Vol. 195, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1993 Pages 400-408

Muscle-specific transcriptional activation by CArG box requires either homophilic or heterophilic interactions of the CArG box binding factors

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Received June 22, 1993

CArG boxes ($CC(A/T)_6GG$ sequences) are present in various promoters and are able to confer two different types of transcriptional responsiveness : serum inducibility and muscle-specific activation.
Inserted upstream from the ubiquitous *HSV* thymidine kinase promoter, multimerized HCA1 boxes (human cardiac α-actin proximal CArG box) behave as strong muscle-specific activating elements.

Transient expression assay was used to determine whether the muscle-specific transcriptional activation by the CArG boxes depends on the presence in the vicinity of other specific *cis*-acting DNA elements. Our results show that no specific association between different regulatory binding sites is required for the myogenic activity of a CArG box and that CArG elements are able to stimulate transcription in myogenic cells through either homophilic or heterophilic interactions of the CArG box binding factors.

CArG boxes are DNA elements sharing the consensus sequences $CC(A/T)_6GG$ and are found in the promoters of two different types of gene : (i) muscle-specific genes such as cardiac or skeletal α-actin genes and the dystrophin gene, for which it plays a positive tissue-specific regulatory role (1, 2, 3, 4, 5, 6). (ii) immediate-early genes, the best studied of which is the *c-fos* proto-oncogene. In this latter gene, the CArG box constitutes the core of the SRE (serum response element) which binds the SRF (serum response factor) protein and has been shown to be crucial for the transcriptional activation of this gene in response to serum factors (7, 8, 9, 10, 11, 12).

Several studies showed that CArG boxes present in the regulatory regions of muscle genes also bind the same ubiquitous factor referred to as SRF (2, 13, 14, 15). Additionally, several *ex vivo* and *in vivo* studies showed that the proximal CArG box of the human cardiac α-actin gene (HCA1) and the *c-fos* SRE are functionally interchangeable for both types of response, namely specific activation in muscle cells and serum inducibility (15, 16).

One hypothesis that could explain how a common DNA element, the CArG box, can be involved in two different types of gene regulation postulates that a CArG

0006-291X/93 $4.00

box alone could be unable to discriminate between these two kinds of transcriptional regulation but could act in combination with other neighbouring or overlapping protein binding sites in the promoter.

For serum responsiveness of the *c-fos* promoter, for example, the p62/TCF (ternary complex factor, or Elk-1 or SAP-1) binds to the 5' side of the SRF binding site (which is an *ets* domain-binding motif : CAGGAT) as part of a ternary complex including SRE and SRF (17, 18, 19). However, the actual role of p62/TCF remains uncertain and could depend on the cell type (20) and on particular SRE elements.

In the case of the human cardiac α-actin (HCA) promoter, Sartorelli et *al.* showed a functional cooperation between protein-DNA complexes formed on three sites of the promoter : the CArG box at -100 bp (HCA1), the Sp1 recognition site at -75 bp and the CANNTG E box at -50 bp, which is a potential binding site for MyoD1 and related myogenic determinant proteins (21).

We have previously reported that multimerized HCA1 boxes are able to behave as strong muscle-specific activating elements, as well as to confer serum responsiveness when inserted upstream from the ubiquitous *Herpes simplex* thymidine kinase (HSV-tk) promoter (16).

The goal of the present paper was to determine whether the muscle-specific transcriptional activation induced by the CArG boxes is dependent on other unique regulatory binding sites present in the promoter region of CArG-dependent genes. Myogenic and non-myogenic cells were transiently transfected with constructions containing one HCA1 copy or several HCA1 copies upstream from different non-muscle promoters containing, outside the TATA box, either several binding sites for transcription factors or no known binding site. Our results show that the muscle-specific activity of a HCA1 box does not require any specific association with particular binding sites, but that, nevertheless, a HCA1 box needs to cooperate either with another CArG box or with different binding sites to be functional in muscle cells.

In addition, we show that multimerized HCA1 boxes are able to confer serum responsiveness on a minimal promoter containing only a TATA box as a known binding site.

