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Importance of the *CArG* box in regulation of β-actin-encoding genes

(Recombinant DNA; promoter; fish; carp; transcriptional control of expression)

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SUMMARY

The β-actin-encoding gene (*Act*) in carp is regulated by several *cis*-acting regulatory elements including the evolutionarily conserved $CC(A/T)_6GG$ (*CArG* box or serum-response element) sequences positioned in the promoter region between the CAAT and TATA boxes and in the first intron. To address the roles of the two *CArG* boxes on gene expression, we replaced them with linker sequences. The *CArG* box in the proximal promoter was not required for promoter activity in tissue-cultured cells, but was required in conjunction with a second *CArG* box in the first intron to give full expression in transgenic embryos. Likewise, the geometry of *cis*-acting transcriptional elements in the proximal promoter was more important for expression of transgenic constructs in developing embryos than in tissue-cultured fibroblasts. Mobility-shift and exonuclease mapping experiments indicated that the same or similar protein factors bind around the two *CArG* boxes, suggesting that interactions between the promoter and the first intron are involved in *Act* regulation.

INTRODUCTION

Genes are transcriptionally regulated by specific binding of *trans*-acting protein factors to *cis*-acting DNA regulatory sequences. Such interactions determine the tissue-specific, developmental stage-specific, and appropriate levels of expression. Thus, reporter genes linked to regulatory sequences of certain genes are generally expressed only in the cell lines or developmental stages in which the endogenous gene itself is transcribed (Atchison et al., 1989; Banerji et al., 1983; De Simone et al., 1987; Godbout et al., 1986; Okazaki et al., 1985; Wabl and Burrows, 1984; Zaller and Eckhardt, 1985) with some exceptions (Jantzen et al., 1987; Morgan et al., 1988). The *Act* genes are regulated by a number of protein factors which bind to *cis*-acting nt sequences in and around the transcriptional unit. The proximal promoter for the human *Act* gene contains a CAAT box, a TATA box, and a *CArG* motif in between the CAAT and TATA boxes. Two more *CArG* boxes have been found, one at 1400 bp upstream from the *tsp*, and the other in the first intron (Kawamoto et al., 1988). These elements are important for regulation of expression of this gene (Ng et al., 1989). A CAAT-binding factor has been identified (Frederickson et al., 1989) and an enhancer-binding factor has been shown to specifically associate with the enhancer

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Abbreviations: aa, amino acid(s); *Act*, β-actin-encoding gene; bp, base pair(s); *CArG* box, $CC(A/T)_6GG$ sequence; *cat*, gene encoding Cm acetyltransferase; Cm, chloramphenicol; EPC, carp epithelial cells; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; *tsp*, transcription start point(s); u, unit(s); wt, wild type.

sequence in the first intron (Kawamoto et al., 1988). Additionally, the *CArG* boxes have been shown to bind serum-response factor (Frederickson et al., 1989) and to be inducible by serum and growth factors (Ng et al., 1989; Orita et al., 1989).

The human and carp *Act* genes are similar in organization and have similar regulatory sequences (Liu et al., 1989; 1990a, b). With carp *Act*, we have previously shown that (*i*) the proximal promoter is active by itself; (*ii*) the first intron contains positive regulatory sequences including the *CArG* motif that enhanced expression in an orientation-, position- and copy number-dependent manner, (*iii*) the first intron has a negative regulatory element in its 5′ region; (*iv*) the *CArG* and TATA boxes in the proximal promoter are required for promoter activity. Although possible interactions between *CArG* motifs in the proximal promoter and first intron have been suggested (Liu et al., 1990b), confirmation experiments have not been performed. Here we report that the proximal *CArG* motif is not required in tissue-cultured cells, but is required for expression in developing animals and that interactions between the promoter and intron *CArG* motifs appear to be important for regulation.

