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ATF-1CREB heterodimer is involved in constitutive expression of the housekeeping Na,K-ATPase α1 subunit gene

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ABSTRACT

Na,K-ATPase α1 subunit is an essential protein for cell growth and homeostasis. The gene coding for the protein is expressed in various types of tissues. We previously demonstrated that the transcription regulatory element of the gene (ARE) is located in the position –102 to –61 from the transcription initiation site. To identify the minimal regions that are essential for the constitutive expression, the sequences of the ARE were analyzed in detail by *in vitro* transcription assays using nuclear extracts from rat kidney, brain and liver. The analyses of various mutations in the promoter demonstrated that the proximal region of the ARE is required for the efficient transcription in every nuclear extract. The factors binding to this region in these nuclear extracts exhibited identical mobility in gel retardation assays. The ATF/CRE core motif is indicated to be important for the factor binding and for the promoter function in all nuclear extracts. The common binding factor in the nuclear extracts was revealed to be an ATF-1/CREB heterodimer by gel retardation assays using specific antibodies. We conclude that the ATF-1/CREB heterodimer is involved in the constitutive expression of the Na,K-ATPase α1 subunit gene.

INTRODUCTION

Na,K-ATPase is a membrane enzyme that extrudes intracellular Na^+ and accumulates K^+ from outside at the expense of ATP, thereby maintaining gradients of Na^+ and K^+ across the plasma membrane. The enzyme is involved in various physiological phenomena such as nutrients transport, cell volume regulation, electrical excitability of nerve and muscle and salt and water transport across kidney and intestinal epithelia. The enzyme consists of two non-covalently linked subunits, α and β, in a 1:1 stoichiometry. The larger α subunit is responsible for catalysis and at least three isoforms (α1, α2 and α3) have been identified in human and rodent (reviewed in refs 21 and 31). The expression of each isoform gene is differentially regulated in various tissues and at various developmental stages (7,24).

The α1 subunit is found in all tissues, and is thought to play an essential role in the maintenance of cell homeostasis. The gene coding for the α1 subunit (*Atp1a1*) is expressed in virtually all tissues so far examined. To understand the molecular mechanism how the gene is expressed in a wide variety of tissues, we analyzed the transcription regulatory elements in the *Atp1a1* promoter by transient transfection assays using several cell lines from different tissue origins (30). Analyses of 5′-sequential deletion mutations revealed that the *Atp1a1* Regulatory Element (ARE; –102 to –62 from the transcription initiation site) acts as a positive regulatory element in all types of cells examined. Elimination of the ARE reduces the level of transcription activity to 10–20%, with some variations depending on the types of cells. The factors that bind to the ARE were identified by gel retardation assays using nuclear extracts from various cells and by screening a cDNA expression library with double-stranded DNA probes containing the ARE sequences (13,30,35). To date, we have identified at least seven ARE-binding factors with distinct binding sequences (14). However, it is not clear which factors would be responsible for the constitutive expression of *Atp1a1*. Analyses of the mutations in the different regions of the ARE, which eliminate the binding site of each factor, showed that none of the mutations fully abolished the activating function of the ARE (30).

In this study, we established an *in vitro* transcription system of *Atp1a1* using nuclear extracts from different tissues of rat to identify the ARE-binding factors which are responsible for the constitutive expression. Most cells in solid tissues are in the resting stage and also are highly differentiated. Therefore, we can exclude the effects of the ARE-binding factors which function only in rapid growing cells. The systematic analyses of the 5′-flanking region of the *Atp1a1* promoter demonstrated that the proximal region is enough for activating function of the ARE in all nuclear extracts. The dominant factor binding to this region was shown to be identical among the nuclear extracts. The core motif of activating transcription factor/cAMP response element (ATF/CRE) in the proximal region of the ARE was required for the factor binding and the transcription activity of the *Atp1a1* promoter. The common factor binding to this region in all nuclear extracts was identified as an ATF-1/CRE binding protein (CREB) heterodimer. Activating transcription factor-1 and CREB are the members of the bZIP family of transcription factors that bind to

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DNA as dimers (reviewed in refs 19 and 23). Both factors are widely expressed in various tissues and seem to play an essential role in the expression of some housekeeping genes. Activating transcription factor-1 and CREB have been shown to be dimerized to each other, although the *in vivo* role of the heterodimer is still unknown. Our results suggest that the ATF-1/CREB heterodimer is involved in the constitutive expression of the housekeeping Na,K-ATPase α1 subunit gene.

MATERIALS AND METHODS

Preparation of nuclear extracts

Nuclear extracts from solid tissues of rat were prepared as previously described (33), except proteinase inhibitors (1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml antipain, 20 μg/ml soybean trypsin inhibitor, 1 μg/ml chymostatin and 5 mM benzamidine), phosphatase inhibitors (10 mM NaF and 1 mM $Na_2M_oO_4$) and 20% low fat milk were added in a homogenizing buffer. All proteinase inhibitors were purchased from Sigma. Nuclear extracts from kidney and liver were prepared from 6 week old Sprague–Dawley rats, while those from brain were prepared from 13 day old rats (33).

