corresponding to the 21-bp long enhancer core sequence within the *Hin*dIII-*Pst*I region of the human c-*myc* gene, with or without systematic 2 bp point mutations, were chemically synthesized (see Fig. 3). They were then inserted into the *Bam*HI site of pSVPCAT, which is a pUC18 vector derivative containing the SV40 *ori*/promoter linked to the bacterial chloramphenicol acetyl transferase gene. The oligonucleotides were inserted in both orientations. All manipulations were performed according to standard techniques. Plasmid pSV2CAT was switched from its original pBR322 vector to pUC18 in order to standardize conditions with the other constructs, and in order to avoid toxic sequences in pBR322 that poison SV40 replication.

2.3. *Replication of plasmid DNA in Cos 1 cells*

To examine autonomous replication of plasmid DNA, Cos 1 cells were transfected with DNA by the calcium phosphate method [16]. 1×10^6 cells were transfected with 10 ng test DNA and cultured, 40 h after transfection, low molecular weight DNAs were extracted according to the Hirt procedure [17]. The DNAs were digested with *Eco*R1 to linearize them, and with *Dpn*I to degrade non-replicated DNA. The digested DNA was then electrophoresed through a 0.8% agarose gel, blotted by the method of Southern [18] and hybridized with ^{32}P-labelled pUC19. Hybridization of the blotted filter with labelled probe was carried out as described previously [12].

2.4. *CAT assays*

Plasmid DNAs were transfected to mouse L cells by the calcium phosphate method [16]. 2 μg of test DNA were used for 1×10^6 cells. Two days after transfection, the cells were harvested, suspended in 200 μl of 0.25 M Tris-HCl (pH 7.8), and disrupted by freeze-thawing three times prior to sonication. CAT assays were carried out using the cell lysate as described previously [14]. 5 μg of a β-galactosidase expression vector (pCMV-β- Gal), carrying a cytomegalovirus promoter linked to the β-galactosidase gene, were co-transfected with the test plasmids to normalize the transfection efficiency. 30 μl of cellular extracts were used for the β-galactosidase assay [19].

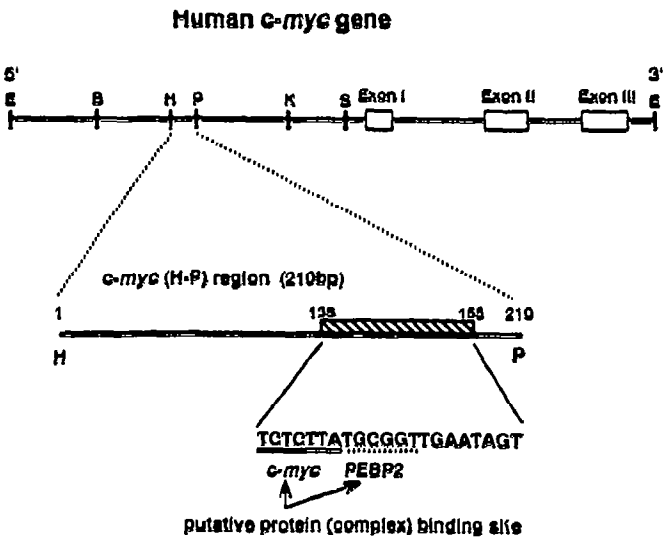

Fig. 1. Schematic drawing of the human c-*myc* gene. Probable protein binding sites within the *Hin*dIII-*Pst*I (H-P) region are shown in the lower panel. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; K, *Kpn*I; S, *Sma*I.

3. RESULTS AND DISCUSSION

3.1. *Stimulation of SV40 DNA replication by the* c-myc *enhancer*

pSVPCAT, which contains the SV40 promoter and origin of DNA replication (*ori*) linked to the bacterial chloramphenicol acetyl transferase (CAT) gene, was used as a control for basal level of viral DNA replication and transcription. The *myc* enhancer sequence (*myc*(H-P) region), and its enhancer core sequence (21 bp oligonucleotide) were introduced into pSVPCAT (Fig. 1).