RESULTS AND DISCUSSION

(a) Function of the *CArG* motif within the proximal promoter

Our previous studies showed that deletion of the *CArG* motif together with the TATA box from the proximal promoter abolished promoter activity (Liu et al., 1990b). To determine whether the loss of promoter activity was due to the deletion of the *CArG* motif, the deletion of the TATA box, or both, we constructed a clone, pRC6dCArGCAT, containing all the proximal promoter elements except that the 29-bp sequence with the *CArG* box between the CAAT and TATA boxes replaced with a 29-bp linker sequence. An intermediate plasmid was generated in the process which had only a 25-bp linker sequence (Fig. 1). We included this construct in our experiments to gain insight into whether the spacing or geometry between the CAAT and TATA boxes is important. In prokaryotic promoters the geometry (facing) of *trans*-acting proteins bound to different sides of the DNA helix substantially affects transcriptional activity (Dunn et al., 1984; Irani et al., 1983; Lee and Schleif, 1989). The wt construct (pRC6CAT) was used as a control. Each construct was transfected into carp EPC cells and microinjected into fertilized zebrafish embryos, and transient expression of the *cat* marker gene was assayed. The results of the *cat* gene assays shown in Fig. 2 and summarized in Table I demonstrated that in cultured EPC cells, pRC6dCArGCAT gave as much *cat* gene activity as

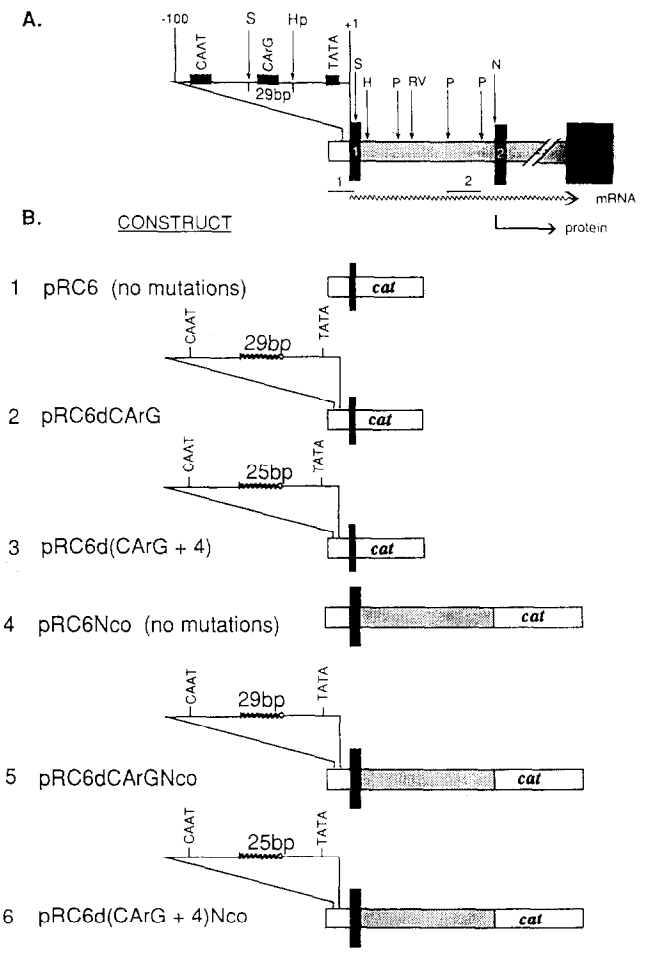

Fig. 1. Constructs used to determine *CArG* box function. (**A**) Organization of the carp β-actin gene with portion of exons 3–5 deleted. Blackened boxes, exons; unfilled rectangles, 5′-flanking sequence of 204 bp containing the proximal promoter elements (Liu et al., 1990b) and introns. Sa, *Sal*I; P, *Pst*I; S, *Sst*I; Hp, *Hpa*II; H, *Hin*dIII; RV, *Eco*RV; N, *Nco*I; +1, *tsp*; −100, 100 bp upstream from the *tsp*. The region between bp +1 and −100 has been expanded. The CAAT, *CArG*, and TATA boxes are indicated. The second line shows probes 1 and 2 (short lines numbered 1 and 2) used for mobility-shift assays (Figs. 3 and 4A). (**B**) Wavy lines, linker replaced regions; the numbers indicate linker lengths. All constructs contained the *cat* reporter gene. To make the *CArG* deletion clones, the *Sst*I fragment of pRC6 (Liu et al., 1990b) was cloned into the *Sst*I site of pUC119 to make pRC6-5′. Clone **3** was constructed by cloning the *Msp*I/*Hin*dIII fragment (the *Msp*I site is located 8 bp downstream from the *CArG* motif) of pRC6 into the *Acc*I/*Hin*dIII sites of pRC6-5′; pRC6d(CArG + 4), is identical to pRC6 except that 29 bp containing the *CArG* motif between the CAAT and TATA boxes was replaced by a 25-bp polylinker sequence in pUC119 from *Sst*I–*Acc*I site. To obtain a plasmid with the same spacing between the CAAT and TATA boxes, clone **3** was digested with *Bam*HI in the replaced polylinker sequence, filled-in by PolIk (BRL) and recircularized, generating clone **2**. Clones **4**, **5**, and **6** were constructed by inserting the corresponding proximal promoter into the *Sal*I site of pSalNcoCAT (Liu et al., 1990b).