In vitro transcription assays

In vitro transcription assays were carried out as previously described (29) with slight modifications. In brief, 30 μg of each nuclear extract was incubated with 400 ng of supercoiled DNA templates at 30°C for 45 min in a total of 20 μl reaction mixture [25 mM HEPES-KOH (pH 7.6), 9% glycerol, 49 mM KCl, 6 mM $MgCl_2$, 0.06 mM EDTA, 0.6 mM dithiothreitol, 0.625 mM each of ATP, GTP, CTP and UTP, 1 U/μl RNase inhibitor (Toyobo)]. For transcription competition assays, 20 ng of pAdMLCAT [a kind gift from Dr Robert G. Roeder, renamed from pMLCAT, containing the adenovirus 2 major late (AdML) promoter](2) was added as an internal control. Four microliters of DNase I mixture [60 mM DTT, 30 mM $CaCl_2$, 20 μg of tRNA and 17.5 U RNase-free DNase I (Boehringer Mannheim)] was added, and incubated at 37°C for 15 min to remove the template DNA. The reactions were stopped by an addition of 276 μl of stop solution containing 25 mM EDTA, 150 mM NaCl, 0.5% SDS, 10 mM Tris–HCl (pH 7.5) and 40 μg of proteinase K (Boehringer Mannheim). Transcripts were analyzed by a primer extension procedure using single-stranded DNA probes as described previously (20). Primer DNAs were synthesized to generate 58- and 68-base transcription signals for the *Atp1a1* (5′-TGCCACGTGTCCGCCTC) and the AdML (5′-GCTTCCTAGCTCCTGAAA) promoters respectively. 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).

Plasmid constructions

pA1LS3LF, pA1LS2LF and pA1LS1LF, which contain the *Atp1a1* promoter region from –102 to +261 with 10 base-pair *Bgl*II linker-substitution mutations within the ARE (30), were digested with *Bgl*II and *Nhe*I. After recessed 3′ ends were filled with Klenow fragment (Toyobo), each plasmid was circularized with T4 DNA ligase (Takara) to generate pA1U-88LF, pA1U-77LF and pA1U-63LF respectively. The constructs were verified by DNA sequencing.

DNA probes and competitors

The –77/–62 wt and –77/–62 LS1 DNA fragments were cut out from pA1U-102LF (30) and pA1LS1LF respectively, by digestion with *Msp*I and *Mlu*I. ARE-P (5′ CTAGAACGGTGACGTGCACGCGTCTAG) and ARE-Pmut (5′ CTAGAACGGTGAGCTGCACGCGTCTAG) were prepared by annealing synthetic oligonucleotides. The core motif of ATF/CRE and the corresponding mutated sequence are underlined.

Gel retardation assays

Gel retardation assays were performed as previously described (12). Antibodies and competitors were incubated with 5 μg each of nuclear extract prior to probes. Approximately 5 fmol of ^{32}P-labeled ARE-P was used as a probe. Anti-ATF-1 monoclonal antibody C41–5.1 was purchased from Santa Cruz Biotechnology. Anti-CREB polyclonal antibody Ab244 was a kind gift from Drs Masatoshi Ogiwara and Marc R. Montminy (38).

Methylation interference and DNase I footprinting experiments

Methylation interference and DNase I footprinting experiments were performed as previously described (12). ARE-P was inserted into *Xba*I site of pBluescript KS(+) to make pKSARE-P. pKSARE-P was 3′ labeled with [α-^{32}P]dCTP by Klenow fragment or 5′ labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase at *Hin*dIII site and digested with *Sac*I for preparing the lower or upper strand probes respectively. In gel retardation steps for both experiments, 1 pmol of *Hin*dIII–*Sac*I fragment from pBluescript KS(+) without insert was added in reaction mixtures to eliminate binding complexes against flanking regions derived from vector sequences.

RESULTS

In vitro transcription assays using nuclear extracts from rat solid tissues

We first established the *in vitro* transcription system of *Atp1a1* using nuclear extracts from rat solid tissues. Although *Atp1a1* is ubiquitously expressed in various tissues, the relative abundance of its mRNA varies in the different tissues (reviewed in refs 21 and 31). The level of *Atp1a1* mRNA in kidney and brain is higher than those in any other tissues, such as liver. To analyze the common molecular mechanism of the *Atp1a1* expression in various tissues, we selected kidney, brain and liver as starting materials for preparing nuclear extracts. Figure 1A shows that the accurately initiated transcripts from the *Atp1a1* promoter were observed in these nuclear extracts (lanes 1, 3 and 5). These transcription activities were all RNA polymerase II dependent, since 2 μg/ml of α-amanitin completely inhibited the transcription (lanes 2, 4 and 6). The transcription start point of each transcript was confirmed to be identical to that of the *Atp1a1* mRNA (data not shown) (37). The AdML promoter was also efficiently transcribed in each nuclear extract (Fig. 4B).

