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Functional cooperativity between two TPA responsive elements in undifferentiated F9 embryonic stem cells

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We have recently identified an enhancer, termed GPEI, in the 5′-flanking region of the rat glutathione transferase P gene, that is composed of two imperfect TPA (phorbol 12-*O*-tetradecanoate 13-acetate) responsive elements (TREs). Unlike other TRE-containing enhancers, GPEI exhibits a strong transcriptional enhancing activity in F9 embryonic stem cells. Mutational analyses have revealed that the high activity of GPEI is mediated by two imperfect TREs. Each TRE-like sequence has no activity by itself but acts synergistically to form a strong enhancer which is active even in the very low level of AP-1 activity in F9 cells. Furthermore, we show that synthetic DNAs containing two perfect TREs in certain arrangements have strong transcriptional enhancing activities in F9 cells and the activity is greatly influenced by the relative orientation and the distance of two TREs.

Key words: c-*jun*–c-*fos* complex/enhancer/F9 embryonic stem cells/glutathione transferase P gene/TPA responsive element

Introduction

Transcriptional regulation is primarily determined by specific interactions between *trans*-acting factors and their *cis*-active DNA elements (for review, see Mitchell and Tjian, 1989). TPA (phorbol 12-*O*-tetradecanoate 13-acetate) responsive element (TRE) is a well-characterized *cis*-acting sequence found in the regulatory regions of many genes which are responsive to TPA (Angel *et al.*, 1987a,b; Lee *et al.*, 1987) and is demonstrated to be the binding site for c-*jun*–c-*fos* complex or a similar protein complex (Bohmann *et al.*, 1987; Angel *et al.*, 1988; Bos *et al.*, 1988; Chiu *et al.*, 1988; Curran and Franza, 1988; Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988; Rauscher *et al.*, 1988; Sassone-Corsi *et al.*, 1988a,b; Schönthal *et al.*, 1988; Cohen *et al.*, 1989; Gentz *et al.*, 1989). Although both c-*jun* and c-*fos* genes are expressed in almost all kinds of cells examined to date, the expression level of these two proteins in F9 cells is quite low. In fact, TRE-containing enhancers such as that of the collagenase gene are inactive in these cells (Angel *et al.*, 1988; Chiu *et al.*, 1988; Sassone-Corsi *et al.*, 1988a). We found, however, that the GPEI enhancer, found in the rat glutathione transferase P gene (Okuda *et al.*, 1987, 1989), containing two imperfect TREs, was active in these cells. We also found that the DNA containing two perfect TREs in a certain arrangement exhibited a strong transcriptional enhancing activity in F9 cells. In this paper we show that TREs which have been considered to be non-functional in F9 cells can be very active through certain spatial combination of two copies and discuss the possible biological significance of this finding.

Results

GPEI enhancer is active in F9 cells

We have recently shown that the activity of the GPEI enhancer found in the 5′-flanking region of the rat glutathione transferase P gene is mediated by two imperfect TREs (Okuda *et al.*, 1989). Furthermore, we found that both two imperfect TREs would bind the c-*jun*–c-*fos* complex as shown by gel shift assay using affinity purified Jun/AP-1 (unpublished observation). In the course of studying the relationship between GPEI and the c-*jun*–c-*fos* complex, however, we came up with rather unexpected results. As shown in Figure 1, lane 1, GPEI exhibited a high activity without co-transfection of the c-*jun* expresson vector in F9 cells which express this protein at an undetectable level. GPEI connected to the glutathione transferase P promoter element showed a 20 times higher chloramphenicol acetyltransferase (CAT) activity than that of enhancer-less plasmid, Δ-56kCAT (Figure 2b, lane 1 versus 3), but the activity was not greatly stimulated by co-transfection with c-*jun* and c-*fos* expression vectors (Figure 1, lanes 2–4). These results are in contrast with those obtained from other TRE-containing enhancers such as that of the collagenase gene (Angel *et al.*, 1988; Chiu *et al.*, 1988; Sassone-Corsi *et al.*, 1988a), i.e. the collagenase enhancer is inactive in F9 cells, but the activity is dramatically elevated by ~300-fold by co-transfection with both c-*jun* and c-*fos* expression vectors (Figure 1, lanes 5–8).

