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Synergism of the ATF/CRE Site and GC Box in the Housekeeping Na,K-ATPase α1 Subunit Gene Is Essential for Constitutive Expression

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Na,K-ATPase α1 subunit gene is constitutively expressed in a wide variety of tissues. Our previous studies revealed that the promoter region between −77 and +17 of the transcription initiation site of the rat Na,K-ATPase α1 subunit gene (*Atp1a1*) is sufficient for the promoter activity. In this region, an ATF/CRE site with an adjacent GC box exists. To elucidate how these sites are involved in the promoter activity, we analyzed effects of point mutations at these sites on transcription by *in vitro* transcription assays using nuclear extracts prepared from various rat tissues. Mutation at either site resulted in dramatic reduction of the promoter activity in all nuclear extracts, while mutation at both sites did not lead to further reduction. These results indicate that the ATF/CRE site and GC box are both essential for promoter activity and show synergistic activation. Electrophoretic mobility shift assay indicated that Sp1 and/or Sp3 bind to the GC box, and ATF1-CREB heterodimer binds to the ATF/CRE site. Since an element, ATF/CRE site-GC box, is conserved in mammalian Na,K-ATPase α1 subunit genes and in other constitutive promoters, we propose that this element is a critical unit for constitutive expression.

Ubiquitous enzyme Na,K-ATPase consists of two subunits α and β. The enzyme maintains Na^+ and K^+ gradient across the cell membrane, and is involved in various physiological phenomena. The α1 subunit, one of the three isoforms of the α subunit, is found in all animal tissues and cells. The gene coding for the α1 subunit is expressed in virtually all tissues, being considered as a housekeeping gene (reviewed in 1, 2). 5′-flanking regions of the Na,K-ATPase α1 subunit genes of rat, human and horse have been isolated (3-5). Nucleotide sequences between the translation initiation site and the position around −100 of the transcription initiation site are highly conserved among these genes (76% identity for 360 bp). An ATF/CRE site (−70 to −63), a GC box (−57 to −48) and a TATA-like sequence (−33 to −27) exist in this region (see Fig. 1). While promoters in typical housekeeping genes have a very high GC content, no apparent TATA sequence, and the presence of multiple transcriptional initiation sites (6), the promoter of the Na,K-ATPase α1 subunit gene has high GC content but has a TATA-like sequence and a single transcription initiation site. To understand molecular mechanism how the gene is expressed in a wide variety of tissues, we analyzed transcription regulatory elements in the promoter of the rat Na,K-ATPase α1 gene (*Atp1a1*) by *in vitro* transcription assays using nuclear extracts prepared from various tissues and cells (7,8) or by reporter gene assays using various cell lines (9), revealing that the region between the position −77 and +17 of the transcription initiation site is essential for the promoter activity. In this paper, we analyzed functions of the ATF/CRE site and GC box in detail and identified binding factors to the GC box. The results suggest that synergism of an ATF/CRE site and an adjacent GC box is essential for constitutive expression of *Atp1a1* and other housekeeping genes.

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Abbreviations: ATF, activating transcription factor; *Atp1a1*, Na,K-ATPase α1 subunit gene; C/EBP, CCAAT/enhancer-binding protein; CRE, cAMP response element; CREB, CRE binding protein; CREM, CRE modulator; EMSA, electrophoretic mobility shift assay; Sp, specificity protein; SREBP, sterol regulatory element binding protein; Stat, signal transducer and activator of transcription.

MATERIALS AND METHODS

Construction of template promoters. ARE-P, ARE-Pmut or ARE-Psym, covering from −75 to −62 of *Atp1a1*, was subcloned into the *Nhe*I site of pA1LS3LF, which contains the region between the position −102 and +261 of the transcription initiation site or the *Atp1a1* promoter (9). The resulting plasmids were digested with *Mlu*I, re-

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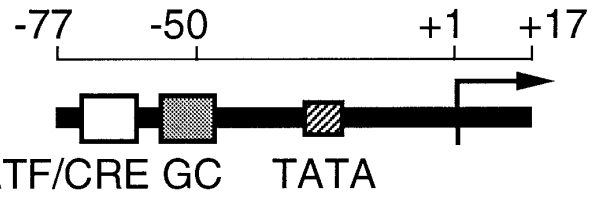

FIG. 1. Promoter elements essential for constitutive expression in *Atp1a1*. The ATF/CRE site, GC box, and TATA-box like sequence are indicated by open, closed and striped boxes, respectively. Numbers indicate the position from the transcription initiation site (arrow).