the wt pRC6CAT, indicating that the TATA box is required for expression, but that the *CArG* box in the proximal promoter is not. In addition, a decrease in spacing between the

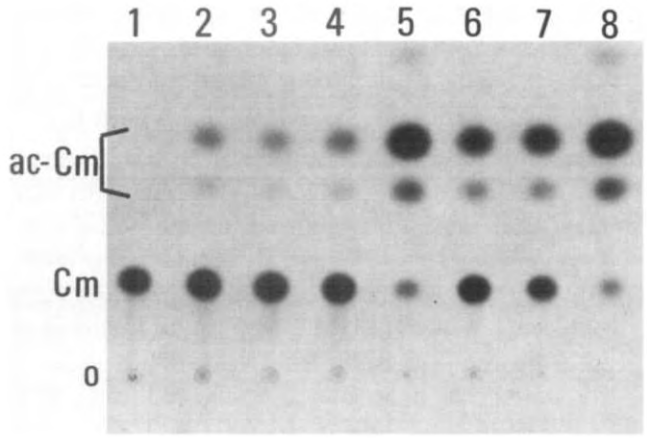

Fig. 2. Typical *cat* gene assays from EPC-transfected cells (Liu et al., 1990c). Lanes: **1**, promoterless control; **2**, clone 1 (Fig. 1B); **3**, clone 2; **4**, clone 3; **5**, clone 4; **6**, clone 5; **7**, clone 6; **8**, 0.07 u of pure *cat* gene enzyme (Sigma). Half the amount of cell extract for pRC6NcoCAT was used in lane **5**. **o**, origin; **Cm**, [^{14}C]-Cm; **ac-Cm**, acetylated forms of [^{14}C]Cm. Carp EPC were transfected by the Ca · phosphate precipitation method (Graham and Van der Eb, 1973) with modifications (Chu and Sharp, 1981). From 30–50% confluent plates of EPC cells were transfected with 24 μg DNA/100 mm dish. Sixty hours after transfection, cells were harvested for analysis of enzymatic activities (Liu et al., 1990b).

CAAT and TATA boxes by 4 bp (pRC6d[CArG + 4] CAT) which alters the rotational geometry by about 140° did not appreciably affect the expression, thereby suggesting that the spacing and facing between the two boxes are not critical for expression in tissue-cultured fish cells.

TABLE I

Expression of *cat* gene constructs[a]

Constructs	*cat* activity	
	Tissue culture	Fish
pCAT	0.1	0.4
pRC6CAT	2	22
pRC6dCArGCAT	2	12
pRC6d[CArG + 4]CAT	3	2
pRC6NcoCAT	100	100
pRC6dCArGNcoCAT	10	27
pRC6d[CArG + 4]NcoCAT	16	2

[a] Expression of *cat* gene in EPC cells and in developing fish. For EPC analysis, four plates were used for each construct and enzyme activities were averaged; the variation between assays was less than 10%. The values were normalised to that of pRC6NcoCAT. For developing embryos, samples of 30 transgenic fish were taken for *cat* gene analysis each day after microinjection for seven days. The values for each construct were integrated over the seven-day period. The values in both columns were normalized to that of pRC6NcoCAT (100% = 0.02 u in tissue culture, 0.004 u in transgenic fish). Microinjection into the central position of the germinal disc of the fertilized eggs of zebrafish was carried out as described (Liu et al., 1990c).

