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Housekeeping Na,K-ATPase α1 Subunit Gene Promoter Is Composed of Multiple *cis* Elements to Which Common and Cell Type-Specific Factors Bind

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Na,K-ATPase α1 subunit gene (ATP1A1) is one of the housekeeping genes involved in homeostasis of Na^+ and K^+ in all animal cells. We identified and characterized the *cis*-acting elements that regulate the expression of ATP1A1. The region between −155 and −49 was determined as a positive regulatory region in five cultured cell lines of different tissue origins (MDCK, B103, L6, 3Y1, and HepG2). The region was divided into three subregions: from −120 to −106 (including the Sp1 binding site), from −102 to −61, and from −58 to −49 (including an Sp1 consensus sequence). Cell type-specific factors binding to the middle subregion (from −102 to −61) were detected by gel retardation analysis, using nuclear extracts prepared from MDCK and B103 cells. Two gel retardation complexes were formed in the B103 nuclear extract, and three were formed in the MDCK nuclear extract. DNA binding regions of these factors were located at −88 to −69 and differed from each other in DNase I footprinting experiments. These factors also showed different binding characteristics in gel retardation competition and methylation interference experiments. The identified *cis* element was named the ATP1A1 regulatory element. The core sequence of this element is found in several other genes involved in cellular energy metabolism, suggesting that the sequence is a common regulatory element responsive to the state of energy metabolism.

Na,K-ATPase is an integral membrane protein which is responsible for forming the electrochemical gradients of Na^+ and K^+ across the plasma membrane of animal cells. This enzyme is one of the major ATP-consuming enzymes. In tissue slices of brain, liver, and kidney, ouabain (a specific inhibitor of Na,K-ATPase)-sensitive respiration amounts to 30 to 45% of total oxygen consumption (38). The ATPase is composed of two nonidentical subunits, α and β, in a 1:1 stoichiometry (3), and three isoforms have been identified for both α and β subunits (9, 14, 23, 33). The expression of each subunit gene is differentially regulated in various tissues and at various developmental stages (28). The α2 and the α3 subunit genes and the β1 and β2 subunit genes are expressed only in restricted tissues (12, 28, 31). The α1 subunit is essential for cell viability, and the gene coding for this subunit is expressed ubiquitously in almost all tissues, although the expression level varies among different tissues (the level of α1 mRNA in kidney is higher than those in any other tissues) (12, 28, 31). Therefore, the α1 subunit gene is a member of the housekeeping genes.

To reveal the molecular mechanisms of the tissue-specific, developmental, and hormonal regulation of Na,K-ATPase gene expression and of the coordinated regulation between the α- and β-subunit genes, we have performed a systematic analysis of the genes encoding α- and β-subunit isoforms, including their 5′-flanking regions where important regulatory functions reside (15, 17, 18, 43). We cloned from the rat chromosome a 13.3-kb DNA fragment that included the Na,K-ATPase α1 subunit gene (ATP1A1) and 5′ flanking region (EMBL/GenBank data library accession number X51461), and identified the transcription initiation site at 262 bp upstream from the translation initiation codon (43).

In this study, we analyzed the 5′-flanking region of ATP1A1 by transient transfection assays in five cultured cell lines and identified the *cis*-acting elements that are involved in both the constitutive expression of the α1 subunit gene and its differential expression in different cell types. In addition, we analyzed factors that bind to the regulatory region of ATP1A1 by gel retardation assays, DNase I footprinting analysis, and methylation interference experiments. An element of ATP1A1 that binds multiple factors was localized to positions −94 to −69.

MATERIALS AND METHODS

Cell culture. Five cultured cell lines, MDCK (canine kidney; CCL34), B103 (rat neuroblastoma), L6 (rat skeletal muscle myoblast), 3Y1 (rat embryo fibroblast, obtained from the Japanese Cancer Research Resources Bank; 0734), and HepG2 (human hepatocellular carcinoma, obtained from the RIKEN Cell Bank; RCB459), were maintained in Dulbecco's modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% (for MDCK, L6, 3Y1, and HepG2 cells) or 15% (for B103 cells) fetal calf serum (GIBCO or Cell Culture Laboratories, Cleveland, Ohio) in a CO_2 incubator (5% CO_2) at 37°C.

Plasmid construction. The *SalI-NcoI* fragment spanning from −1537 of the 5′-flanking region (numbering with reference to the major transcription initiation site, designated +1) to +261, just before the translation initiation site in the first exon, was excised from ATP1A1 (43). The cohesive end of the *NcoI* site was deleted with mung bean nuclease to prevent reconstruction of the translation initiation codon (ATG at +263), and the *SalI* site was filled in with Klenow fragment. The blunt-ended DNA fragment was coupled with a *Hin*dIII linker (dCAAGCTTG) and ligated to *Hin*dIII-digested pSVOA/LΔ5′ (6a), a vector containing a luciferase gene. The resulting plasmid, which had the ATP1A1 sequence from −1537 through +261, just upstream of the

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*Hin*dIII site at the 5′ end of the luciferase-coding sequence of pSVOA/LΔ5′, was called pA1LF-1.