moving −102 to −62 of pA1LS3LF, and were re-ligated to construct pA1U-75wwLF, pA1U-75mwLF, pA1U-75swLF, respectively. ARE-P (5′-CTAGAACGGTGACGTGCACGCGTCTAG), ARE-Pmut (5′-CTAGAACGGTGAGCTGCACGCGTCTAG) and ARE-Psym (5′-CTAGAACGGTGACGTCAACGCGTCTAG) were prepared by annealing synthetic oligonucleotides. The ATF/CRE site and the corresponding mutated sequences are underlined. DNA fragment GC-Pmut was ligated into pA1LS3LF after being digested with *Mlu*I and partially with *Bsp*MI to construct pA1LS3LF-GCmut. GC-P (5′-CGCGTGGGCGGAGCCA) and GC-Pmut (5′-CGCGTGGATGGAGCCA) were prepared by annealing synthetic oligonucleotides. The GC box and the corresponding mutated sequence are underlined. ARE-P, ARE-Pmut or ARE-Psym was subcloned into the *Nhe*I site of pA1LS3LF-GCmut, and the resulting plasmids were digested with *Mlu*I and were re-ligated to construct pA1U-75wmLF, pA1U-75mmLF pA1U-75smLF, respectively. All of the constructs were verified by DNA sequencing.

In vitro transcription assays. *In vitro* transcription assays were carried out as previously described (7). Transcripts were analyzed by a primer extension procedure. The products were resolved on denaturing polyacrylamide gel electrophoresis and were detected by autoradiography. For quantification of individual transcripts, autoradiograms were scanned and analyzed using the Discovery Series (PDI). Nuclear extracts from rat tissues were prepared as previously described (7).

Electrophoretic mobility shift assay (EMSA). EMSA was performed as previously described (7). Approximately 5 fmole of ^{32}P-labeled GC-P was used as a probe. Polyclonal antibodies against Sp1 (PEP2) and Sp3 (D-20) were purchased from Santa Cruz Biotechnology.

RESULTS

ATF/CRE site and GC box act synergistic on Atp1a1 transcription. 5′-flanking region of *Atp1a1* is shown in Fig. 1. Our previous studies have shown that the region between the position −77 and +17 is sufficient for constitutive expression of *Atp1a1* on the basis of *in vitro* transcription assays (7,8). Both the ATF/CRE site (−70 to −63) and GC box (−57 to −48) exist in this region (Fig. 1), implying their roles in constitutive expression. To clarify this point, we analyzed the transcriptional activity of the *Atp1a1* promoters (−75 to +261) with point mutation on either the ATF/CRE site or GC box (Fig. 2A) by *in vitro* transcription assays using nuclear extracts from rat kidney, brain and liver. These mutations abolished binding of factors to the ATF/CRE site or GC box (7; data not shown).

Fig. 2B and C show the results of *in vitro* transcription assays. Point mutation in ATF/CRE (mw) reduced the transcriptional activity to one tenth in all three nuclear extracts. The result suggests that binding factors to the ATF/CRE site are critical in all tissues. This observation is consistent with our previous results (7). Point mutation in the GC box (wm) reduced transcriptional activity to one tenth in kidney and liver nuclear extract and one fifth in brain. The result indicates that binding factors to the GC box are also critical for the promoter activity in all tissues. When we introduced mutations in both ATF/CRE site and GC box (mm), the transcriptional activity was reduced to about 5-10% of the wild-type promoter, which was comparable with that in the single mutation at either the ATF/CRE site or GC box. These results suggest that the ATF/CRE site and GC box act synergistic on *Atp1a1* transcription.

Symmetrization of the ATF/CRE site did not compensate for the GC box mutation. The *Atp1a1* pro-