To address the function of the evolutionarily conserved *CArG* motif in the developing animal, the DNA constructs were each microinjected into 500 fertilized zebrafish eggs. Samples of 30 eggs were randomly taken from each injected batch daily after injection for seven days (days two–eight postinjection). Assays of *cat* gene activity were performed to monitor *cat* gene expression and the total integrated expression was calculated (Table I). The wt proximal promoter (pRC6CAT) directed *cat* gene activity at only twice the rate of the *CArG*$^-$ clone (pRC6dCArGCAT), indicating that the *CArG* motif is required for full promoter activity. However, the spacing/facing between the CAAT and TATA boxes appeared to be important in the developing animal for expression. The expression of PRC6d[CArG + 4]CAT was 9% that of pRC6CAT, suggesting that alteration of the geometry between the CAAT and TATA boxes is essential for full transcriptional activity. In several S1-nuclease and RNase-protection assays for the different mRNAs from the transgene constructs we were unable to detect *cat* mRNAs, which must exist at levels less than 0.5% that of endogeneous *Act* mRNAs in zebrafish embryos (Z.L., unpublished observation). Accordingly, our conclusions are based on the assumption that transcriptional initiation is correct in fish embryos as it is in transfected fish cells (Liu et al., 1990b).

(b) Deletion of the *CArG* motif within the proximal promoter reduces enhancement by intron elements

Our previous results (Liu et al., 1990b) suggested interactions between the *CArG* sequences in the proximal promoter and the first intron might regulate *Act* gene expression. Thus, if interactions between the two *CArG* boxes are essential for function, deletion of the *CArG* box within the proximal promoter should result in loss of enhancement of expression by the intronic element. To test our hypothesis, we made the constructs numbered 4–6 (Fig. 1B) by linking intron-1 of the *Act* gene to constructs 1–3 (Fig. 1B) and either transfected them into carp EPC cells or microinjected them into fertilized zebrafish embryos. Expression of the *cat* gene was determined for each construct. In the cultured cells, although no effect was observed with deletion of the proximal *CArG* box for promoter activity (constructs 1 and 2 above), a tenfold difference in *cat* gene activity was observed between the wt promoter plus intron (pRC6NcoCAT) and the *CArG*$^-$ promoter plus intron (pRC6dCArGNcoCAT) (Fig. 2 and Table I). The 4-bp space alteration between the CAAT and TATA boxes did not further reduce the expression of pRC6dCArGNcoCAT (Fig. 2 and Table I). However, in the developing animal, deletion of the proximal *CArG* motif reduced activity fourfold and alteration of the spacing further lowered expression another tenfold. These differences in effects in tissue-cultured cells and the developing animal may be due

to differences in the presence, concentration and/or quality of the *trans*-acting protein factor(s). Similar results showing differences in relative expression in tissue-cultured cells and transgenic animals have been obtained with rat albumin (Heard et al., 1987; Herbomel et al., 1989; Tronche et al., 1989; Pinkert et al., 1987) and elastase I (Swift et al., 1989) genes.

(c) Specific interaction of cell nuclear factors with β-actin gene regulatory sequences

We used two probes for mobility shift assays (Fried and Crothers, 1981) to detect the protein factors which could be involved in the regulation of β-actin gene expression. Probe 1, the proximal promoter bp −204 to bp +68, contained the CAAT, *CArG*, and TATA boxes that comprised the regulatory elements of the pRC6 clone (Fig. 1A). In the experiment shown in Fig. 3, nuclear extracts were prepared from the EPC cells and mobility-shift binding reactions were performed. In absence of the nuclear extract, the 3′-labelled fragment migrated as a discrete band (lane 1). When the crude nuclear extract was added to the binding reaction, a single, major, shifted band was prominent (lane 2). The specificity of the binding was confirmed by competition tests. When either a 25-fold or 50-fold molar excess of unlabelled probe fragment was included, the intensity of the shifted band decreased (lanes 3 and 4). To resolve further which sequences in the 272-bp probe the protein factor bound, we used 25-fold and 50-fold molar excesses of the unlabelled intron fragment containing the *CArG* motif as a competitor. As shown in Fig. 3 (lanes 5 and 6), the intronic fragment competed as well as the proximal promoter fragment. This suggested that the protein factor(s) was binding to the *CArG* motif. This conclusion was supported by the inability of the promoter fragment lacking the *CArG* box to compete with the protein