Replication activities of these plasmids were tested in monkey Cos 1 cells, expressing SV40 T antigen. Replication of the SV40 system is absolutely dependent upon the presence of SV40 T antigen and SV40 *ori* sequence. Accordingly, pUCCAT, which only carries the CAT gene in pUC19, could not replicate; pSVPCAT was able to replicate efficiently; pSV2CAT replicated, too, although less efficiently than pSVPCAT. This observation is consistent with reports published so far that negative autoregulation occurs between *ori* and enhancer [22]. Some researchers also found that this phenomenon correlates with the observation that an enhancer binding protein, AP2, traps T antigen after SV40 DNA accumulates to some extent [23]. Previously, pBR322-derived pSV2CAT constructs were reported to yield low replication levels because of toxic sequences present in the vector [24]. To obviate this problem, we used a pSV2CAT construct based on the pUC18 vector (which does not carry those toxic sequences), like the other constructs used in these experiments. Incidentally, all of the constructs contained the same portion of pUC18, so that all replication activities observed were due to the SV40-derived sequences and not to side effects of neighbouring vector regions. The test plasmid p*myc*(H-P)CAT, carrying the *myc*(H-P) region, replicated in Cos 1 cells (Fig. 2, lane 6), which was due to ARS function present in the *myc*(H-P) region, as previously reported [12,14]. Replication activity of p*myc*-O-CAT, carrying the 21-bp long enhancer core sequence, was not detected under the conditions used here, where only more than 1,000 copies of replicated molecules per cell give rise to a significant signal (Fig. 2, lane 7). The difference in replication efficiency between p*myc*(H-P)CAT and p*myc*-O-CAT is probably due to an as yet unidentified auxiliary function of the sequences surrounding the core 21 bp (Ono, Ariga, and Iguchi-Ariga, manuscript in preparation).

Both p*myc*(H-P)PCAT and p*myc*-O-PCAT, which carry the c-*myc* enhancer sequences in addition to the SV40 *ori*/promoter, could replicate 5–10-times better than pSVPCAT (Fig. 2, lanes 1, 3 and 4). It is apparent that the replication activities of p*myc*(H-P)PCAT and

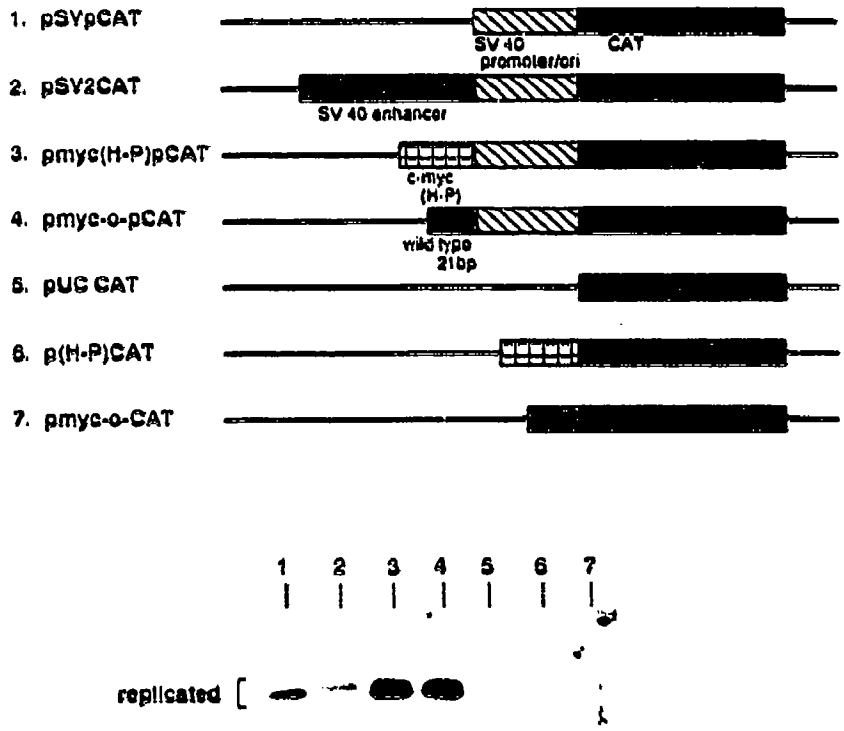

Fig. 2. Effect of the c-*myc* enhancer on SV40 DNA replication. Test plasmids (shown in the upper part) were transfected to Cos I cells and their replication activities were examined as described in Materials and Methods. The results of Southern blot hybridizations are shown in the lower panel. Lane numbers correspond to the plasmid numbers shown in the upper panel.