For 5′-promoter deletion analysis, a nested set of ATP1A1 promoter fragments was isolated from pA1LF-1, blunt ended with Klenow fragment, coupled with a *Hin*dIII linker, and cloned into the *Hin*dIII site of pSVOA/LΔ5′. The following restriction sites were used for construction of the 5′ sequential deletion mutations of ATP1A1: *Bgl*II (−1100) site for pA1U-1100LF, *Xho*I (−375) site for pA1U-375LF, *Rsa*I (−201) site for pA1U-201LF, *Nhe*I (−155) site for pA1U-155LF, *Pvu*II (−102) site for pA1U-102LF, *Mlu*I (−61) site for pA1U-61LF, *Bsp*MI (−49) site for pA1U-49LF, and *Pma*CI (+53) site for pA1U+53LF.

Internal deletion mutations of ATP1A1 were constructed as follows. pA1LF-1 was cut with *Hin*dIII at the 5′ end (−1537) of the ATP1A1 sequence, blunt ended with Klenow fragment, and self-ligated. An *Nhe*I site was generated at the 5′ end (−1537) of the ATP1A1 sequence in the resulting plasmid, pA1LF-1(O/H). pA1LF-1(O/H) was digested with BAL31 nuclease from the *Xho*I (−375) site of the ATP1A1 sequence. The digested ends were filled in with Klenow fragment and coupled with a *Hin*dIII linker. The *Hin*dIII fragments were excised from the digests (spanning from the BAL31-digested end to +261 of ATP1A1). pA1LF-1(O/H) was digested with BAL31 nuclease from the *Mlu*I (−61) site of the ATP1A1 sequence, filled in with Klenow fragment, and coupled with *Hin*dIII linker. The *Hin*dIII fragments (spanning from the BAL31-digested end to +261 of ATP1A1) and that of pA1LF-1(O/H) described above were ligated. Finally, the constructed plasmids were cut with *Nhe*I and self-ligated. After sequencing of these plasmids, suitable deletion mutations of ATP1A1 (pA1ID2LF, pA1ID3LF, and pA1ID4LF) were selected. For pA1ID1LF, the *Hin*dIII fragment (from −49 to +261) of ATP1A1 excised from pA1U-49LF was ligated into pA1LF-1(O/H) cut with *Mlu*I, filled in with Klenow fragment, and coupled with a *Hin*dIII linker. The constructed plasmid was cut with *Nhe*I and self-ligated.

Linker substitution mutations were introduced into ATP1A1 by oligonucleotide-directed mutagenesis (42). pA1LF-1(O/H) was cut with *Nhe*I, filled in with Klenow fragment, and cut with *Mlu*I (−61). Six oligonucleotides spanning from *Pvu*II (−102) to *Mlu*I (−61), including a 10-bp mutation with a *Bgl*II linker (underlined), were synthesized. Plasmids pA1LS1LF (5′-CTGGAGCCGGTGTCAGGTTG CTCCGGTAATCAGATCTGACA and 5′-CGCGTGTCAG ATCTGATTACCGGAGCAACCTGACACCGGCTCCAG), pA1LS2LF (5′-CTGGAGCCGGTGTCATCAGATCTGAG TAACGGTGACGTGCA and 5′-CGCGTGCACGTCACC GTTACTCAGATCTGATGACACCGGCTCCAG), and for pA1LS3LF (5′-CTGGTCAGATCTGAAGGTTGCTCCGGT AACGGTGACGTGCA and 5′-CGCGTGCACGTCACCG TTACCGGAGCAACCTTCAGATCTGACCAG) were annealed and ligated into pA1LF-1(O/H) described above.

β-Galactosidase (β-Gal) expression vectors pSV2βGAL and pEF-BOS/βGAL were constructed as follows. For pSV2βGAL, CDM8-βGAL (a *Hin*dIII-*Pst*I fragment that contains the β-Gal coding region from pCH110 ligated into CDM8) was cut with *Bam*HI and filled in with Klenow fragment and then cut with *Hin*dIII, and the resulting β-Gal fragment was excised and ligated into the *Hin*dIII and *Sma*I sites of pSV2/LΔ5′ (6a). For pEF-BOS/βGAL, the *Hin*dIII-*Bam*HI fragment of CDM8-βGAL was filled in with Klenow fragment, coupled with an *Xba*I linker, and ligated into the *Xba*I site of PEF-BOSCAT (24). These plasmids were used as internal controls for luciferase gene expression.

For the probe used in gel retardation assays, DNase I footprinting analysis, and methylation interference experiments, pUC119/ATP1A1 was constructed as follows. The *Pvu*II-*Mlu*I fragment (from −102 to −58) of ATP1A1 was filled in with Klenow fragment and ligated into the *Hin*cII site of pUC119. All plasmids were purified by modified lysozyme-Triton extraction followed by CsCl gradient centrifugation (4).

DNA transfection and luciferase chloramphenicol acetyltransferase (CAT), and β-Gal enzyme assays. Each 16 or 15 μg of plasmid was cotransfected with 4 μg of pSV2CAT (20), 5 μg of pSV2/βGAL, or pEF-BOS/βGAL into 2×10^5 to 3×10^5 cells in 9-cm-diameter dishes by the calcium phosphate coprecipitation method (4). At 48 h after transfection, the cells were harvested with 1 ml of 100 mM potassium phosphate (pH 7.8)–1 mM dithiothreitol. Cell extracts were prepared by three cycles of freeze-thawing in 50 to 60 μl of 100 mM potassium phosphate (pH 7.8)–1 mM dithiothreitol.