To confirm the positive role of the ARE in the *Atp1a1* promoter function, we performed systematic analyses of the 5′-flanking region using deletion mutations of the promoter (30). As shown

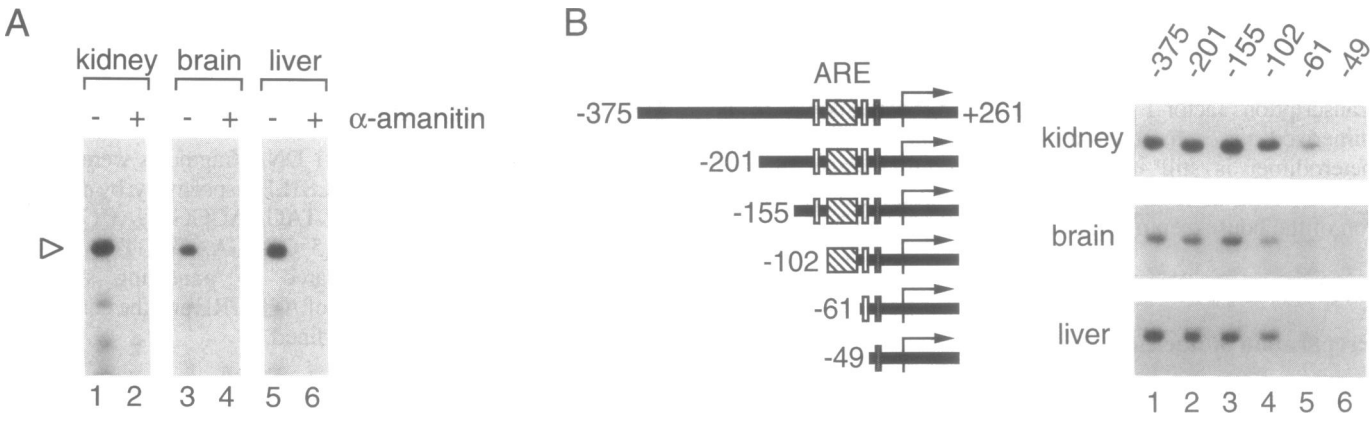

Figure 1. Requirement of the ARE for the *in vitro* transcription from the *Atp1a1* promoter using nuclear extracts from various tissues of rat. (**A**) RNA polymerase II specific transcription from the *Atp1a1* promoter using nuclear extracts from rat tissues. pA1U-155LF was transcribed using rat kidney (lanes 1 and 2), brain (lanes 3 and 4) and liver (lanes 5 and 6) nuclear extracts in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of α-amanitin (2 μg/ml). The position of accurately initiated transcripts is indicated by an arrowhead. (**B**) Effects of 5′-sequential deletion mutations of the *Atp1a1* promoter on transcription activity. Transcripts from pA1U-375LF (lane 1), pA1U-201LF (lane 2), pA1U-155LF (lane 3), pA1U-102LF (lane 4), pA1U-61LF (lane 5) and pA1U-49LF (lane 6) were analyzed. The terminal nucleotide position of each promoter is indicated in left panel. ARE, Sp1 sites and TATA-box like sequence are indicated by striped, open and shadowed boxes respectively. Transcription initiation sites are indicated by arrows.

in Figure 1B, shortening the upstream sequence to –155 did not lead to a significant decrease in transcription activity in any of the nuclear extracts (lanes 1–3). Deletion up to –102 decreased transcription activity to less than half of that up to –155 in these nuclear extracts (lane 4). However, the deletion of ARE (–102 to –62) caused a dramatic loss (80–85%) of transcription efficiency in all nuclear extracts (lane 5). Further deletion up to –49 reduced transcription activity almost to undetectable level (lane 6). These results indicate that the positive regulatory elements responsible for the *Atp1a1* promoter function are confined to the region between –155 and –50 in all nuclear extracts. Furthermore, as was found in transient transfection assays (30), the ARE is the most essential element for the promoter function in all nuclear extracts. Thus, we conclude that a *cis*-acting element for the constitutive expression of *Atp1a1* is located in the ARE.

The proximal region of the ARE is required for the promoter function

To accurately map the minimal regions required for the promoter function in each nuclear extract, we prepared a set of fine deletion mutations in the ARE to test in *in vitro* transcription assays. Figure 2A shows the results of the *in vitro* transcription assays using pA1U-88LF, pA1U-77LF and pA1U-63LF (containing sequences from –88, –77 and –63 to +261 respectively) as templates. The transcription activity of pA1U-88LF was relatively low compared with that of pA1U-102LF (lane 2). The transcription activity of pA1U-77LF showed similar activity to that of pA1U-102LF in all nuclear extracts (lane 3). Weak positive and negative regulatory elements might be located in the distal (between –102 and –89) and middle (between –88 and –78) regions of the ARE respectively. However, further deletion up to –63 dramatically decreased the activity near to the level of that of pA1U-61LF in all nuclear extracts (lanes 4 and 5). A strong positive regulatory element might exist in the proximal region (between –77 and –64) of the ARE.

To confirm the positive role of the ARE-proximal region in the promoter function, we next examined the transcription activities of the promoters which contain linker-substitution mutations in different regions of the ARE (30). Figure 2B shows the results of *in vitro* transcription assays using pA1LS1LF, pA1LS2LF and pA1LS3LF as templates. Only the LS1 but not the LS2 or LS3 mutation decreased transcription activity (lanes 4–6). The magnitude of reduction in transcription activity by the LS1 mutation was similar to that by deletion up to –61 (lanes 2 and 4).