p*myc*-O-PCAT (Fig. 2, lanes 3 and 4) reach much higher values than those one would obtain by simply adding p*myc*(H-P) or p*myc*-O (lanes 6 and 7) to the SV40 sequences (lane 1). Therefore, the high replication efficiencies of p*myc*(H-P)PCAT and p*myc*-O-PCAT do not seem to be simply due to the additive effects of ARS activities of the *myc* sequences and of the SV40 *ori*/ promoter. This sounds all the more reasonable if we consider that in the absence of the SV40 *ori*/promoter, p*myc*(H-P) works as an ARS much better than p*myc*-0, while little difference is observed between the activities of the combinations of either of the two with the SV40 *ori*/promoter. These results rather indicate that the c-*myc* enhancer, be it the whole (H-P) region or its 21-bp long core sequence, stimulates SV40 DNA replication.

3.2. *Effect of various mutations within the 21 bp core sequence on promotion of SV40 DNA replication*

As a matter of course, we set out to study what nucleotides within the 21 bp core are responsible for promotion of SV40 replication by systematic point mutations. Incidentally, there are two possible protein binding sites within the 21 bp core sequence (Fig. 1): TCTCTTA is the c-*myc* protein complex binding site [13,25–27] and TGCGGT (adjacent to TCTCTTA) is the consensus target for the polyoma enhancer binding protein 2 (PEBP2) [28]. Oligonucleotides containing various mutations within the 21 bp core sequence were synthesized and inserted into pSVPCAT in either orientation. Using these pSVPCAT/21 bp clones, replication assays were carried out (Figs. 3 and 4).

As for the clones with oligonucleotides inserted in 'up-to-down' orientation (indicating that the c-*myc* sequences were inserted from upstream to downstream, towards the SV40 promoter/*ori*), introduction of any mutation decreased to some extent the activity promoting SV40 *ori* replication (Fig. 3). Especially the substitution of AA for TT in TCTCTTA and CG for GC in TGCGGT strongly reduced the replication activity of the respective plasmid (Fig. 3A, lanes 5 and 7) to the levels of pSVPCAT. We can therefore say that TT and GC are very important for promotion of SV40 replication by the *myc* sequence. These data were confirmed with other kinds of mutations within either of the two sites, which also weakened the replication promoting activity (Fig. 3B).

For the clones with mutated oligonucleotides in 'down-to-up' orientation, on the other hand, variations in replication activity were scarcely observed (Fig. 4). These results suggest that the replication-promoting activity is dependent on the orientation of the sequence.