Luciferase activity was measured as described previously (6a, 18), using a luminometer (model 1251; LKB Instruments). CAT activity was assayed as described previously (10, 17). The acetylated chloramphenicol was separated by thin-layer chromatography and analyzed by a radioanalytic imaging system (AMBIS System, Inc., San Diego, Calif.). β-Gal activity was assayed as described previously (11). Most constructs were tested in four to six independent transfection experiments.

Gel retardation assay. Nuclear extracts were prepared from MDCK and B103 cells as described by Dignam et al. (7). The gel retardation assay was performed as described by Kawakami et al. (16). The *Pvu*II-*Mlu*I fragment (from −102 to −58) from ATP1A1 or the *Eco*RI-*Hin*dIII fragment (95 bp) from pUC119/ATP1A1 was 3′ labeled with [^{32}P]dCTP with Klenow fragment and used for the probe. Annealed oligonucleotides described above (LS1, LS2, and LS3) were used as competitors. Other competitor fragments were as follows: a *Hin*dIII (−155)-*Mlu*I (−61) fragment of pA1U-155LF for wild-type ATP1A1 and a *Hin*dIII (−49)-*Pma*CI (+53) fragment of pA1-49LF for the ATP1A1 TATA-like box; a *Hae*II (−359 and −280) fragment of pmMDHneo-6.5 for mouse mitochondrial malate dehydrogenase (32); a *Sac*II (−102)-*Ban*II (−13) fragment of pUC/NFIL6PRO for human NF-IL6 (1); a *Hae*III (−1447)-*Hin*fI (−1323) fragment of pUC19-serine dehydratase for rat serine dehydratase (26); a *Alu*I (−356)-*Hin*cII (−244) fragment of pHRR68 for human thymidylate synthase (41); and a *Bgl*I (−59)-*Sma*I (−11) fragment of pKSEo for human ATP synthase β subunit (27).

DNase I footprinting and methylation interference analyses. DNase I footprinting and methylation interference experiments were performed as described by Kawakami et al. (16).

(i) **DNase I footprinting experiments.** pUC119/ATP1A1 was digested with *Hin*dIII, 5′ labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase, and cut with *Eco*RI for use as the upper-strand probe. The *Eco*RI-*Hin*dIII fragment from pUC119/ATP1A1 was 3′ labeled with [α-^{32}P]dCTP with Klenow fragment for use as the lower-strand probe. The binding reaction mixture (150 μl) contained 140 fmol (for the upper strand) or 190 fmol (for the lower strand) of probe and 18 μl (90 μg of protein) or 9 μl (45 μg of protein) of the MDCK or B103 nuclear extract, respectively, and 20 μg of poly[d(I-C) · d(I-C)]. After incubation for 30 min at 30°C, 30 μl of DNase I (4.5 μg/ml) in 18 mM $MgCl_2$–1 mM $CaCl_2$ was added, and the mixture was incubated for 40 s at 25°C. Reactions were terminated with 2 μl of 0.5 M EDTA, and the samples were analyzed on nondenaturing gels. Bands corresponding to the unretarded probe and to the C1, C2, and C3

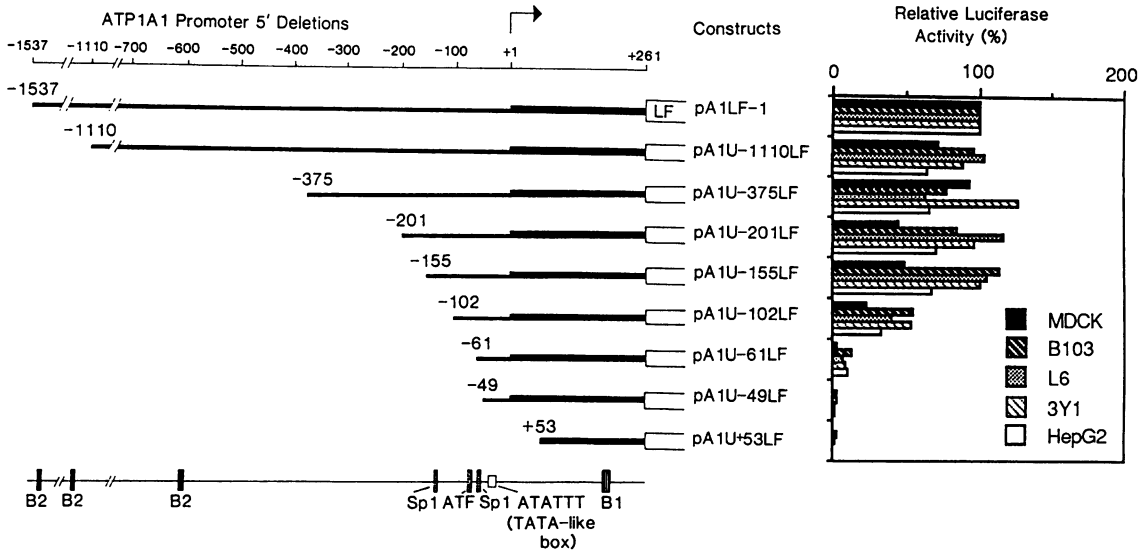

FIG. 1. Reporter gene assays of 5' sequential deletion mutations of the ATP1A1 promoter, constructed as described in Materials and Methods. The terminal nucleotide of the deletions is indicated. Each of the plasmids (16 μg) was cotransfected with 4 μg of pSV2CAT into MDCK, B103, L6, 3Y1, and HepG2 cells. Luciferase activity was normalized with respect to the CAT activity in the same cell lysates. The lower line shows the location of a sequence element common to ATP1A1 and ATP1B1 or ATP1B2 gene (B1 and B2) and the consensus sequences of Sp1, ATF/CRE, and a TATA-like box. The luciferase activity of pA1LF-1 was taken as 100; the standard deviation did not exceed 25%.