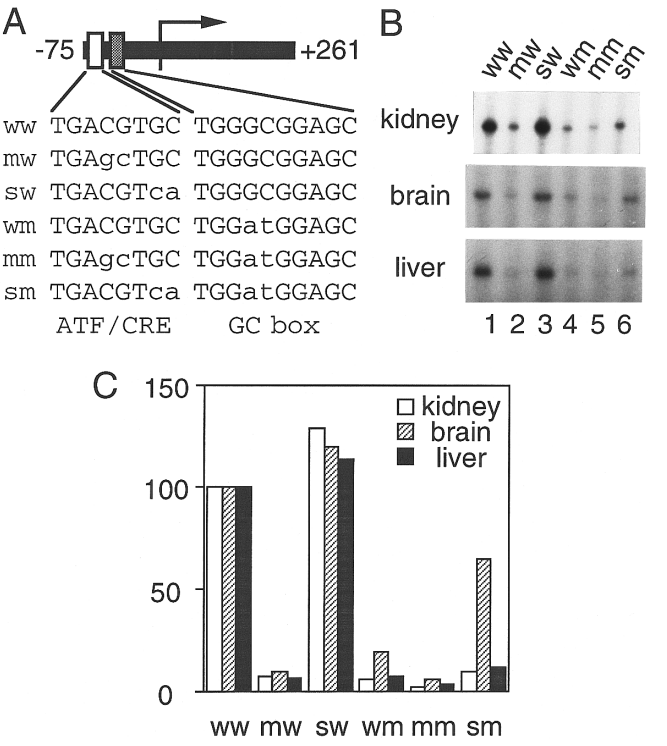

FIG. 2. Transcriptional synergy of the ATF/CRE site and GC box. (A) Scheme of the *Atp1a1* promoters with point mutation used in *in vitro* transcription assays. The introduced point mutations are indicated with lowercase letters. (B) *In vitro* transcription using rat kidney, brain and liver nuclear extracts. (C) Relative transcriptional activity of mutant promoters was compared with the wild-type promoter. Results of *in vitro* transcription assays shown in (B) were quantitated as described in Materials and Methods. The activity of the wild-type promoter was taken as 100.

moter has an asymmetrical ATF/CRE sequence. Previous studies have shown that ATF/CREB proteins have lower binding affinity to an asymmetrical ATF/CRE site than a symmetrical site (10,11), suggesting that a symmetrical ATF/CRE site has stronger transcriptional activity than an asymmetrical site. We next substituted GC dinucleotide in the wild-type *Atp1a1* promoter with CA to make the ATF/CRE site symmetrical (see Fig. 2A sw). The transcriptional activity of this promoter (sw) was increased by 10-30% than that of the wild-type (Fig. 2B,C). The result indicates that symmetrization of the ATF/CRE site enhances the promoter activity, though the extent was small.

Next we introduced the GC box mutation on the promoter with a symmetrical ATF/CRE site (see Fig. 2A sm) and tested the promoter activity. Mutation in the GC box reduced the transcriptional activity to about one tenth of the sw promoter in kidney and liver nuclear extracts (Fig. 2B,C). The reduction was comparable with that in the promoter with an asymmetrical ATF/CRE site (wm). The result indicates that increase in DNA binding affinity for ATF/CREB proteins cannot compensate for the function of the GC box in kidney and liver. On the other hand, only 50% reduction of the transcriptional activity by the GC box mutation was observed in brain nuclear extract (Fig. 2B,C). This observation suggests that contribution of the GC box in constitutive expression is relatively smaller in brain.

Sp1 and Sp3 bind to the GC box of the Atp1a1 promoter. Although we have previously shown that a binding factor to the ATF/CRE site in nuclear extracts prepared from rat kidney, brain and liver were identified as an ATF1-CREB heterodimer (7), we have not identified binding proteins to the GC box. To analyze binding factors to the GC box, we performed EMSA using GC-P which contains nucleotide sequence between the position −61 and −46 as a probe (see Materials and Methods). Two major shifted complexes were observed in all three nuclear extracts (Fig. 3, open and closed arrowheads). Binding of factors in these complexes was shown to be sequence specific on the basis of oligonucleotide competition assays (data not shown). To identify these binding factors, we performed EMSA in the presence of specific antibodies against Sp1 and Sp3. Anti-Sp1 antibody abolished the upper shifted complex and produced super-shifted complex in kidney and liver nuclear extracts (Fig. 3, lanes 2,10). In brain nuclear extract, trace amount of super-shifted complex appeared when we used anti-Sp1 antibody, and the formation of the upper shifted complex showed marginal decrease (Fig. 3, lane 6). These observations indicate that the upper shifted complex in all nuclear extracts contains Sp1, while another upper complex which does not contain Sp1 exists in brain. Anti-Sp3 antibody interfered the formation of the lower shifted complex and produced super-shifted complex in all nuclear extracts (Fig. 3, lanes 3,7,11). This observation indicates that the lower complex in kidney, liver and brain nuclear extracts contains Sp3. These results were confirmed by EMSA using both anti-Sp1 and anti-Sp3 antibodies (Fig. 3, lanes 4,8,12). We conclude that Sp1 and Sp3 are major binding proteins to the GC box in the *Atp1a1* promoter in kidney and liver.