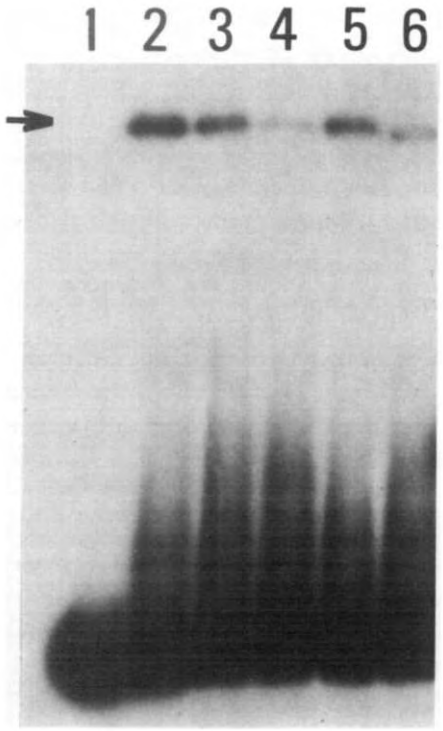

Fig. 3. Sequence-specific binding of protein factors to the promoter region as revealed by a mobility shift assay. Plasmid pRC6 fragment (probe 1, Fig. 1A) was end-labelled. Nuclear extract was derived from EPC cells. Plasmid pUC119 digested with *Msp*I and poly(dI/dC) were used as a nonspecific competitor. Lanes: **1**, no extract; **2–6**, assays contain 10 μg (Bradford, 1976) of nuclear extract; **2**, no specific competitor; **3** and **4**, with 25-fold and 50-fold molar excess of unlabelled pRC6 fragment as competitor, respectively; **5** and **6**, with 25-fold and 50-fold molar excess of unlabelled probe 2 fragment (Fig. 1A). Carp EPC cell nuclear extracts were prepared according to Dignam et al. (1983) with 2 μg/ml of the protease inhibitor leupeptin and aprotinin. The 20 μl binding reactions (Gustafson et al., 1988) contained 2 μg pUC119 and 2 μg poly(dI/dC) as non-specific competitors, 5–10 μg of crude nuclear extracts (Bradford, 1976), 0.1–1.0 ng of 3′-end labelled probe, and appropriate specific competitors. After incubation at room temperature for 10 min the reaction mixtures were loaded onto 6% (29:1) polyacrylamide-bisacrylamide gels.

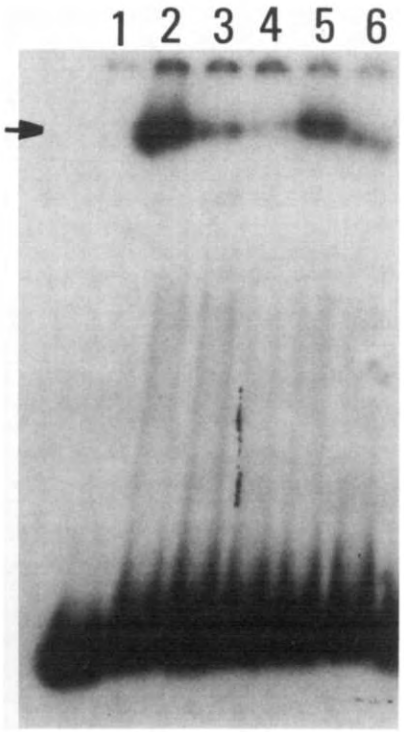

Fig. 4. Mobility shift assay with the fragment of the first intron containing the positive regulatory element (probe 2, Fig. 1A). Conventions are the same as in Fig. 3. Lanes: **1**, no extract; **2–6**, with 10 μg of the nuclear extract each. Lanes: **2**, no specific competitor; **3** and **4**, with 25-fold and 50-fold molar excess of the unlabelled intron fragment (probe 2) as competitor; **5** and **6**, with 25-fold and 50-fold molar excess of the unlabelled probe 1 fragment as competitor.

factor binding (data not shown). The competition tests indicated that the protein factors bound at the *CArG* boxes in the proximal promoter and in the intron are probably similar factors. The results left open the question whether the same or similar proteins bind to the two *CArG* sequences in a fashion analogous to the CAAT-binding protein gene family (Raymondjean et al., 1988).