The analyses of the linker-substitution mutations combined with those of the deletion mutations revealed that the activation function of the ARE was primarily attributed to the proximal region in all nuclear extracts. This observation was distinguished with that in our previous study using cultured cells (30). Contribution of the proximal region of the ARE in the promoter function is relatively small in cultured cells (30). The reason for this discrepancy is probably that the factors binding to the other regions of the ARE also act as positive regulatory factors for the expression of *Atp1a1* in certain cultured cells. Consequently, the results suggest that the binding factors to the proximal region of the ARE are responsible for the constitutive expression of *Atp1a1*.

Characterization of the factors binding to the ARE-proximal region

To identify the DNA-binding factors to the proximal region of ARE, a double-stranded oligonucleotide DNA, ARE-P, corresponding to the ARE-proximal region (–75 to –57) was synthesized and used as a probe in gel retardation assays. Figure 3A shows the retarded complexes of ARE-P in the nuclear extracts. A single gel retardation complex was observed in the kidney nuclear extract, while several additional complexes were observed in others (lanes 1, 4 and 7). The complex of identical mobility with the single complex in the kidney nuclear extract was dominant in both brain and liver nuclear extracts. The formation of the common migration complex and the two

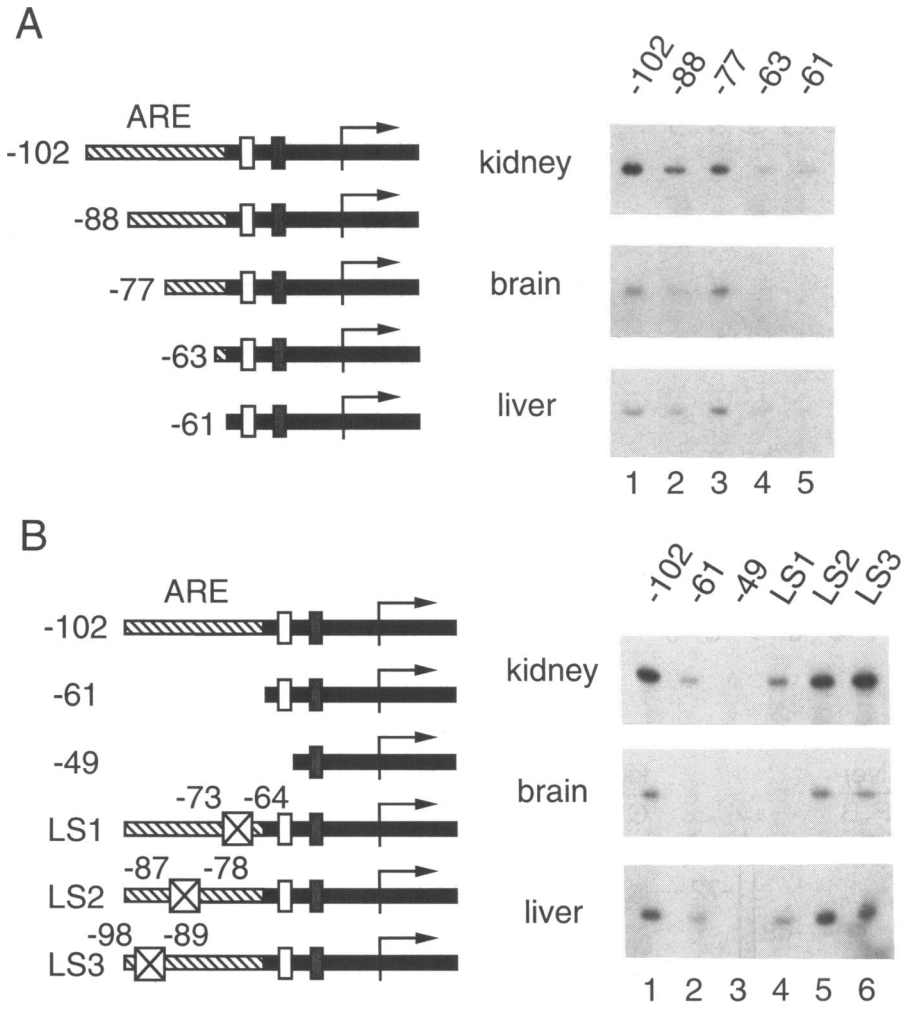

Figure 2. The proximal region of the ARE is required for the transcription in the nuclear extracts. (**A**) Transcription from the promoters which have 5′ deletion mutations in the ARE. Transcripts from pA1U-102LF (lane 1), pA1U-88LF (lane 2), pA1U-77LF (lane 3), pA1U-63LF (lane 4) and pA1U-61LF (lane 5) were analyzed. (**B**) Transcription from the promoters which have linker-substitution mutations in the ARE. Transcripts from pA1U-102LF (lane 1), pA1U-61LF (lane 2), pA1U-49LF (lane 3), pA1LS1LF (lane 4), pA1LS2LF (lane 5) and pA1LS3LF (lane 6) were analyzed. Crossed boxes in left panel indicate the positions of linker-substitutions.

additional complexes in the brain and liver nuclear extracts were all competed by a DNA fragment covering –77 to –62 (–77/–62 wt) but not by its LS1 mutation (–77/–62 LS1)(lanes 2–3, 5–6 and 8–9). This indicates that all these complexes were specific for the ARE-proximal region. We focused our studies on the common migration complex in these nuclear extracts in the following experiments.