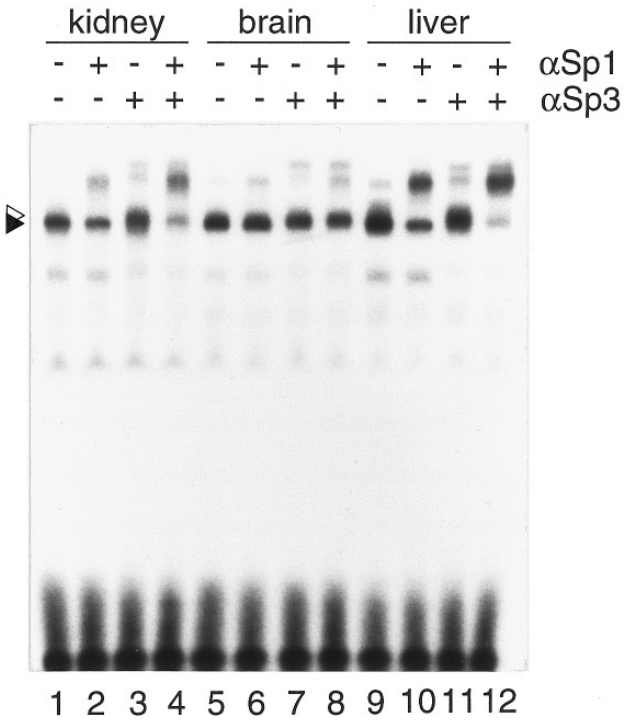

FIG. 3. Sp1 and Sp3 in nuclear extracts prepared from rat tissues bind to the GC box. EMSA was performed using nuclear extracts prepared from rat kidney (lanes 1-4), brain (lanes 5-8) and liver (lanes 9-12), and GC-P as a probe. Five hundred ng each of antibody against Sp1 (lanes 2,4,6,8,10,12) and/or Sp3 (lanes 3,4,7,8,11,12) was added before incubation. Open and closed arrowheads indicate the upper and lower shifted complexes, respectively.

DISCUSSION

The results in this study clearly indicate that synergism of the ATF/CRE site and GC box is essential for the promoter activity of *Atp1a1*. Nomoto *et al.* (12) reported that both the ATF/CRE site and GC box of *Atp1a1* are protected in rat kidney, brain and liver nuclei on the basis of *in vivo* footprinting analysis. Their observation suggests that synergism of the ATF/CRE site and GC box is also required *in vivo*. Sequence alignment of 5′-flanking regions of mammalian Na,K-ATPase α1 subunit genes indicates that the ATF/CRE site and GC box also exist in human and horse genes

	ATF/CRE	bp	GC box
r NaK α1	TGACGTgc	5	TGGGCGGAGC
h NaK α1	TGACGTtt	7	TGGGCGGGGC
ho NaK α1	TGACGTgc	7	TGGGCGGGGC
h H4 histone	TGACGaaA	7	aGGGCGGGGa
h HMGcoAR	TGACGTag	16	TGGGCGGttg**
r HMGcoAR	TGACGTag	16	TGGGCGGttg**
ha HMGcoAR	TGACGTag	16	TGGGCGGttg**
h β-pol*	TGACGTCA	7	TGGGCGGGGC**
b β-pol*	TGACGTCA	7	GGGGCGGtGC**
m β-pol*	TGACGTCA	12	GGGGCGGGGC**
h Rb*	TGACGTtt	0	cGGGCGGAAg
h cyclin D3*	cGACGTCc	11	GGGGCGGGGC**
r cyclin D3*	TGACGTCg	11	GGGGCGGGAC**
m cyclin D3*	TGACGTag	11	GGGGCGGGGC**
r PP2Aα	TGACGTCA	6	cGGGCGGccg**
h α-pol*	gcACGTCA	13	GGGGCGGAGC
consensus	TGACGTCA		GG/TA GGCG GGGC/TAAT

FIG. 4. ATF/CRE site-GC box in various promoters. bp indicates numbers of nucleotides between the ATF/CRE site and GC box. The GC box is located upstream of the ATF/CRE site in promoters indicated by asterisks. Orientation of the GC box is reversed in promoters with double asterisks. Consensus sequences of ATF/CRE site (55) and GC box (56) were shown as references. Nucleotide sequences which does not match to the consensus sequences are indicated with lowercase letters. Source: rat Na,K-ATPase α1 (r NaK α1; 5); human Na,K-ATPase α1 (h NaK α1; 4); horse Na,K-ATPase α1 (ho NaK α1; 3); human H4 histone (h H4 histone; 38); human 3-hydroxy-3-methylglutaryl coenzyme A reductase (h HMGcoAR; 40); rat 3-hydroxy-3-methylglutaryl coenzyme A reductase (r HMGcoAR; 46); chinese hamster 3-hydroxy-3-methylglutaryl coenzyme A reductase (ha HMGcoAR; 39); human DNA polymerase β (h β-pol; 41); bovine DNA polymerase β (b β-pol; 47); mouse DNA polymerase β (m β-pol; 42); human retinoblastoma (h Rb; 43); human cyclin D3 (h cyclin D3; 48); rat cyclin D3 (r cyclin D3; 50); mouse cyclin D3 (m cyclin D3; 49); rat protein phosphatase 2Aα (r PP2Aα; 44); human DNA polymerase α (h α-pol; 45).