complexes were excised, and DNAs were isolated and analyzed on a 9 or 12% (lower strand of MDCK) polyacrylamide–8 M urea sequencing gel.

(ii) Methylation interference experiments. pUC119/ATP1A1 was digested with *Eco*RI or *Hin*dIII, 5' labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase, and cut with *Eco*RI for use as the upper-strand probe and with *Hin*dIII for use as the lower-strand probe. Partially methylated probes were prepared and used for gel retardation experiments (90 μl of the binding reaction mixture). Bands corresponding to the unretarded probe and to C1, C2, and C3 were excised from the gel after autoradiography. DNAs recovered from the gel slices were cleaved at modified guanine residues with piperidine at 90°C for 30 min and resolved on a 9% polyacrylamide–8 M urea gel.

RESULTS

Identification of regulatory elements in the 5'-flanking region of ATP1A1. To examine the molecular mechanism of the expression of ATP1A1 in various tissues, we analyzed the *cis* elements responsible for the ATP1A1 promoter activity in five cell lines from different tissues (MDCK, B103, L6, 3Y1, and HepG2), in which endogenous ATP1A1 expression was confirmed by Northern (RNA) analysis (data not shown). A series of 5' sequential deletion mutants of an ATP1A1-luciferase chimeric construct was prepared and tested for promoter activity by the transient transfection assay. The luciferase activity of the longest construct (wild type, pA1LF-1) was 9 to 30% of that of pSV2A/LΔ5' in these cells. As shown in Fig. 1, shortening the upstream sequence to −375 (pA1U-1110LF and pA1U-375LF) did not lead to a significant decrease in luciferase activity in any of the cell lines. In the next two shorter constructs, pA1U-201LF and pA1U-155LF, the activity decreased to half of that in MDCK cells but not in the other cells, suggesting that a positive regulatory region may exist between −375 and −201 only for MDCK cells. Deletion to position −102 (pA1U-102LF) decreased luciferase activity to less than half of that of pA1U-155LF in all cell lines. With deletion up to position −61 (pA1U-61LF), luciferase activity decreased to a very low level (three- to eightfold less than that in pA1U-102LF). Further deletion to position −49 (pA1U-49LF) reduced luciferase activity to the background level (as that of pSVOA/LΔ5') in all cell lines. These results indicate that major positive elements responsible for the promoter activity are confined to the region from −155 to −49 in all cell lines tested.

To locate the positive regulatory element(s) between −155 and −49, we constructed three internal deletion mutants (Fig. 2). Deletion from −58 to −49 (pA1ID1LF) led to a 10 to 25% decrease in luciferase activity compared with that of pA1U-155LF. An Sp1 binding core sequence exists in the deleted region (−56 to −51), indicating that Sp1 acts as a positive regulator of ATP1A1 transcription in all cell lines. The luciferase activity levels of pA1ID2LF and pA1ID3LF were higher than that of pA1U-102LF, while deletion to position −61 (pA1U-61LF) reduced luciferase activity to three- to eightfold less than that of pA1U-102LF in all cells (Fig. 1). These results suggested the existence of multiple positive and/or negative regulatory elements in the region from −155 to −61.

To precisely locate the regulatory elements, we made three linker substitution mutations, pA1LS1LF, pA1LS2LF, and pA1LS3LF, having a *Bgl*II linker substitution at positions −73 to −64, −87 to −78, and −98 to −89, respectively, (Fig. 3), and tested their promoter activities. Two results were obtained from these experiments. First, luciferase activity decreased in some of these mutations, although the decrease was not as conspicuous and consistent as for pA1U-61LF. This finding again indicated the existence of multiple regulatory elements. Second, the decreasing pattern of luciferase activity was different among the cell lines tested. Roughly the original level was maintained in MDCK