MATERIALS AND METHODS

Cells :

The human hepatoma HepG2 cell line (22) and the mouse fibroblastic Ltk$^-$ cell line (23) were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% (v/v) fetal calf medium (FCS). The mouse embryonic C3H10T1/2 cell line (24, 25), the T4 azamyoblast subclone, derived from 5-azacytidine treated C3H10T1/2 cells (26, 27), and the subclone C2C12 (28), derived from the mouse myogenic C2 cell line (29), were cultured in DMEM plus 20% FCS (v/v). Cells were grown at 37°c under a humidified atmosphere of air plus 7% CO_2.

Transfections :

The serum conditions were standardized for all cell lines : the growth medium was replaced 4 hours before transfection by DMEM plus 10% (v/v) FCS, and 16-18

hours after transfection by DMEM plus 2% FCS. For serum stimulation, L cells were grown and transfected as previously described (16).

Transfections and transient chloramphenicol acetyl transferase (CAT) assays were carried out as previously described (16, 30, 31, 32) with the systematic use of the pRSV/L plasmid (encoding a firely luciferase cDNA) as a transfectability standard (33). Results are expressed as a percentage of the activity generated by the KS-SV2CAT plasmid, in which the CAT gene is driven by the SV40 early promoter and enhancer. We have analyzed the activation induced by CArG boxes by calculating the ratio of CAT activities driven by the HCA1 constructs (CArG boxes binding SRF) and M1 construct (mutated CArG boxes not binding SRF (2)).
In the case of the serum stimulation experiments, for each construct, the mean induction value of stimulated *versus* unstimulated cells was calculated. The ratio of the results obtained for HCA1 and M1 constructs gave the stimulation increase induced by serum response via HCA1 boxes.

Plasmids :
- For plasmids containing multiple copies of CArG boxes, double-stranded oligonucleotides HCA1 or M1 (2) were kinased, self-ligated and submitted to partial digestion by BglII restriction enzyme. Resulting fragments, containing tail-to-tail ligated CArG sequences, were inserted either into the BamHI site of plasmid -54 PK CAT, or into the SmaI site of plasmid PBLCAT2 after filling in with the DNA polymerase Klenow fragment (34). The -105 tk HCA1 4x and -105 tk M1 4x constructs have previously been described (16).
- For plasmids containing only one copy of the CArG box, double-stranded oligonucleotides HCA1 or M1 were blunted and inserted into the blunted BamHI site of plasmids PBLCAT2 and -54 PK CAT.
Integrity and orientation of each construct were tested by sequencing.
The PBLCAT2 plasmid contains the fragment (-105 to +51) of the *Herpes simplex* virus thymidine kinase gene (HSV-tk) ligated to the CAT reporter gene (35). The -54 PK CAT plasmid contains the fragment (-54 to +11) of the rat liver pyruvate kinase promoter ligated to the CAT reporter gene (36).

RESULTS

Cis-activation of a minimal PK promoter and of a -105 tk promoter by 1, 2 and 4 copies of HCA1 CArG boxes.

Cardiac α-actin CArG boxes (HCA1), or mutant boxes (M1) were inserted in 1, 2 or 4 copies in front of either the -54 PK CAT construct or the -105 HSV tk CAT construct. The -54 PK promoter contains a TATA box, but no other known cis-acting element (37), while the -105 tk promoter possesses two binding sites for Sp1 (at -100 and -50 bp), one site for C/EBP at -83 bp and a TATA box at -23 bp (38, 39). In addition, there is a putative E box with a consensus CANNTG sequence at position -64 bp.

Table1 and Fig.1 show that a single copy of the HCA1 element upstream from the tk promoter stimulates its activity by 25-35 fold in myogenic cells (T4 and C2C12) but not in non-myogenic cells (HepG2 and Ltk$^-$). Interestingly, a stimulation similar to that in myogenic cells was noted in 10T1/2 cells which are mesodermic cells inducible to differentiate in myoblasts on 5-azacytidine treatment (27). In contrast, the -54 PK promoter has a scarcely detectable activity when ligated to either a mutant M1 or a wild type HCA1 CArG box in myogenic and non-myogenic cells .