GPEI activity in F9 cells is mediated by two imperfect TREs

To determine which sequence was responsible for the high activity of GPEI in F9 cells we tested several mutant DNAs of GPEI fragments for their transcriptional enhancing activity and responsiveness to c-*jun* production. As already reported, the effective enhancer element of GPEI which works in rat hepatoma dRLh84 cells is an imperfect palindrome composed of two TRE-like sequences (Okuda *et al.*, 1989). We also found that the same *cis*-active element was active in HeLa cells upon transfection, as in hepatoma cells (data not shown). Therefore, we first examined the effect of mutations in and around the downstream TRE-like sequence in F9 cells. As shown in Figure 2b, mutations at positions −1 and +4 of the downstream TRE-like sequence did not seriously affect the activity of GPEI, whereas a mutation at position +1 abolished its activity completely. These results are exactly the same as those obtained previously from

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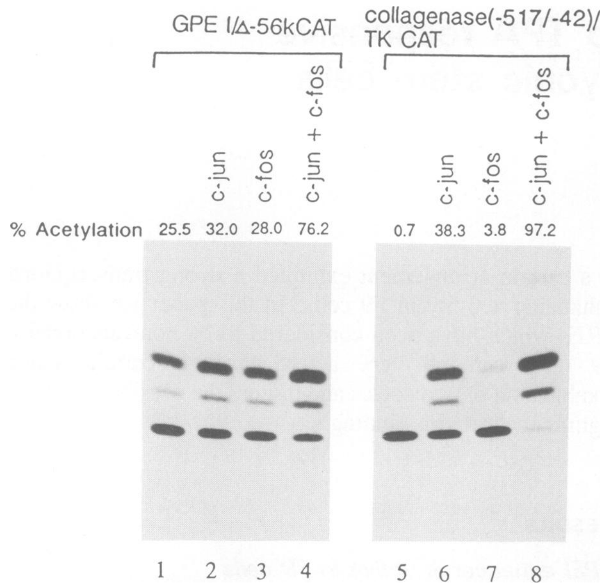

Fig.1. Effect of c-*jun* and c-*fos* expressions on the GPEI and collagenase enhancer activities in F9 cells. Rat c-*jun* and human c-*fos* cDNAs were cloned into the β-actin expression vector (pHβApr-1) and pcDSRα, respectively. The plasmid constructs which were transfected into F9 cells are indicated on the top of each lane. Transfection and CAT assay were done as described in 'Materials and methods'. The conversion of [^{14}C]chloramphenicol to the acetylated forms is also indicated at the top panel.

analyses using hepatoma and HeLa cells (Okuda *et al.*, 1989 and unpublished observation). We also found that the activities of these single-point mutants were not influenced by overexpression of c-*jun* protein (Figure 2b). Next, we examined the effect of mutations of the upper TRE-like sequence. As shown in Figure 2c, all triple-point mutations (tpm) except for tpm 3 reduced the enhancing activity measured without c-*jun* production. In particular, tpm 5 and 6 had much less activity than intact GPEI. These results, again, are quite similar to those obtained from hepatoma and HeLa cells (Okuda *et al.*, 1989 and unpublished observation), but the effect of tpm 5 or 6 was significantly greater in F9 cells than in hepatoma and HeLa cells. Interestingly, unlike the intact GPEI enhancer, triple-point mutants which had a much more greatly reduced activity than the intact GPEI enhancer were remarkably stimulated by the expression of the c-*jun* protein. These results indicate GPEI activity in F9 cells is also mediated by two imperfect TREs acting synergistically. The downstream TRE-like sequence seems to be functionally dominant over the upstream sequence since disruption of the downstream TRE-like sequence completely abolished the GPEI activity, whereas the mutants whose upper TRE-sequence was disrupted were significantly responsive to overproduction of the c-*jun* protein, though they were clearly less active than intact GPEI. This functional dominance of the downstream TRE-like sequence is in good agreement with the higher binding affinity of this sequence to Jun/AP-1 than the upstream TRE-like sequence (Okuda *et al.*, 1989).