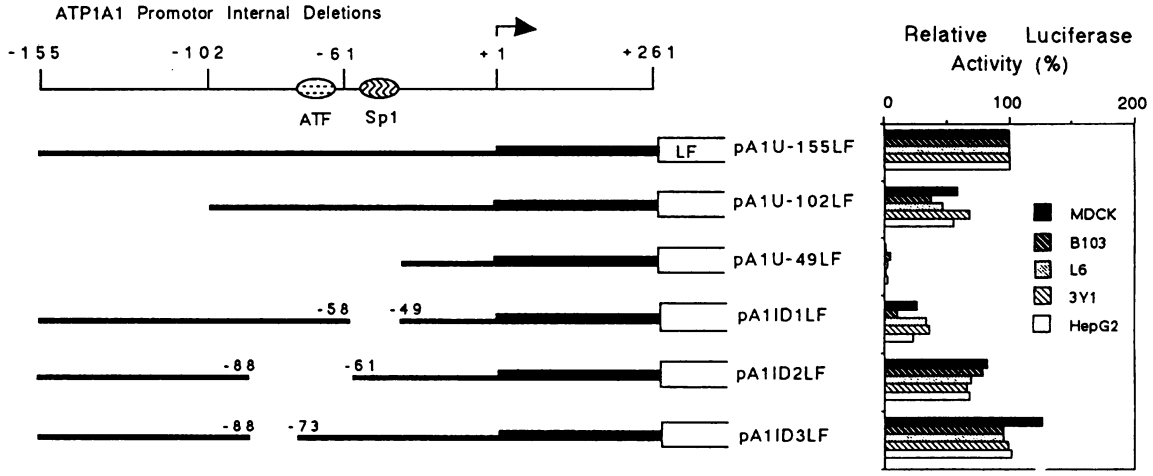

FIG. 2. Reporter gene assays of internal deletion mutations of the ATP1A1 promoter. The deletion mutations of ATP1A1 were constructed with the use of BAL31 nuclease. The terminal nucleotides are indicated. Each of the plasmids (16 μg) was cotransfected with 4 μg of pSV2/βGAL into cells. Luciferase activity was normalized with respect to the β-Gal activity in the same cell lysate. The upper line shows the location of the consensus sequence of Sp1 and ATF/CRE. The luciferase activity of pA1U-155LF was taken as 100; the standard deviation did not exceed 16%.

cells for the three linker substitution mutations, while the level was reduced to about 50% in B103 cells. The pattern in L6 and HepG2 cells was similar except that one mutation (pA1LS2LF) gave approximately the original level. In contrast, in 3Y1 cells, one linker substitution mutation (pA1LS1LF) lowered the luciferase expression. On the other hand, pA1ID6LF gave 3.5- to 10-fold-higher luciferase activity than did pA1U-61LF in all cell lines. The existence of cell type-specific, multiple positive and/or negative regulatory elements in the region from −155 to −61 is a plausible explanation for these results.

Identification of the *trans*-acting factors binding to the ATP1A1 promoter. Assuming the presence of cell-type-specific multiple transcription regulatory factors binding to the region between −155 to −61 of ATP1A1, we tried to find DNA-binding factors by using a gel retardation assay. DNA fragments from −155 to −102 and from −102 to −58 of ATP1A1 were used as probes. When the DNA fragment from −155 to −102 of ATP1A1 was used as a probe, three complexes were observed in MDCK and B103 nuclear extracts. The patterns of retardation were similar for the two nuclear extracts (data not shown). The binding region of the complexes was from −120 to −106, and the binding factors were identified as Sp1 from the following three observations. First, the DNA sequence from −122 to −114 or from −116 to −108 fits perfectly with the Sp1 consensus sequence [(G/T)(G/A)GGC(G/T)(G/A)(G/A)(G/T) (8)]. Second, formation of the gel retardation complexes was competed for with a 50-fold excess of the ATP1A1 fragment (from −102 to −42) which contained the Sp1 binding core sequence (GGGCGG;

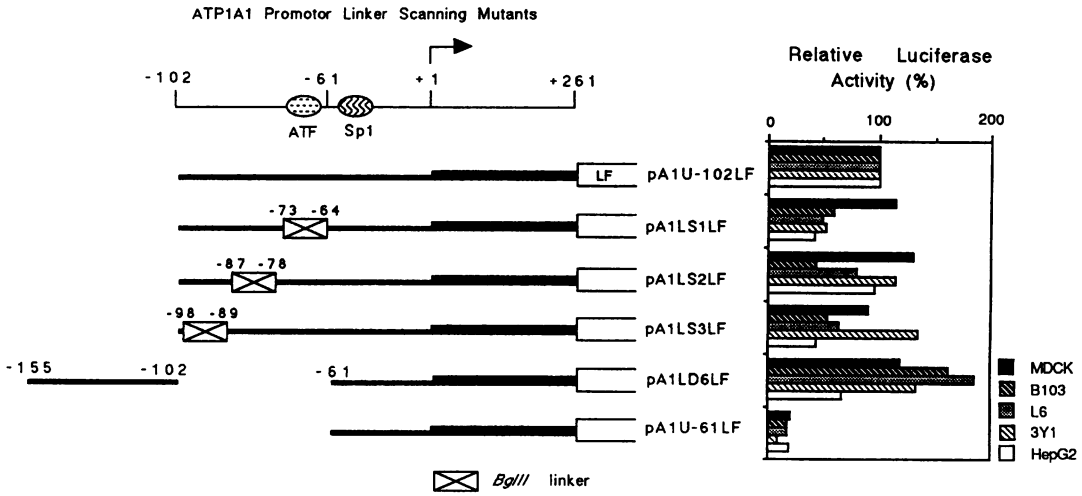

FIG. 3. Reporter gene assays of linker substitution and internal deletion mutation of the ATP1A1 promoter. The *Bgl*II linker was introduced into the positions indicated as described in Materials and Methods. The terminal nucleotides are indicated. Each of the plasmids (15 μg) was cotransfected with 5 μg of pSV2/βGAL or pEF-BOS/βGAL into MDCK, B103, L6, 3Y1, and HepG2 cells. Luciferase activity was normalized with respect to the β-Gal activity in the same cell lysate. The upper line shows the location of the consensus sequence of Sp1 and ATF/CRE. The luciferase activity of pA1U-102LF was taken as 100; the standard deviation did not exceed 20%.