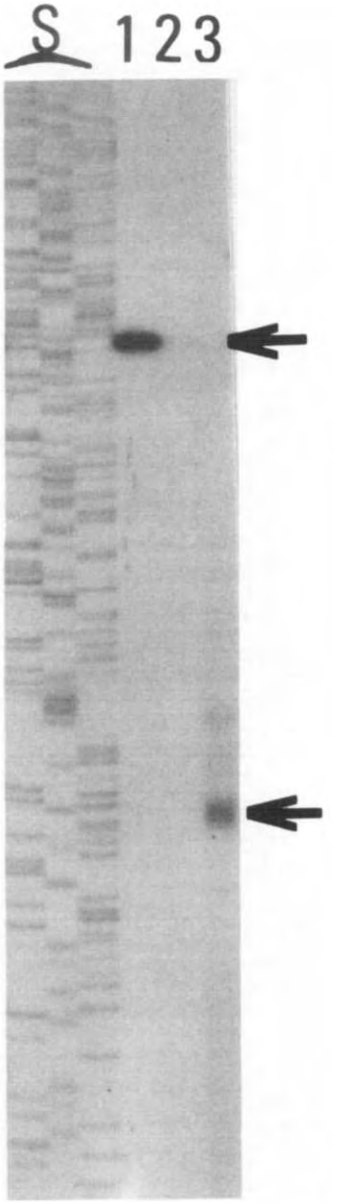

Fig. 5. Exonuclease mapping. **S**, sequencing ladder of pUC119 polylinker sequence with G, A, T. Lanes: **1**, probe 1 (upper arrow) as shown in Fig. 1A; **2**, probe 1 with phage λ exonuclease treatment; **3**, probe 1 with 10 μg of the nuclear extract and treated with phage λ exonuclease; lower arrow, 151-bp protected fragment. The binding reaction was performed as described in Fig. 3 after which 2 μl of 10 × buffer (670 mM glycine-KOH pH 9.4/25 mM $MgCl_2$) and 5 u of phage λ exonuclease (BRL) were added. The reaction was incubated at 37°C for 10 min and stopped by phenol extraction. The extracted DNA was analyzed by electrophoresis on a sequencing gel.

The second probe we used for identification of *trans*-acting regulators was the intronic fragment used above as competitor (Fig. 1A). As with probe 1, the addition of nuclear extract to the binding reaction gave a single shifted band (Fig. 4, lane 2) whose specificity was confirmed by competition with 25-fold and 50-fold molar excesses of unlabelled probe 2 fragment. Probe 1 fragment was able to compete, but not as well, on a molar basis, as the intronic fragment.

We only detected a single shifted band with the proximal promoter probe although at least two more known regulatory motifs are located in this fragment. To confirm the protein factors bind at the *CArG* box in the proximal promoter as shown with competition test, we performed exonuclease mapping. The probe was 3′-labelled at one end downstream from the gene. After the binding reaction was completed, phage λ exonuclease was added which digests from the 5′ ends of linear DNA. If a protein binds to a certain region, the exonuclease should stop at the site where the protein factor binds. As shown in Fig. 5, a fragment of 151 bp was protected. The 5′ end of the protected fragment matched precisely the 5′ end of the *CArG* box in the proximal promoter, demonstrating that the protein factor we detected with probe 1 bound at the *CArG* box. Additional DNaseI protection experiments were conducted which showed that the *CArG* region was protected by extracts in the nuclear extracts described above (Z.L., unpublished observation).

(d) Conclusions

Consistent with the synergistic function of the proximal promoter and the intronic elements, mobility-shift competition tests (Figs. 3 and 4) indicated that the same class of *trans*-acting protein factor(s) bind both regions with *CArG* boxes which favours a DNA looping mechanism (Ptashne, 1988). The interactions between the *CArG* boxes may be accomplished by the same factor. When the *CArG* box in the proximal promoter was deleted, the interaction cannot occur and transcriptional activity was low. When the intronic element containing the *CArG* box was inverted expression was low (Liu et al., 1990b). The data suggest that either promoter and intronic interactions may still occur at the cost of disrupting the normal spacial geometry of the transcriptional complex (Bornstein et al., 1988) or that the interaction might not occur due to an altered stereostructure (see Takahashi et al., 1986). Similarly, interactions between two *CArG* boxes in the proximal regulatory region of the human cardiac α-acting-encoding gene (Miwa and Kedes, 1987), and between elements in the promoter and enhancer of the prolactin-encoding gene (Crenshaw et al., 1989) and the *rpL32* gene (Atchison et al., 1989;

Chung and Perry, 1989), have been suggested to account for the synergistic functions of similar elements.

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