A methylation interference experiment was performed to identify the G residues that come into direct contact with the proteins forming the common migration complex. ARE-P was cloned into pBluescript KS(+) for preparation of the lower and upper strand probes. Gel retardation assays against these probes showed virtually identical patterns to those against ARE-P (data not shown). Figure 3B shows the results of methylation interference experiments of the complexes in the nuclear extracts. Methylation of –67 G strongly interfered with complex formation with the lower strand probe in all nuclear extracts (lanes 2, 4 and 6). On the other hand, methylation of three G residues at positions –71, –69 and –66 interfered with complex formation with the upper strand probe (lanes 8, 10 and 12). The profiles of G residue contacts in the common migration complex were virtually identical among all nuclear extracts, except that a weak inhibition of the complex formation was found in the brain nuclear extract by methylation of –72 G or –64 G (lane 10) and in the liver nuclear extract by methylation of –63 G, –61 G, –72 G or –64 G (lanes 6 and 12).

We next performed DNase I footprinting experiments to identify the binding region of the common migration complex in each nuclear extract (Fig. 3C). For the lower strand probe, the region corresponding to the sequence from –69 to –61 were protected from DNase I digestion in all nuclear extracts (lanes 3, 5 and 7). For the upper strand probe, the region corresponding to the sequence from –72 to –62 was weakly protected in all nuclear extracts (lanes 10, 12 and 14).

The results of the methylation interference and DNase I footprinting experiments are summarized in Figure 3D. Three of four G residues which are involved in the complex formation reside in the ATF/CRE core motif (TGACG)(19) between –70 and –66 of the *Atp1a1* promoter. The ATF/CRE motif exists in the