3.3. *Transcription functions of the* c-myc *enhancer*

As described previously [13], the 21 bp core sequence

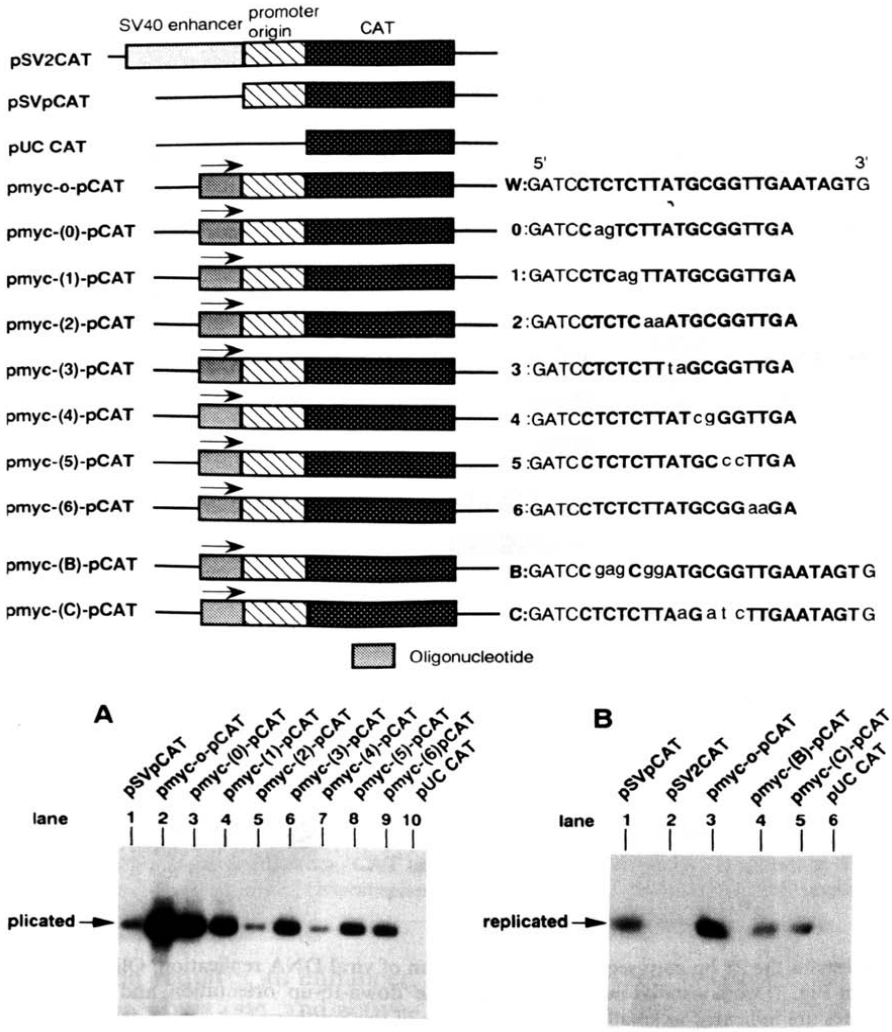

Fig. 3. Effect of various mutations in the 21 bp core sequence on promotion of viral DNA replication. Oligonucleotides with various mutations (upper part) were inserted into pSVPCAT in the 'up-to-down' orientation, and replication assays were carried out as in Fig. 2. The mutated nucleotides are indicated with small letters. The results of the Southern blot hybridizations are shown in the lower part. (A) Results obtained with systematic two-point mutations. (B) Results obtained with more drastic mutations affecting either the TCTCTTA (*myc*) site or the TGCGGT (PEBP2 consensus) site.

of the c-*myc* gene showed enhancer activity on transcription from the SV40 promoter (Fig. 5A, lanes 1 and 9). Therefore, in parallel with replication assays, the effects of mutations within the 21 bp on CAT activity were also examined (Fig. 5). CAT reporter plasmids were transfected into mouse L cells and CAT assays were carried out. The choice of L cells was determined by the fact that they do not produce SV40 T antigen, so the various reporter plasmids cannot replicate. This consideration enabled us to compare the various transcriptional activities at a semi-quantitative level. Besides, L cells had been used for studies on the enhancement of transcription from the c-*myc* gene's promoter under the control of the *myc*(H-P) region [15].

It is interesting to note that mutation of TT in TCTCTTA, and of GC or GG in TGCGGTT reduce transcription to the basal level of pSVPCAT (Fig. 5A, lanes 4, 6 and 7). Mutation of TT in TGCGGTT has, if at all, a little enhancing effect (Fig. 5A, lane 8). More strikingly, mutations within TC or AT of TCTCTTAT actually increase the transcription levels (Fig. 5A, lanes 2, 3 and 5). When the oligonucleotides were inserted in 'down-to-up' orientation to the SV40 promoter, enhancing effect was hardly observed even with the non-mutated 21 bp sequence (Fig. 5B): the clones carrying the oligonucleotides, with or without mutations, showed CAT activities equal or lower than that of pSVPCAT. It is thus suggested that stimulation of transcription from the SV40 *ori*/promoter by the *myc* enhancer is orientation dependent, as in the case of replication promotion. A similar property was also reported in the case of TATA box whose orientation affected transcription of the immunoglobulin λ and heavy-chain genes [25].