Effect of distance between two imperfect TREs on the activity of the GPEI enhancer in F9 cells

To further characterize the mode of synergistic action, we made one internal deletion mutant and several insertion mutants between the two imperfect TREs (Figure 3a) and

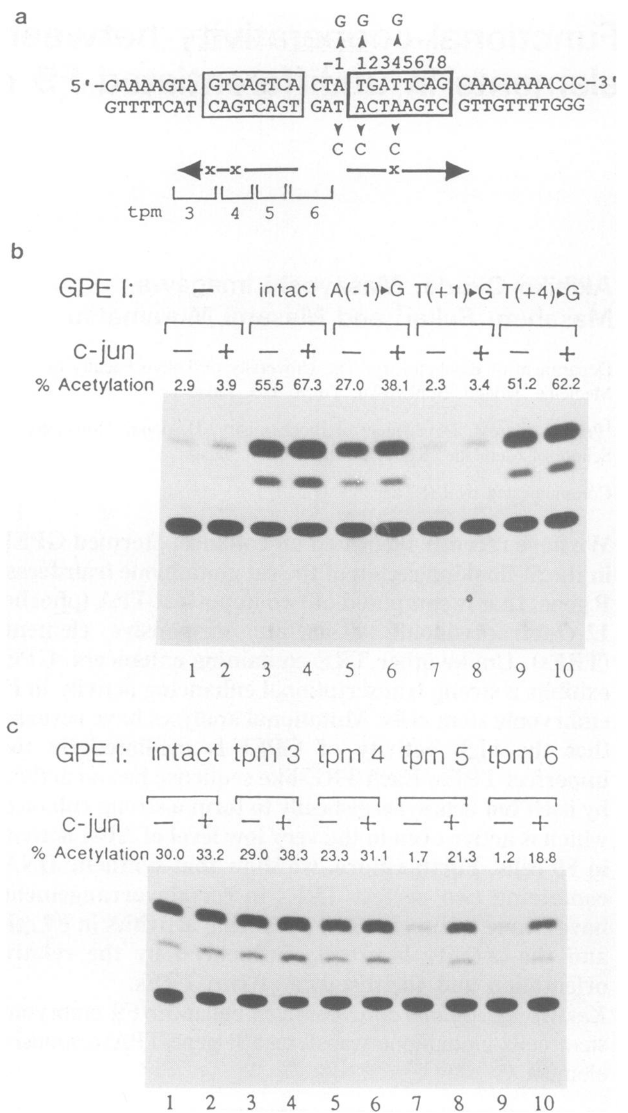

Fig. 2. Two imperfect TREs mediate the activity of GPEI in F9 cells. **(a)** Nucleotide sequence of GEPI enhancer. The upstream and downstream TRE-like sequences are boxed with thin and heavy lines, respectively. X indicates the position of a nucleotide which is different from that of the consensus TRE. The details of the construction of nucleotide substitution mutants were described previously (Okuda *et al.*, 1989). **(b and c)** Effect of mutation of the upstream (c) and downstream (b) TRE-like sequences on the transcriptional enhancing activity of GPEI and responsiveness to c-*jun* production. Each mutant is cloned into the *Sal*I–*Kpn*I site of Δ-56kCAT. Transfection with (+) and without (−) c-*jun* expression vector and CAT assay were done as described in Materials and methods. The conversion of [^{14}C]chloramphenicol to the acetylated forms is indicated at the top panel.

tested their activities by CAT assay as described above. The results of one assay of these constructs are shown in Figure 3b. Though a 2 bp deletion or 2 bp insertion did not affect the enhancing activity appreciably, mutants having more than a 4 bp insertion exhibited much lower activity than with the intact GPEI enhancer. They, however, did not lose all the GPEI activity. To confirm that the activities of insertion mutants were also mediated by two imperfect TREs we tested a single-point mutation which disrupted the upstream TRE-like sequence of the 2 and 6 bp insertion mutants (MTREu-(+2)-TREd, MTREu-(+6)-TREd). As shown in Figure 3b, lanes 6 and 9, these mutants were found to lose all the enhancing activity of GPEI, demonstrating unequivocally that