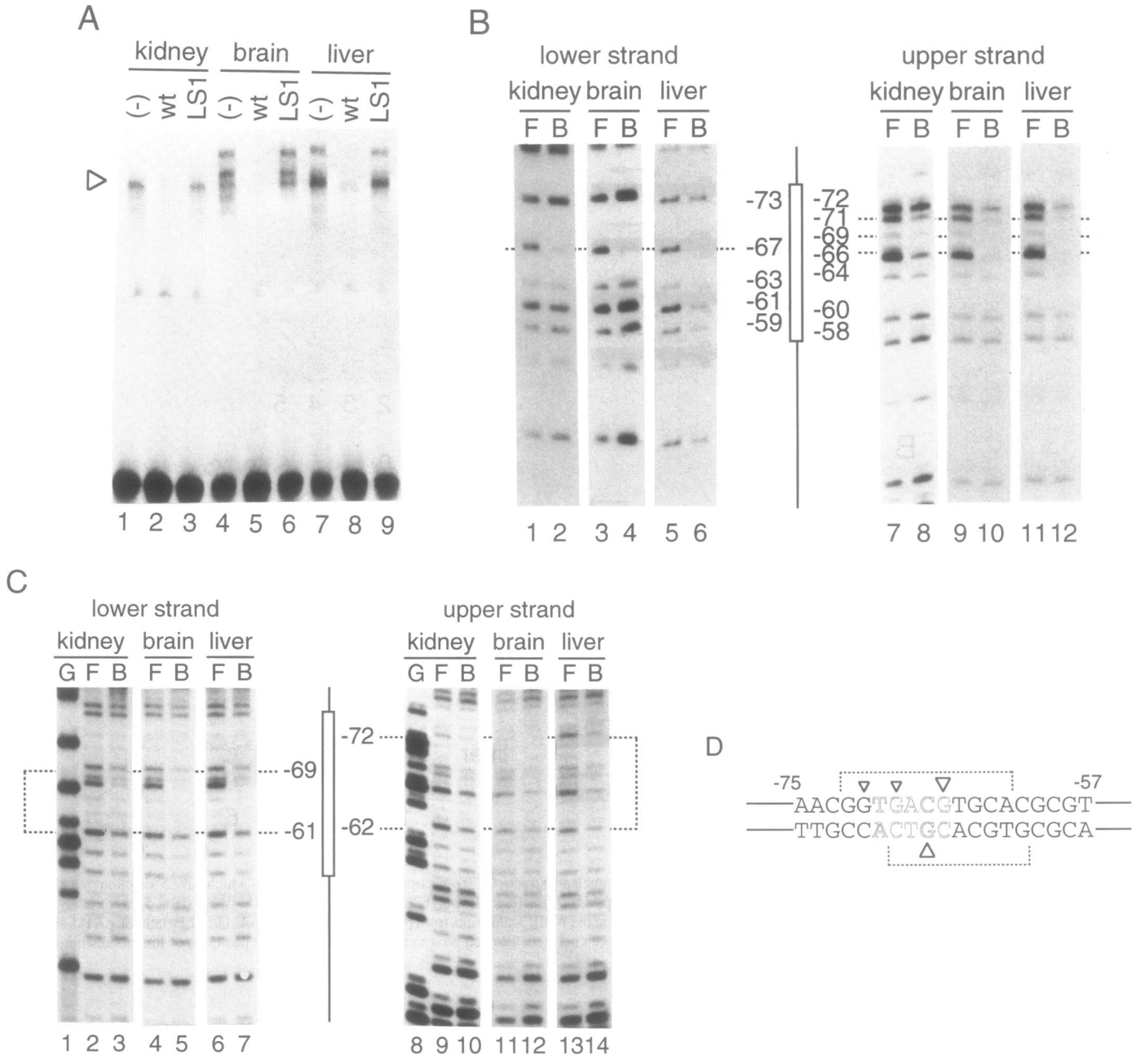

Figure 3. Characterization of DNA-binding factors to the proximal region of the ARE. (**A**) Gel retardation assays. Probes were incubated with the nuclear extracts in the absence of competitor (lanes 1, 4 and 7) or in the presence of 1 pmol of –77/–62 wt (lanes 2, 5 and 8) or –77/–62 LS1 (lanes 3, 6 and 9). An arrowhead indicates the position of the common migration complex in the nuclear extracts. (**B**) Methylation interference experiments. The lower strand (lanes 1–6) and upper strand (lanes 7–12) probes were methylated and used for gel retardation assays. Free probes (F) and the probes in the common migration complex (B) were isolated from the gel, cleaved by piperidine, and further analyzed by gel electrophoresis. The positions of guanine residues whose methylation interfered with complex formation are indicated by broken lines. (**C**) DNase I footprinting experiments. The lower strand (lanes 2–7) and upper strand (lanes 9–14) probes were incubated with each nuclear extract followed by DNase I treatment. Free probes (F) and the probes in the common migration complex (B) were isolated from the gel and further analyzed by gel electrophoresis. The regions protected from DNase I digestion are indicated by broken line brackets. G is a guanine ladder marker (lanes 1 and 8). (**D**) Summary of methylation interference and DNase I footprinting experiments. The G residues whose methylation interfered with complex formation are marked by arrowheads. The degree of interference is indicated by the size of the arrowhead. The regions protected from DNase I digestion are shown by broken line brackets. The nucleotides in the ATF/CRE core motif are depicted by the outlined characters.

region protected from DNase I digestion by complex formation. These results suggest that a member of the ATF/CREB family of transcription factors present in these nuclear extracts should bind to this region of the ARE and play a role in the constitutive expression of *Atp1a1*.

Role of the ATF/CRE motif in the *Atp1a1* transcription

To confirm that the factor we identified above is a member of the ATF/CREB family, we next analyzed the effects of mutation in the ATF/CRE motif on complex formation in the nuclear extracts

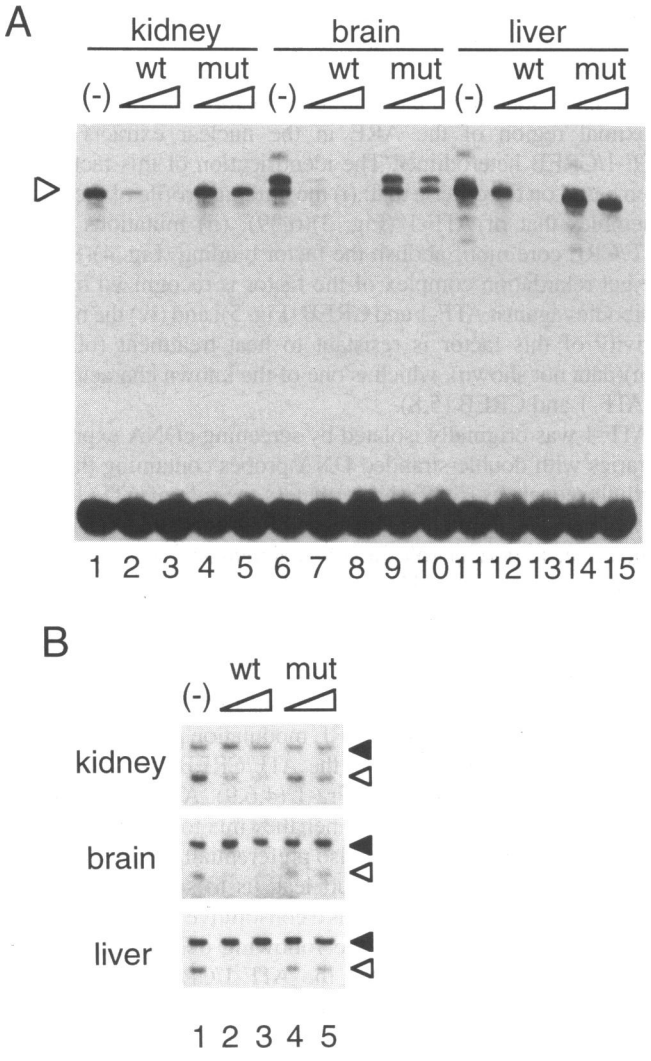

Figure 4. Role of the ATF/CRE motif in the promoter function. (**A**) Effect of mutations in the ATF/CRE core motif on DNA-binding activity. Each nuclear extract was incubated with probes in the absence of competitor (lanes 1, 6 and 11), or in the presence of ARE-P (lanes 2–3, 7–8 and 12–13) or ARE-Pmut (lanes 4–5, 9–10 and 14–15). Competitors were added at a 40-fold (lanes 2, 4, 7, 9, 12 and 14) or 200-fold (lanes 3, 5, 8, 10, 13 and 15) molar excess to the probe. An arrowhead indicates the position of the common migration complex in the nuclear extracts. (**B**) Transcription competition assay using the oligonucleotide DNA containing the sequences corresponding to the ARE-proximal region. pA1U-102LF and pAdMLCAT were transcribed in the absence (lane 1) or in the presence of ARE-P (lanes 2 and 3) or ARE-Pmut (lanes 4 and 5). Competitors were added at a 20-fold (lanes 2 and 4) or 60-fold (lanes 3 and 5) molar excess to pA1U-102LF. The positions of specific transcripts from pA1U-102LF and pAdMLCAT are shown by open and closed arrowheads respectively.