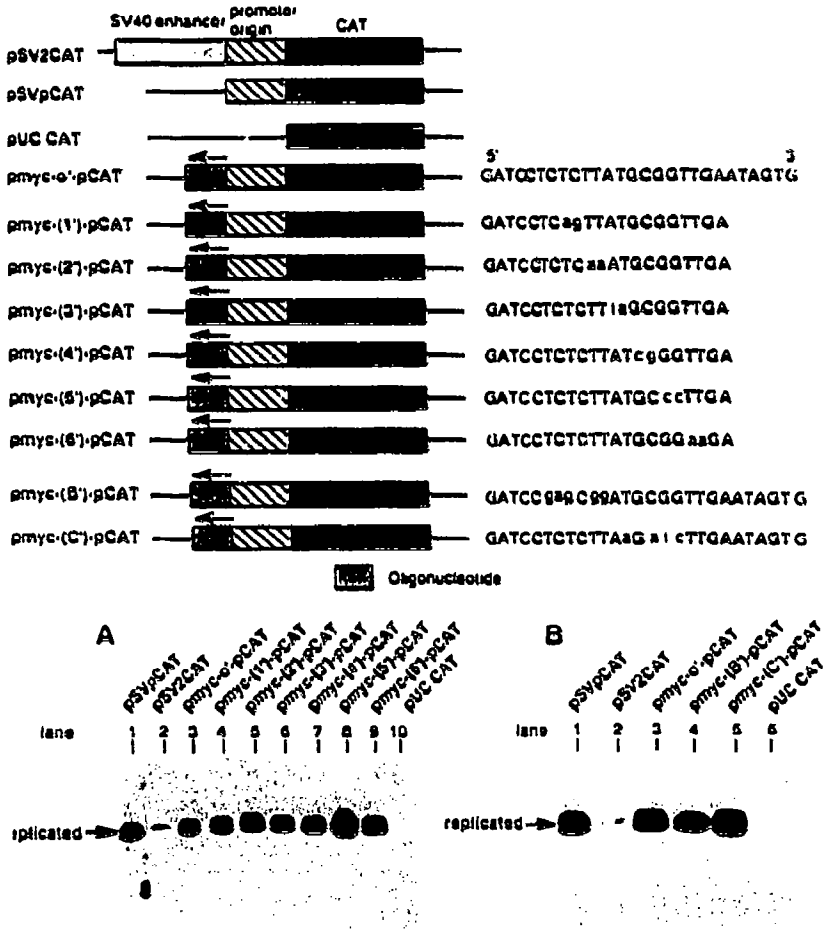

Fig. 4. Effect of various mutations in the 21 bp core sequence on promotion of viral DNA replication. Oligonucleotides with various mutations (upper part; the same series as in Fig. 3) were inserted into pSVPCAT in the 'down-to-up' orientation, and replication assays were carried out as in Fig. 2. The mutated nucleotides are indicated as small letters. The results of the Southern blot hybridizations are shown in the lower part. (A) Results obtained with systematic two-point mutations. (B) Results obtained with more drastic mutations affecting either the TCTCTTA (*myc*) site or the TGCGGT (PEBP2 consensus) site.

To summarize, the sites important for full enhancement of transcription overlap with those required for promotion of replication (see Table I). In fact, promoting activities on replication and transcription were negatively affected by the same mutations. At the present stage, we cannot say whether the same sequences are by chance involved in two different, independent mechanisms that respectively activate transcription and replication, or whether transcription activation function is actually required for promotion of replication. Interestingly, it seems that transcriptional activation requires the integrity of the sequence TGCGGT, whereas the mutation of crucial sites within TCTCTTA actually increase transcriptional activity beyond that yielded by the wild-type 21 bp *myc*(H-P) enhancer core. As previously reported, TCTCTTA is the target of a complex that includes the c-*myc* protein, or a protein with similar epitopes [13,26,27]. These observations correlate with the finding that transcription from the c-*myc* promoter was activated by the *myc*(H-P) sequence when the cells were so treated as to express only moderate amounts of c-*myc* protein (under serum-free conditions), while higher amounts (with serum) actually resulted in a decrease in transcriptional activity [15].

Taken together, these results indicate that the transcriptional enhancer present in the c-*myc* gene also stimulates SV40 DNA replication. This substantiates previous results of ours, that the 21 bp core of the c-*myc* enhancer concertedly regulates transcription and replication [13]. In the presence of T antigen, the SV40 enhancer increases transcription from the SV40 *ori*/promoter when placed either upstream or downstream thereof. By contrast, the SV40 enhancer is not strictly required for replication (reviewed in [29]), although its presence can affect its extent in a position and orientation-dependent manner [30]. It is therefore suggested that in the SV40 system, the enhancer functions dominantly on transcription. In the polyomavirus system, on the other hand, the viral enhancer sequence activates transcription from *ori*/promoter and is also required for