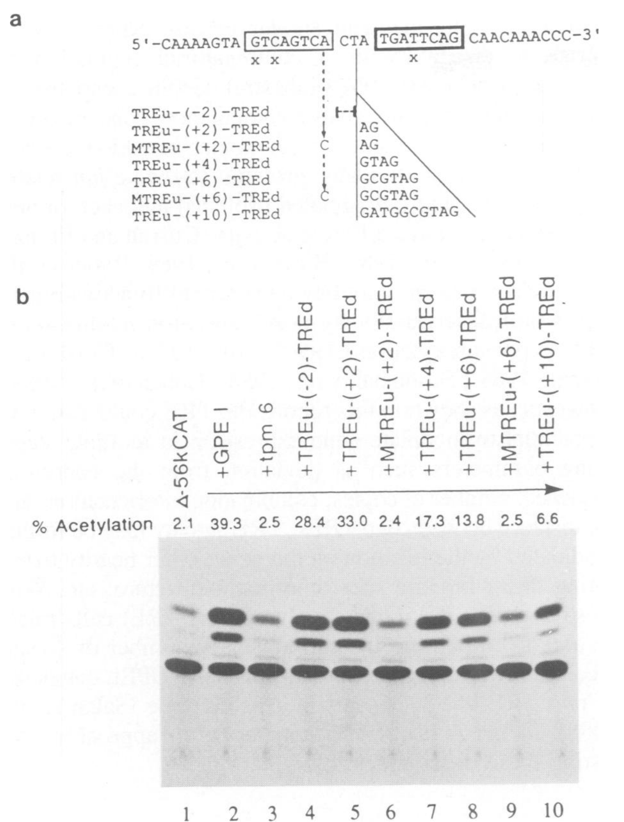

Fig. 3. The GPEI activity is influenced by the distance separating two imperfect TREs in F9 cells. (**a**) Schematic representation of internal deletion and insertion mutations between two imperfect TREs of the GPEI enhancer. The upstream and downstream TRE-like sequences are boxed with thin and heavy lines, respectively. I---I means the position of nucleotides which have been removed. Sequence of inserted nucleotides is derived from the fifth intron of the glutathione transferase P gene (Okuda *et al.*, 1987) which was found to have no activity to modulate gene transcription (data not shown). Each mutant was cloned into *Sal*I–*Kpn*I site of Δ-56kCAT. (**b**) Effect of internal deletion and insertion mutations on GPEI activity in F9 cells. Transfection and CAT assay were done as described in Materials and methods. The conversion of [^{14}C]chloramphenicol to the acetylated forms is indicated at the top panel.

the upstream TRE-like sequence is also working as a TRE unit in the palindromic complex.

Synthetic DNA containing two perfect TREs exhibits differential activities by their relative orientation

From the analysis of the GPEI enhancer, we suspected that, although one copy of TRE was not functional in F9 cells, this element might be active when two copies of TRE were placed close to each other. Several DNAs containing two perfect TREs were synthesized, in which two TREs were assembled in various directions to each other (Figure 4). The TRE is not a palindrome by itself, which was confirmed by Angel *et al.* (1987b); although mutation of T(+1) nucleotide eliminated all the TRE activity, mutation of both A(+7) and G(+8) reduced it to ~25–33% of the consensus TRE. These synthesized DNAs were inserted into the *Hin*dIII–*Bam*HI site of pBLCAT2 having a herpes simplex virus thymidine kinase promoter (Angel *et al.*, 1987a) and assayed for transcriptional enhancing activity after transfection into F9 cells. As shown in Figure 4, first line, the DNA fragment in which two TREs were arranged to form a palindrome exhibited the strongest (~80 times) enhancing

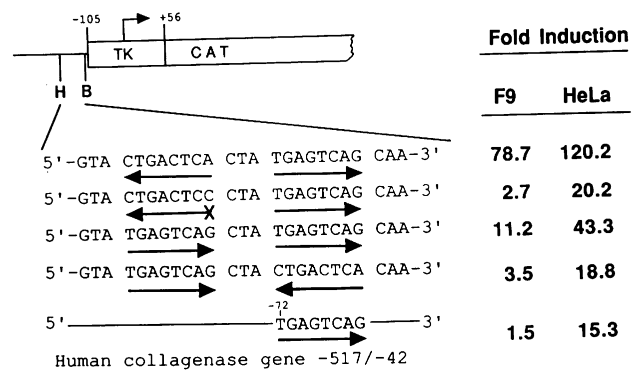

Fig. 4. Differential enhancing activity of synthetic DNA containing two perfect TREs by their relative orientation in F9 and HeLa cells. Synthetic DNAs shown and flanked by *Hin*dIII and *Bam*HI sites at the 5′ and 3′ ends, respectively, were cloned into these sites of pBLCAT2 plasmid. Direction of the TRE is indicated below each sequence by an arrow. X: mutation of the first nucleotide of TRE. Various constructs (4 μg each), together with 8 μg of pUC18 plasmid, were transfected into F9 and HeLa cells as described in Materials and methods.