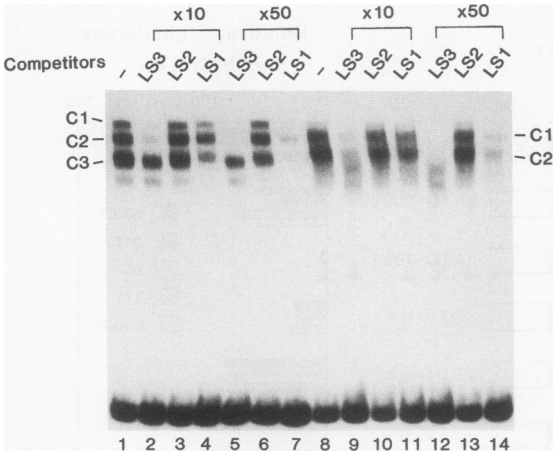

FIG. 4. Gel retardation analysis of DNA-binding factors of ATP1A1, using nuclear extracts from MDCK and B103 cells. The ^{32}P-labeled *Pvu*II-*Mlu*I (−102 to −61) fragment (15.7 fmol) of ATP1A1 was incubated with 1 μl of MDCK nuclear extract (5 μg of protein; lanes 1 to 7) or 1 μl of B103 nuclear extract (5 μg of protein; lanes 8 to 14). C1, C2, and C3 indicate the positions of the complexes observed. As a competitor, 157 fmol of LS1, LS2, or LS3 (lanes 2 to 4 and 9 to 11) or 785 fmol of LS1, LS2, or LS3 (lanes 5 to 7 and 12 to 14) was added.

data not shown). Third, specific gel retardation complexes were formed when the bacterially expressed truncated Sp1 (38a) instead of the nuclear extracts was used for the gel retardation assay, while no specific complex was formed with the probe DNA fragment from −102 to −58 of ATP1A1 (data not shown).

We found DNA-binding factors with the DNA fragment from −102 to −58 of ATP1A1 used as a probe (Fig. 4). Three specific gel retardation complexes (C1, C2, and C3) were formed in the MDCK nuclear extract, while two complexes (C1 and C2) were formed in the B103 nuclear extract. To examine whether these complexes are formed on the same DNA element, we performed the gel retardation competition assays with use of ATP1A1 fragments (from −102 to −58) containing linker-substituted mutations (LS1, LS2, and LS3 in Fig. 4; see also Materials and Methods and Fig. 3). The LS3 fragment, possessing a *Bgl*II linker substitution at positions −98 to −89, competed for formation of the C1 and C2 complexes in both MDCK and B103 nuclear extracts but had only a marginal effect on formation of the C3 complex in the MDCK nuclear extract (Fig. 4, lanes 2, 5, 9, and 12). The LS2 fragment, with the *Bgl*II linker substitution at positions −87 to −78, competed for none of the complexes (Fig. 4, lanes 3, 6, 10, and 13). The LS1 fragment, in which the *Bgl*II linker substitution was from positions −73 to −64, competed for formation of all three complexes in both extracts (Fig. 4, lanes 4, 7, 11, and 14). These results suggest that C1 and C2 have the same binding specificity, while that of C3 is different from those of C1 and C2. These findings were confirmed by the DNase I footprinting and methylation interference experiments described below.

Interaction of the binding factors with the ATP1A1. Having found that specific gel retardation complexes were formed in the region from −102 to −58 of ATP1A1, we examined the binding sequence and the guanine residue contacts of the complexes. Figure 5 shows the pattern of DNase I footprinting and methylation interference of the C1, C2, and C3 complexes with the MDCK and B103 nuclear extracts used for Fig. 4. Differences in DNA binding between the C1, C2, and C3 complexes were observed in the DNase I footprinting experiment. For upper-strand DNA, the binding regions corresponded to the sequence from −88 to −69 (region I in Fig. 5A) of the C1 and C2 complexes of MDCK and B103 and from −88 to −79 of the C3 complex of MDCK (region II in Fig. 5A), including the region protected weakly (from −80 to −79, broken-line brackets of region II). For lower-strand DNA, the binding sites were from −90 to −71 (regions III and IV in Fig. 5A) of all complexes, although the region from −77 to −71 (broken-line brackets of region IV) of the C3 complex of MDCK cells was protected weakly. These regions did not include the consensus binding sequence of the activation transcription factor (ATF) (from −71 to −65). The hypersensitive sites were observed at position −94 in the C1, C2, and C3 complexes (upper strand) of MDCK and about 20 base downstream (located in the multilinker of the pUC119) from the 3′ side of the binding region in the C1 and C2 complexes of B103.

The patterns of methylation interference on these complexes were clearly different (Fig. 5B). Methylation of eight guanine bases (indicated by closed triangles in Fig. 5B and C) interfered with formation of the C1 and C2 complexes. Methylation of eight guanine bases (four of them different in position in C1 and C2; indicated by open triangles in Fig. 5B and C) interfered with C3 complex formation. The positions of the guanine residues which interfered with C1 and C2 formation ranged from −86 to −72, while those of C3 in the MDCK nuclear extract were from −94 to −81.