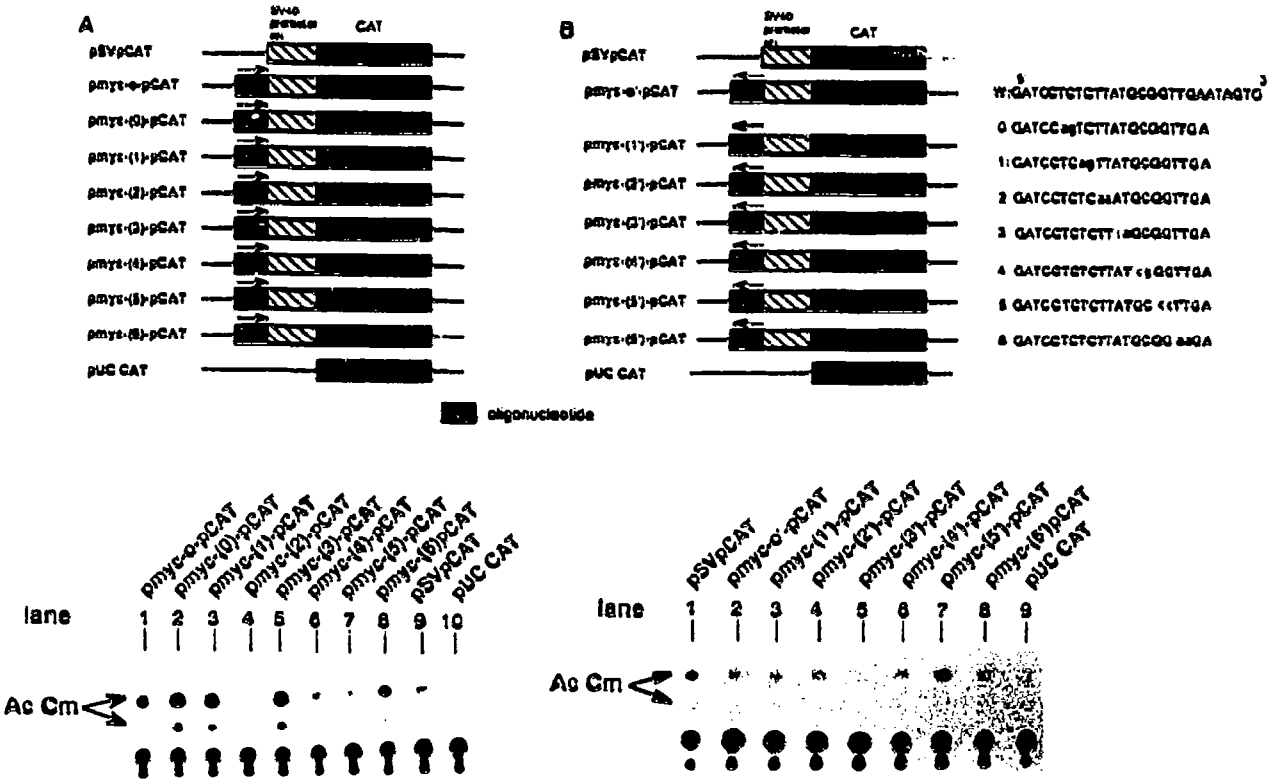

Fig. 5. Effect of various mutations in the 21 bp on transcriptional activity. The various CAT plasmids used in Figs. 3 and 4 (shown here in A and B, respectively), and pCMV-β-gal were co-transfected to L cells. 48 h after transfection, cell extracts were prepared, and β-galactosidase assays were first carried out to normalize the transfection efficiency. CAT assays were then performed as described in Materials and Methods. Cm and Ac-Cm indicate the positions of chloramphenicol and acetylated chloramphenicol, respectively.

replication. Polyoma replication requires an enhancer, but not necessarily the polyoma's own one, and activation takes places in a position- and orientation-independent manner [3]. In contrast with the 'SV40-type' enhancer, the 'polyoma-type' enhancer thus affects both replication and transcription. From the results obtained here, we deduce that the enhancer sequence identified in the *Hin*dIII–*Pst*I region upstream of the human c-*myc* gene is middle way between the two systems, in that it promotes both transcription and replication like a 'polyoma-type' enhancer, but its action is orientation-dependent, like an 'SV40-type' enhancer.

Table I

Effects of mutations on enhancement of transcription and promotion of SV40 DNA replication ('up-to-down' orientation)

REPLICATION PROMOTION	— — ⇓ — ⇓ — —
↑	
mutation introduced	ag ag aa ta cg cc aa
↑	↑ ↑ ↑ ↑ ↑ ↑ ↑
WILD TYPE SEQUENCE	TC TC TT AT GC GG TT
↓	↓ ↓ ↓ ↓ ↓ ↓ ↓
mutation introduced	ag ag aa ta cg cc aa
↓	
TRANSCRIPTION ACTIVATION	⇑ ⇑ ⇓ ⇑ ⇓ ⇓ —

⇑, higher than wild type
⇓, lower than wild type
—, same as wild type

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