activity. This construct had ~2 times higher activity as GPEI connected to pBLCAT2 (data not shown). The activity was, however, almost eliminated by a single-point mutation at the first nucleotide of the upper TRE (second line). The DNA fragment containing two tandem repeats of the TRE apparently exhibited a lesser activity (third line). The two TREs arranged in opposite directions to that of the GPEI was least active (fourth line). The TRE-containing collagenase enhancer had no significant activity in F9 cells (fifth line) which is consistent with the data described above and those obtained by others (Angel *et al.*, 1988; Chiu *et al.*, 1988; Sassone-Corsi *et al.*, 1988a). We also examined the enhancing activity of col-TRE×5/TKCAT containing five copies of perfect TRE at 18 bp intervals (center to center) (Angel *et al.*, 1987b). However, this construct exhibited, only ~4 times higher activity than control pBLCAT2 in F9 cells (data not shown). This is apparently due to the long distance between TREs which prevents the functional cooperativity observed for DNA shown in Figure 4. We also examined the transcriptional enhancing activity of these DNA fragments in HeLa cells. The right side column in Figure 4 shows that all these synthetic DNAs exhibit strong transcriptional enhancing activity in HeLa cells and the effect of combinations on the activity of these DNAs is not so marked as in F9 cells, although the order of the enhancing activity is essentially the same as in F9 cells. We also confirmed that all the constructs shown in Figure 4 exhibited ~300-fold higher activity than the control plasmid pBLCAT2 in F9 cells after co-transfection of both c-*jun* and c-*fos* expression vectors (data not shown). These results together indicate that the effect of the combination of two TREs, such as in a palindrome, is particularly significant in the cells with low AP-1 activity.

Discussion

We have recently shown that the GPEI enhancer found in the rat glutathione transferase P gene (Okuda *et al.*, 1987) contains a TRE-like sequence (Sakai *et al.*, 1988). For maximal activity, however, GPEI requires an adjacent upstream sequence of ~19 bp in addition to the TRE-like sequence. In the upstream sequence there is also a TRE-

like sequence which is further degenerated from the consensus TRE compared with the downstream one (Okuda *et al.*, 1989). Unlike other TRE-containing enhancers, GPEI exhibited a strong transcriptional enhancing activity in F9 cells having a very low AP-1 activity. From this observation we first thought that there might be another *cis*-acting element in GPEI that was active in F9 cells. Mutational analyses, however, indicated that the GPEI activity in F9 cells was mediated by the two imperfect TREs. We also found that synthetic DNA containing two perfect TREs exhibited strong transcriptional enhancing activities in F9 cells and the activity was greatly influenced by the combination of their orientations.

The mechanism by which DNA composed of two TREs exhibits differential activities according to their relative orientation is not clear at present. We found that the binding pattern and the affinity of DNA fragments containing two perfect TREs (Figure 4) to affinity purified Jun/AP-1 were not significantly different from each other in the gel shift assay (data not shown). Furthermore, the specific binding activity of the nuclear extract from F9 cells to these fragments is $<10\%$ of that of the nuclear extract from HeLa cells and there are no significant differences among these DNA fragments in the binding activity (unpublished observation). We therefore feel that there must be another mechanism than cooperative binding to the target DNA, as demonstrated for some other *trans*-acting factors such as the phage lambda repressor (Hochshild and Ptashne, 1986) and the glucocorticoid receptor (Schmid *et al.*, 1989), for the synergistic action of two regulatory elements *in vivo*. TRE is a binding site for *jun–jun* homodimeric and *jun–fos* heterodimeric complexes, but most transcription activations are thought to be mediated by the latter complex (Chiu *et al.*, 1988; Halazonetis *et al.*, 1988.; Kouzarides and Ziff, 1988; Sassone-Corsi *et al.*, 1988a,b; Schönthal *et al.*, 1988; Cohen *et al.*, 1989; Gentz *et al.*, 1989). Since TRE is not a palindrome by itself (Angel *et al.*, 1987b), one can imagine that the direction of binding of the *jun–fos* complex to TRE would be obligatorily determined. Because the first T nucleotide of TRE is essential for the binding of this complex (Angel *et al.*, 1987b) and the *jun* protein appears to play a major role in binding with TRE, it is tempting to speculate that the *jun* protein lies closer to the first T nucleotide than *fos* and the two *jun* proteins juxtaposed by palindromic arrangement of the two TREs interact with each other to elicit higher enhancing activity. Then, the modulation of the enhancing activity by two TREs would be due to the mode of protein–protein contacts determined by the arrangements of this element. We also showed that the effect of the combination of two TREs was much more prominent in F9 cells than in HeLa cells having a significant amount of Jun/AP-1 (Lee *et al.*, 1987; Angel *et al.*, 1987b). Jun D is the only protein which is expressed in F9 cells in a detectable amount among the *jun* family (Ryder *et al.*, 1989). It is therefore possible that, unlike the c-*jun* protein (Angel *et al.*, 1988; Chiu *et al.*, 1988; Sassone-Corsi *et al.*, 1988a), *jun D* has a unique property that exhibits its activity only when two TREs are present close to each other on the same DNA just like a protein which binds to the core A motif of the SV40 enhancer (Ondek *et al.*, 1988), although *jun D* has been shown to bind to DNA containing a single copy of TRE (Nakabeppu *et al.*, 1988). This possibility is currently under investigation.