Identification of the minimal sequence for C1, C2, and C3 complex formation. To determine the minimal sequence requirement for the formation of the complexes, we performed a gel retardation competition assay with use of DNA fragments of the 5′-flanking regions of several other genes (Fig. 6), each containing the GGTTGCT core sequence (from −87 to −81 of ATP1A1). The results of methylation interference experiments indicated that all of the guanines of both the upper and lower strands in the GGTTGCT sequence interfered with the formation of C1, C2, and C3 (Fig. 5). Figure 6A shows the results of a gel retardation competition assay with the MDCK nuclear extract. DNA fragments from mouse mitochondrial malate dehydrogenase (lanes 4 and 10) and from human NF-IL6 (lanes 6 and 12) competed for formation of the C1 and C2 complexes. Fragments from rat serine dehydratase (lanes 2 and 8), human thymidylate synthase (lanes 3 and 9), human ATP synthase β subunit (lanes 5 and 11), and ATP1A1 (including the GGTTGCA sequence from −38 to −32; lanes 7 and 13) were ineffective. None of the DNA fragments competed for formation of the C3 complex. The DNA fragments of mouse mitochondrial malate dehydrogenase and human NF-IL6 also competed for formation of the C1 and C2 complexes of the B103 nuclear extract, while the other fragments did not (data not shown). The sequences around the GGTTGCT element of these fragments are shown in Fig. 6B. The two guanines at the third and fourth residues from the 3′ end of the core GGTTGCT sequence, whose methylation interfered with formation of the C1 and C2 complexes (Fig. 5B), were conserved among the ATP1A1, malate dehydrogenase, and human NF-IL6 genes but not among the other genes (Fig. 6B). These results indicated that both of the guanines at the third and fourth residues downstream from the 3′ end of the core GGTTGCT sequence are essential for formation of the C1 and C2 complexes (Fig. 6B, line 8). Although guanine at the sixth, thymine at the fifth, and cytosine at second residues upstream of the 5′ end of the core GGTTGCT