(Fig. 4; 3-5), demonstrating importance of both the ATF/CRE site and GC box in constitutive and ubiquitous expression of the Na,K-ATPase α1 subunit gene.

We propose an idea that transcription factors responsible for the synergism of the ATF/CRE site and GC box in the *Atp1a1* promoter are ATF1-CREB heterodimer and Sp1/Sp3. In this study, we have shown that binding proteins to the GC box are Sp1 and/or Sp3 in rat tissues (Fig. 3). Sp1 and Sp3 are ubiquitous transcription factors which bind to the GC box with the zinc finger domain (13,14). Cooperation with other transcription factors, such as GATA-1, YY1, Stat1, SREBP, is required for Sp1 to function in transcription of some promoters (15-18). It is interesting to note that an unidentified zinc-dependent DNA binding protein which binds to G-rich sequence cooperate with CREB to activate the human thyroglobulin enhancer (19). On the other hand, we have previously demonstrated that a major binding factor to the ATF/CRE site is ATF1-CREB heterodimer in various rat tissues (7). ATF1 and CREB are members of the ATF/CREB family, which belong to the basic leucine zipper superfamily (20). Some members of the ATF/CREB family can form heterodimer with each other or with other members of the basic leucine zipper superfamily, for example, ATF2-ATF3, ATF2-cJun, ATF4-cFos and ATF2-C/EBPα (21-23). In case of ATF1 and CREB, they can only heterodimerize with each other or with transcription repressor CREM (20). We suggest that ATF1-CREB heterodimer but not other ATF/CRE site-binding activities is responsible for the synergism at least in kidney, since ATF1-CREB heterodimer is the only binding activity in kidney nuclear extract (7).

Expression of *Atp1a1* is activated by intracellular cAMP (24,25) or Ca^{2+} (26) levels, and by mineralocorticoid (27-30) or glucocorticoid (30,31). Since phosphorylation of either ATF1 or CREB by the cAMP- or the Ca^{2+}/calmodulin-dependent protein kinase have been shown to enhance ATF/CRE site-dependent transcription (32-35), transcriptional activation of *Atp1a1* may be mediated by phosphorylation of ATF1-CREB heterodimer. Indeed, we have previously shown that phosphorylation of ATF1-CREB heterodimer enhances transcription of the *Atp1a1* promoter *in vitro* (11). Requirement of the synergism between the ATF/CRE site and GC box in the cAMP- or Ca^{2+}-mediated induction of *Atp1a1* expression remains to be elucidated.

Only brain nuclear extract showed low sensitivity to the GC box mutation. One possible explanation for this observation is that binding proteins to the ATF/CRE site in brain may act without synergistic effects of GC box binding proteins. CREB homodimer is a candidate for such protein, since we have previously observed that it exists in brain but not in kidney or liver nuclear extract (7). Another possibility is that GC box binding proteins in brain has weaker transcriptional activity than those in kidney or liver. We detected smaller amount of Sp1 in brain nuclear extract compared with kidney and liver (Fig. 3), implying that Sp1 may mainly contribute to efficient synergism of the ATF/CRE site and GC box.

GC box is the most well known element for constitutive transcription, and exists in many housekeeping gene promoters (6). While multiple GC boxes have strong transcriptional activity, single GC box has only weak activity (36). An adjacent single GC box addition to the *cis*-regulatory element is required for transcription activation by Stat1 or SREBP (17,18). These observations suggest that synergism of a GC box and an adjacent *cis*-element is required for efficient transcription. On the other hand, the basal activity of an ATF/CRE site depends on flanking sequences (37), implying

that transcription factors binding adjacent to an ATF/CRE site may be required for constitutive expression. Kanei-Ishii and Ishii (37) have shown that an ATF/CRE site linked to the human c-Ha-ras-1 promoter, which has four GC boxes, resulted in constitutive expression in all cell types they tested. We propose that an ATF/CRE site with an adjacent GC box is essential for efficient constitutive transcription. Indeed some constitutive promoters have an ATF/CRE site-GC box element (Fig. 4; 38-50), and the elements are known to be essential for transcription of these genes (41-43,47,48,51-54). We suggest that ATF/CRE site-GC box is an essential element for constitutive expression in various genes.

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