Table 1 : **Cis-activation of a minimal Pk promoter and a -105 tk promoter by 1, 2 and 4 copies of HCA1 CArG boxes**

	HepG2		Ltk-		10T1/2		T4		C2C12	
	CAT activity % (range)	activation x	CAT activity % (range)	activation x	CAT activity % (range)	activation x	CAT activity % (range)	activation x	CAT activity % (range)	activation x
-105 tk HCA1 4x	8.38 n=7 (6.5-15.8)	1	3.83 n=6 (1.4-7.3)	1.9	12.79 n=3 (12.1-13.3)	31	40.8 n=5 (21.3-59.3)	281	150.4 n=6 (91-217)	284
-105 tk M1 4x	8.50 n=9 (5.6-11.0)	1	2.04 n=8 (0.7-3.8)	1	0.41 n=6 (0.25-0.73)	1	0,14 n=8 (0.01-0.35)	1	0.53 n=10 (0.28-1.32)	1
-105 HCA1 1x	17.79 n=2 (15.4-20.1)	1.4	6.39 n=2 (5.7-7.0)	1.9	14.92 n=2 (12.1-17.7)	34	13.80 n=4 (6.0-18.1)	35	40.88 n=2 (40.7-41.1)	25
-105 tk M1 1x	13.08 n=2 (13.0-13.1)	1	3.41 n=2 (3.0-3.8)	1	0.44 n=2 (0.4-0.5)	1	0.39 n=4 (0.09-0.66)	1	1.61 n=2 (1.5-1.7)	1
-54 PK HCA1 4x	1.26 n=8 (0.6-2.2)	52	0.28 n=9 (0.06-0.44)	16.5	1.42 n=5 (0.8-2.9)	[11.0]	8.67 n=5 (5.8-13.0)	[210]	22.90 n=6 (9.8-33.7)	[1500]
-54 PK HCA1 2x	0.98 n=8 (0.70-1.35)	41	0.03 n=2 (0.02-0.03)	1.5	0.20 n=6 (0.01-0.07)	[1.5]	1.34 n=5 (0.9-2.2)	[33]	2.34 n=6 (0.8-4.1)	[160]
-54 PK M1 4x	0.02 n=8 (0.00-0.11)	1	0.02 n=5 (0.00-0.05)	1	0.13 n=6	1	0.04 n=5	1	0.01 n=6	1
-54 PK HCA1 1x	0.16 n=4 (0.16-0.19)	2.1	0.22 n=4 (0.20-0.24)	2	-		-		0.04 n=3 (0.03-0.05)	1.3
-54 PK M1 1x	0.08 n=4 (0.06-0.09)	1	0.11 n=8 (0.04-0.19)	1	-		-		0.03 n=6 (0.01-0.13)	1

Mean CAT activity of each construct standardized by the luciferase activity and expressed as a percentage of the corresponding activity of the KS-SV2CAT plasmid; n, number of independent measurements. Activation values (fold) are expressed relative to the activity of the corresponding M1 constructs. For these latter plasmids an activation value of 1 has therefore been assigned. Numbers in square-brackets are probably underestimated since the -54 PK M1 4x construct activity is around background level.

When four HCA1 copies were multimerized upstream from the tk promoter, the activation was 280 fold in C2C12 and T4 cells and was 31 fold in 10T1/2 cells, confirming previous results (40).

With the -54 PK promoter, the activation appeared considerable (more than 1000 fold in C2C12 cells and 200 fold in T4 cells), although its exact level was difficult to determine because of the very low activity of the minimal promoter by itself. A slight but significant HCA1-dependent *cis*-activation was also observed in non-myogenic cells, similar to that observed in 10T1/2 cells. A constitutive activating role of a CArG element has previously been described, *e.g.* in the *Xenopus* cytoskeletal γ-actin promoter (41).

These results indicate that oligomerized CArG boxes are on their own able to stimulate a minimal promoter in myogenic cells. However, a single HCA1 element