using gel retardation competition assays. A synthetic oligonucleotide DNA, ARE-Pmut, was prepared to use as a competitor. ARE-Pmut has the same sequences as ARE-P except for a substitution of TGACG to TGAGC in the ATF/CRE core motif. The mutation introduced in these residues is known to abolish the binding of ATF-1 and CREB (8). Figure 4A shows the results of gel retardation competition assays using ARE-P and ARE-Pmut in the nuclear extracts. When we added ARE-P as a competitor, formation of the common migration complex was almost completely inhibited in all nuclear extracts (lanes 2–3, 7–8 and 12–13). Formation of the additional complexes in the brain and liver nuclear extracts was also inhibited by the ARE-P addition. On the other hand, the addition of ARE-Pmut had little influence on the formation of either the common or the additional complexes (lanes 4–5, 9–10 and 14–15). These results suggest that the intact ATF/CRE core motif is required for the formation of the common migration complex and they further support the idea that the factor in the complex is a member of the ATF/CREB family.

To reveal the contribution of the ATF/CRE binding factors to transcriptional activity, we performed transcription competition assays using ARE-P and ARE-Pmut (Fig. 4B). The addition of ARE-P resulted in a dramatic reduction of the *Atp1a1* specific transcription in all nuclear extracts (lanes 2 and 3). While the addition of ARE-Pmut had little effect on the transcription activity (lanes 4 and 5). In each case, transcription from the AdML promoter was not competed by the oligonucleotide DNA. Normalizing the transcription activity with that of the AdML promoter in each reaction, the activity of the *Atp1a1* promoter was reduced to 20% by the addition of 60-fold molar excess ARE-P and to 60–75% by that of ARE-Pmut. These results show that elimination of the factors binding to the ATF/CRE motif reduces *Atp1a1* transcription specifically in all nuclear extracts.

Identification of the factors binding to the ATF/CRE motif

Among various members of the ATF/CREB family so far identified, two well-characterized transcription factors, ATF-1 and CREB, are ubiquitously expressed in all types of cells. To prove the involvement of ATF-1 and/or CREB in the *Atp1a1* transcription, we used specific antibodies against ATF-1 and CREB for gel retardation assays (Fig. 5). An antibody against ATF-1 supershifted the common migration complex (indicated by open arrowheads in lanes 1, 4 and 7) in all nuclear extracts (lanes 2, 5 and 8). The common complex was also supershifted by an antibody against CREB (lanes 3, 6 and 9). These results indicate that the common complex consists of both ATF-1 and CREB and suggest that it is formed by an ATF-1/CREB heterodimer. A second major gel retardation complex formed only in the brain nuclear extract (indicated by a closed arrowhead in lane 4) was supershifted by the antibody against CREB but not by the antibody against ATF-1 (lanes 5 and 6). The most probable candidate for this complex is a CREB homodimer. The minor slow migrating complex formed in the brain and liver nuclear extracts was supershifted with neither the anti-ATF-1 nor the anti-CREB antibody. Since formation of this complex was abolished by the mutation in the ATF/CRE core motif (Fig. 4A), it might be formed by another member(s) of the ATF/CREB family.

DISCUSSION

Transient transfection assays using a reporter gene are an elegant method for the identification of a *cis*-acting regulatory element in the promoter of a selected gene. These assays require the availability of representative cultured cells for the specific purpose of each study. *In vitro* transcription assays using nuclear extracts from solid tissues are also a powerful method for studying gene regulatory mechanism. Tissue-specific regulatory

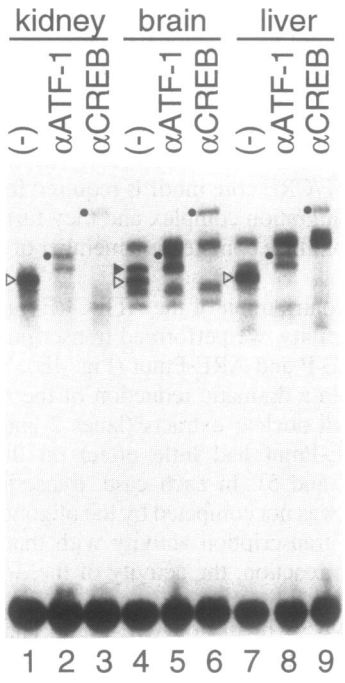

Figure 5. Gel retardation assays using antibodies against ATF-1 and CREB. Each nuclear extract was incubated without antibody (lanes 1, 4 and 7) or with antibodies against ATF-1 (lanes 2, 5 and 8) or CREB (lanes 3, 6 and 9) prior to the addition of probes. Open arrowheads indicate the position of the common migration complex in the nuclear extracts. A closed arrowhead indicates the position of second major complex observed only in the brain nuclear extract. Dots indicate the positions of supershifted complexes.

elements in certain gene promoters have been identified by this method (3,32). Since the cells in solid tissues are mostly in the resting stage, we can exclude the influence of the factors only function in rapid growing cells. In this study, we applied *in vitro* transcription assays using nuclear extracts from solid tissues to reveal the common regulatory mechanism of the housekeeping gene *Atp1a1* in various tissues.