In any event, the data shown in this paper raise an intriguing possibility of a combinatorial regulation by enhancer sequences in the orchestral genomic responses to inter- and intracellular signals including those concerned with cellular proliferation and differentiation. Jun/AP-1 are now known to consist of many proteins (c-*jun*, c-*jun*-related proteins, c-*fos* and c-*fos*-related proteins) and each protein is considered to have different activity (Curran and Franza, 1988; Cohen *et al.*, 1989; Hirai *et al.*, 1989; Ryder *et al.*, 1989). Each protein may have a potential to activate gene expression differentially by post translation modifications such as phosphorylation (Angel *et al.*, 1987a; Curran and Franza, 1988.; Schönthal *et al.*, 1988;. Cohen *et al.*, 1989). However, as shown in this report, the TRE could also have a potential to modulate gene expression in multiple steps, using parameters such as deviation from the consensus sequence, number of copies, combination of orientations and the distance between two TREs. TRE activity may be further modulated by the location on the gene, other nearby *trans*-acting factor binding sites, chromatin structure, etc. With these two sets of parameters (Jun/AP-1, TRE) cells might be able to respond to the extraordinary number of signals received. The identification of an enhancer, GPEI, composed of two TRE-like sequences in a palindrome (Sakai *et al.*, 1988; Okuda *et al.*, 1989) may open up approaches for testing these possibilities.

Materials and methods

Plasmid constructions

Rat c-*jun*(Sakai *et al.*, 1989) and human c-*fos* (Sassone-Corsi *et al.*, 1988a) cDNAs were cloned into the *Bam*HI–*Hin*dIII site of β-actin expression vector (pHβApr-1) (Gunning *et al.*, 1987) and the *Eco*RI site of pcDSRα (Takebe *et al.*, 1988) respectively. The construction of the reporter plasmids, GPEI/Δ-56kCAT and collagenase (-517/-42)TKCAT has been described elsewhere (Angel *et al.*, 1987a; Okuda *et al.*, 1989).

Preparation of synthetic DNA fragments

The synthesis and purification of oligonucleotides were carried out as described previously (Okuda *et al.*, 1989). The different oligonucleotides were mixed, phosphorylated and ligated with T4 DNA ligase in the presence of the *Sal*I–*Kpn*I fragment of Δ-56kCAT containing the CAT gene which is under the control of the glutathione transferase P promoter (-56 to $+59$) as described previously (Okuda *et al.*, 1989) or the *Hin*dIII–*Bam*HI fragment of pBLCAT2 (Angel *et al.*, 1987a). Aliquots of ligation mixture were used directly to transform *Escherichia coli* competent cells, HB101. DNA sequences were confirmed according to Sanger *et al.* (1977).

Cell culture and DNA transfection

Embyronic carcinoma F9 and HeLa cells were grown in minimal essential medium-alpha and Dulbecco's modified Eagle's medium, respectively, both supplemented with 10% fetal calf serum. Cells were transfected by the calcium phosphate co-precipitation technique as described by Chen and Okayama (1987). Four micrograms of the various plasmid constructs were used for transfection assay. When <12 μg of specific DNA was used per 10 cm culture dish, pUC18 plasmid DNA was added to give 12 μg of total DNA. CAT activity was determined according to Gorman *et al.* (1982).

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