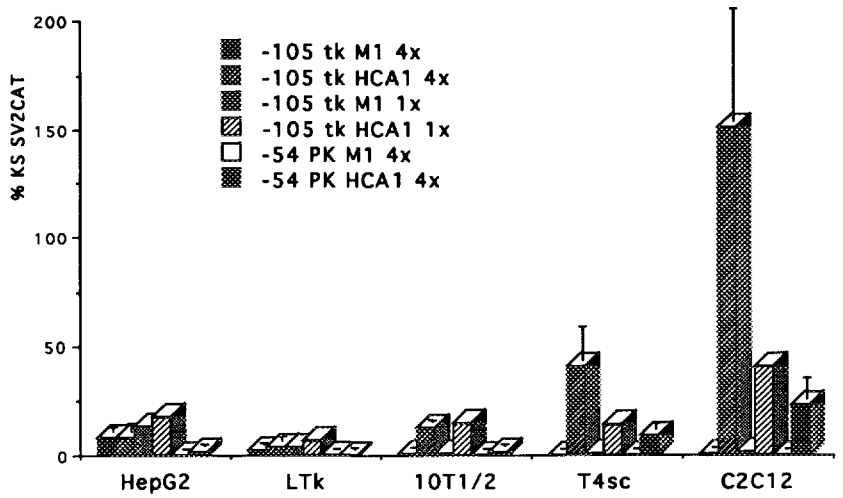

Fig.1. Schematic representation of CAT activity (as a percentage of the KS-SV2CAT plasmid expression) obtained by transfection experiments in different cell lines.

needs, to be active, to cooperate with other cis-acting elements, either identical (oligomers upstream from the -54 PK promoter) or different (monomer upstream from the -105 tk promoter).

Inefficiency of oligomerized CArG boxes in a remote, downstream position.

Since oligomerized CArG boxes are able to behave as strong activating elements, we wondered whether this effect was dependent on their position with respect to the start site of transcription. Therefore, we inserted dimers of HCA1 or M1 boxes 3' to the -105 tk construct, downstream from the CAT gene. The -105 tk (HCA1 2x)3' and -105 tk (M1 2x)3' constructs were similarly active in the different myogenic

Table 2 : **Inefficiency of oligomerized CArG boxes in a remote, downstream position**

	HepG2 CAT activity % (range)	Ltk- CAT activity % (range)	10T1/2 CAT activity % (range)	T4 CAT activity % (range)	C2C12 CAT activity % (range)
-105 tk (HCA1 2x)3'	3.35 n=6 (2.3-4.6)	6.95 n=8 (3.2-10.1)	1.26 n=2 (0.84-1.69)	0.83 n=6 (0.35-1.23)	2.49 n=7 (1.1-3.6)
-105 tk (M1 2x)3'	18.14 n=4 (14.5-22.7)	8.54 n=4 (6.7-11.1)	1.28 n=2 (1.1-1.4)	1.10 n=4 (0.4-2.0)	4.32 n=5 (1.4-9.5)

Mean CAT activity of each construct standardized by the luciferase activity and expressed as a percentage of the corresponding activity of the KS-SV2CAT plasmid; n, number of independent measurements.

Table 3 : **HCA1 CArG box-dependent serum responsiveness**

	+FCS/-FCS	activation
-105 tk HCA1 4x	13.71	x 6.7
-105 tk M1 4x	2.03	x 1
-54 PK HCA1 4x	35.18	x 30.0
-54 PK M1 4x	1.17	x 1

L cells were transfected by different plasmids, then starved and either stimulated or not by serum as previously described (16). For each construct, the mean induction value of stimulated relative to unstimulated cells and the stimulation increase (fold) induced by serum response via HCA1 boxes are indicated. Results represent 4 independent experiments.

and non-myogenic cells tested (table 2). For unknown reasons we even observed a negative effect of the 3' HCA1 copies in HepG2 cells (table 2).

HCA1 CArG box-dependent serum responsiveness.

It has been well established in our laboratory (16) and by other groups (15) that CArG boxes, either *c-fos* SRE or muscle CArG, are able to confer serum inducibility when inserted upstream from homologous and heterologous promoters. Table 3 shows that this response does not require any specific cooperation between a CArG box and another type of *cis*-acting DNA element since it was very well reproduced with the -54 PK promoter ligated to 4 HCA1 copies.