We report here that the proximal region of the ARE is required for efficient transcription from the *Atp1a1* promoter in the nuclear extracts from various tissues. The proximal region of the ARE contains the ATF/CRE core motif, which is conserved within the rat, horse and human genes encoding the Na,K-ATPase α1 subunit (11,28,37). The binding factors which recognize the ATF/CRE motif are required for the efficient transcription in all nuclear extracts (Fig. 4B). The ATF/CRE motif is present in many viral and cellular promoters (reviewed in ref. 19), and has both inducible and constitutive enhancer activities that depend on flanking sequences and types of cells (10). In some housekeeping genes, such as the DNA polymerase β and the retinoblastoma genes, the ATF/CRE motif was also demonstrated to be required for the gene expression (27,36). Moreover, the ATF/CRE motif is known to be essential for the basal transcriptional activity of many promoters (1,15,18,25). There are many different proteins which can bind to the ATF/CRE motif. The protein responsible for the basal activity is still ambiguous, although more than a dozen members of the ATF/CREB family have been cloned so far (reviewed in ref. 23). The ATF/CREB family belongs to a large super-family of DNA-binding proteins known as the bZIP proteins, which bind as dimers to their target DNA sequences (reviewed in ref. 17).

We have identified the common factor that binds to the proximal region of the ARE in the nuclear extracts as an ATF-1/CREB heterodimer. The identification of this factor has been based on the criteria that: (i) the binding profile of the factor resembles that of ATF-1 (Fig. 3)(6,39); (ii) mutations in the ATF/CRE core motif abolish the factor binding (Fig. 4)(8); (iii) the gel retardation complex of the factor is recognized by both antibodies against ATF-1 and CREB (Fig. 5); and (iv) the binding activity of this factor is resistant to heat treatment (60°C, 5 min)(data not shown), which is one of the known characteristics of ATF-1 and CREB (5,8).

ATF-1 was originally isolated by screening cDNA expression libraries with double-stranded DNA probes containing the ATF binding sequences (6,39). Nevertheless the role of ATF-1 is not known yet. The failure to identify cell lines that lack ATF-1 may indicate an important role in cell maintenance. The functional activity of purified ATF-1 was demonstrated by *in vitro* transcription assays using the adenovirus E4 promoter (5,34). In JEG3 cells, an ATF-1/GAL4-fusion protein can mediate cAMP-dependent transcription, however this is likely to reflect the effect of a heterodimerization with endogenous CREB (9). ATF-1 can heterodimerize with CREB or CRE modulation protein (CREM), but not with other members of the ATF/CREB family, such as ATF-2, ATF-3, c-*jun*, c-*fos* or Fra-1 (4,6,9). ATF-1 and CREB selectively form a heterodimer when they mix together (34). This implies that the heterodimer is also preferentially formed *in vivo*.

The observations in this report lead us to speculate that the ATF-1/CREB heterodimer acts as a constitutive activator *in vivo*. This hypothesis arises from the following observations. First, Wada *et al.* (34) reconstituted the ATF-1/CREB heterodimer (E4TF3) *in vitro* and showed that the activity of ATF-1/CREB heterodimer was higher than those of the ATF-1 or CREB homodimer in basal transcription from the adenovirus E4 promoter. Second, monomeric CREB, prepared as a fusion protein with the DNA-binding region of the B-cell specific activator protein, was demonstrated to be a highly efficient constitutive activator (16). In light of the inefficient DNA-binding activity of CREB in a monomeric form, it is suggested that the property of this artificial monomeric CREB might reflect that of a heterodimeric complex containing CREB.

CREB is known to be activated by distinct signaling molecules, such as cAMP, Ca^{2+} and transforming growth factor β, and also to function as a constitutive activator (reviewed in ref. 19). But how this regulatory diversity occurs is unknown. Formation of an ATF-1/CREB heterodimer provides one mechanism for generating diversity. The function of the heterodimer may differ qualitatively or quantitatively from either of the two homodimers. Rehfuss *et al.* (26) reported that CREB but not ATF-1 activates basal transcription of the rat somatostatin gene through an ATF/CRE motif in F9 cell. This is consistent with our hypothesis because endogenous ATF-1 in F9 cells is highly abundant compared with CREB (22) and the expression of exogenous CREB but not ATF-1 should increase the amount of heterodimers. Thus, it is apparent that the relative proportion of ATF-1 and CREB may be important in the regulations of promoter activity. An *in vitro* transcription analysis using the homo- and hetero-dimers of ATF-1 and CREB reconstituted from recombi-

nant proteins will provide significant information regarding the functional differences among the dimerization forms.

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