DISCUSSION

The mechanisms by which the CArG boxes seem to be able to confer two very different types of responsiveness on various promoters - inducibility by serum and growth factors on the one hand, activation in myogenic cells on the other hand - remain rather elusive. Working with the human cardiac α-actin gene promoter, Sartorelli et *al.* have indicated that cooperation between the HCA1 CArG box, a Sp1 element and a E box (binding site for MyoD1 and related factors) was essential for both promoter activity and muscle-specificity (21). In a previous paper, our laboratory has shown that cloned upstream from the -105 tk promoter, oligomerized CArG boxes, either *c-fos* SRE or HCA1, were able to confer a myogenic cell-specific activation (16) while such constructs were not transactivated in fibroblasts when co-transfected with a MyoD1 expression vector (Phan-Dinh-Tuy, unpublished). These results suggest that cooperation between the CArG boxes and a functional E box is not indispensable to the specific *cis*-activating effect of the CArG boxes in myogenic cells. In line with this interpretation, it is known that CArG-dependent CAT constructs are already active when transfected in proliferating myoblasts (16, 40), while MyoD1

is considered to be inactive in these cells (42). To address this problem, we decided to test the effect of a single or oligomerized HCA1 CArG box(es) on a minimal promoter containing only a TATA box, without any other *cis*-acting element. Indeed, the -105 tk promoter used in earlier investigations has been shown to contain binding sites for transcription factors, such as Sp1 and C/EBP, and also includes a putative E box.

We show here that a single HCA1 box is able to stimulate the tk promoter in myogenic cells, but has no effect on the minimal PK promoter which contains only a TATA box as a known binding site. We can therefore hypothesize that protein(s) bound to a single CArG element in myogenic cells need to cooperate with other DNA binding proteins, present on the tk promoter but not on the PK promoter, to be active. However, the fact that multimerized CArG boxes stimulate very strongly the -54 PK promoter in myogenic cells suggests that this cooperation can be replaced by homologous interactions between identical complexes. This observation is also relevant for serum inducibility since multimerized HCA1 boxes can confer the serum response on either the PK or the tk promoter. Similarly, it has been demonstrated that the activity of a glucocorticoid response element also requires the interaction between several binding sites for either other glucocorticoid receptors (homologous interactions) or different proteins, e.g. Sp1, CP1, OTF, NF1, or CACC box binding protein (heterologous interactions) (43).

In any case, it is evident that the cis-activating effect of CArG boxes on myogenic cells does not absolutely require a specific heterologous cooperation with Sp1 or MyoD1 binding sites. However, accurate regulation of the promoter activity in different cell contexts could nevertheless depend on the combined influence of different cis-acting elements cooperating with CArG boxes. This could explain the difference observed in the activity of -54 PK HCA1 4x and -105 tk HCA1 4x constructs in the 10T1/2 cells, the latter being much more active in those cells than in hepatoma cells or fibroblasts, while the former is not differentially activated. We have recently suggested that the mechanisms responsible for CArG-dependent transcriptional activation occur very early and begin to be operative in the 10T1/2 cells that are known to be pre-determined to differentiate into myoblasts, adipocytes or chondrocytes on 5-azacytidine treatment (40). It is conceivable that this phenomenon depends on transacting factors present at a very low concentration in 10T1/2 cells and active only on CArG boxes interacting with another element (such as a Sp1 binding site).

Additionally, we show that the position of the CArG boxes with respect to the start site of transcription is also important since two HCA1 elements had no effect on the tk promoter when inserted downstream from the reporter CAT gene. The ability of a DNA binding protein to activate transcription when bound in a remote position has been shown to depend, in particular, on the nature of its activation domain. Seipel et al. have suggested that the factors with acidic, serine-threonine-rich activation domains, which is the case for SRF (11), could be active from an "enhancer" position

while factors with glutamine and proline-rich domains were mainly active from a promoter position (44). If this rule is also valid for SRF, and if SRF is, indeed, bound to CArG boxes in myogenic cells, our results could suggest that this factor does not directly contact the initiation complex but, rather, could interact with it through an adaptator (45).

In conclusion, we confirm in this paper that CArG boxes have on their own the ability to stimulate neighbouring promoters in myogenic cells. Several lines of evidence indicate that SRF is needed for this effect (14, 46). Further investigations are required to determine the mechanisms responsible for this CArG-dependent, muscle-specific transcriptional activation involving such an ubiquitous factor.

Acknowledgments : This work was supported by grants from the Association Française contre les Myopathies, the Ministère de la recherche et de l'espace. We are grateful to H. Gilgenkrantz for critical reading of this manuscript and to A.Strickland for his careful revision of the text.

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