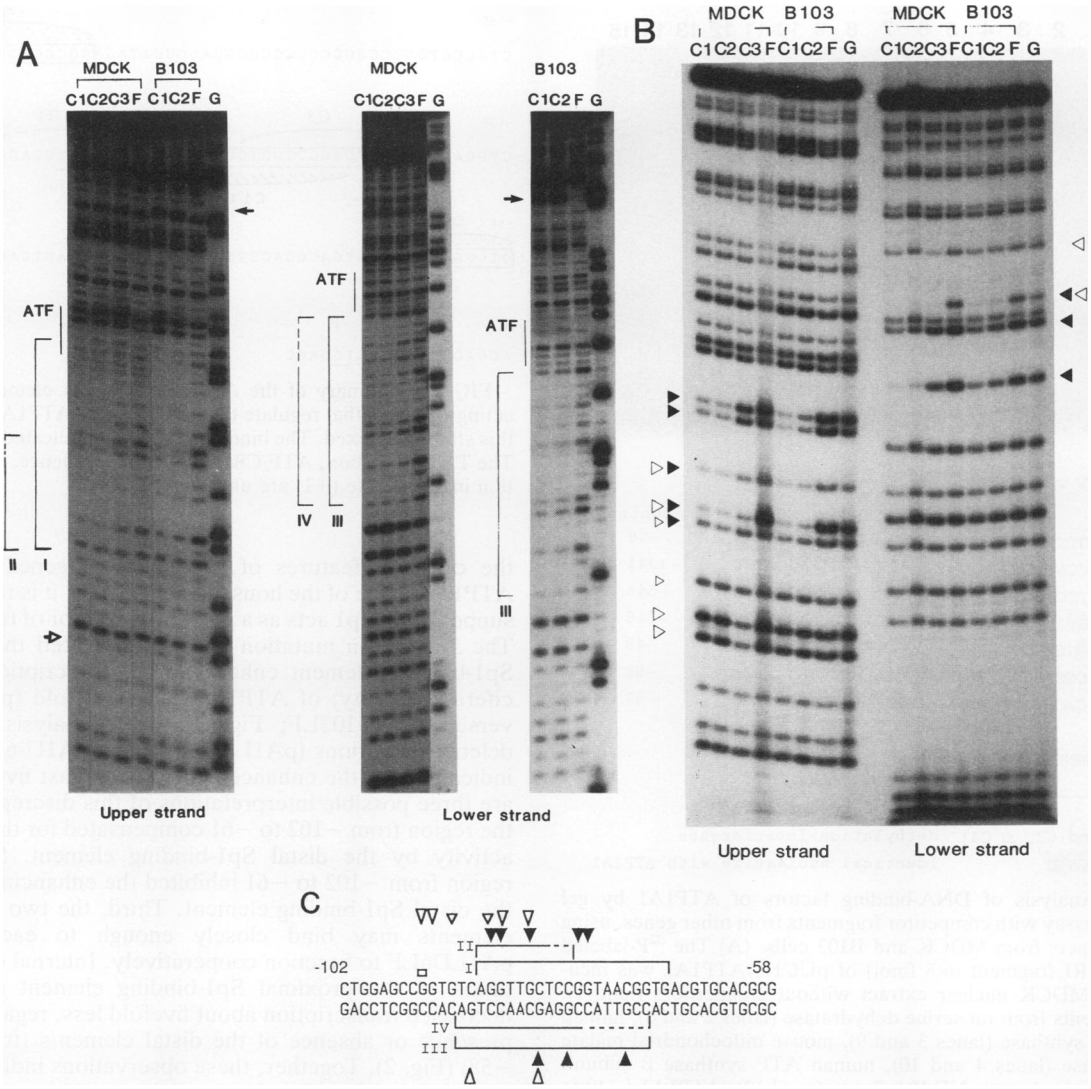

FIG. 5. DNase I footprinting and methylation interference analysis of MDCK and B103 nuclear extracts with the ATP1A1 promoter probe (from −102 to −58). (A) DNase I footprinting. The gel retardation complexes corresponding to C1, C2, and C3 (MDCK), C1 and C2 (B103), and the unretarded probe (F) shown in Fig. 4 were analyzed by DNase I footprinting. The protected regions are indicated. Region I, footprints of the C1 and C2 complexes of MDCK and B103; region II, footprint of the C3 complex of MDCK; region III, footprints of the C1 and C2 complexes of MDCK and B103; region IV, footprints of the C3 complex of MDCK. The open arrow denotes positions of hypersensitive sites in the C1, C2, and C3 complexes of MDCK, and closed arrows denote those in the C1 and C2 complexes of B103. A Maxam-Gilbert guanine ladder is shown in lane G. (B) Methylation interference. C1, C2, and C3 (MDCK), C1 and C2 (B103), and the unretarded probe (F) were isolated and analyzed. G residues of the C1 and C2 complexes of MDCK and B103 that are interfered with are indicated by closed arrowheads; those of the C3 complex of MDCK are indicated by open arrowheads. (C) Summary of DNase I footprinting and methylation interference experiments. The protected regions are shown. The G residues that are interfered with are marked by triangles (▲, C1 and C2 of MDCK and B103; △, C3 of MDCK). The hypersensitive site of the C1, C2, and C3 complexes (open square) of MDCK is shown.

sequence were conserved between ATP1A1 and malate dehydrogenase (Fig. 6B), the DNA fragment of malate dehydrogenase did not compete for formation of the C3 complex (Fig. 6A). This result indicated that the guanines at the fourth and seventh residues upstream of the 5′ end of the core GGTTGCT sequence and some other residues might be essential for formation of the C3 complex (Fig. 6B, line 9).

DISCUSSION

We identified the *cis*-acting elements that control expression of ATP1A1 and the factors that bind to the elements of the ATP1A1 promoter (summarized in Fig. 7). The region between −155 and −49 was a positive regulatory region in all of the cell lines tested. The region was composed of at least three segments: from −155 to −102 (including an Sp1 binding site from −120 to −106), from −102 to −61 (none of the consensus sequences of transcription factors so far identified was found in the region), and from −58 to −49 (including the Sp1 binding core sequence, GGGCGG, from −56 to −51). The results of internal deletion mutation and linker substitution mutation experiments (Fig. 2 and 3) suggest that all of these elements are involved in control of ATP1A1 expression in a mutually compensating manner.

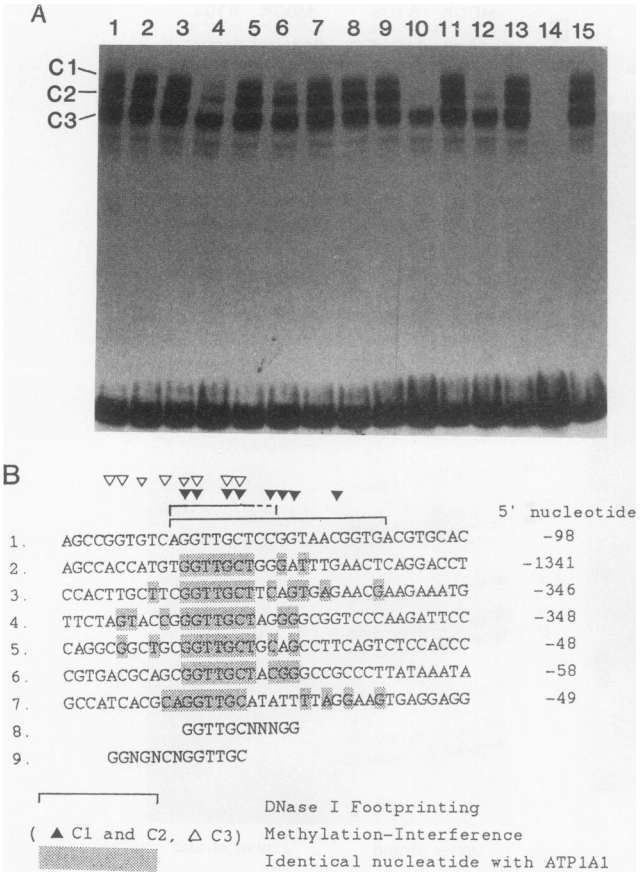

FIG. 6. Analysis of DNA-binding factors of ATP1A1 by gel retardation assay with competitor fragments from other genes, using nuclear extracts from MDCK and B103 cells. (A) The ^{32}P-labeled *Hin*dIII-*Eco*RI fragment (6.8 fmol) of pUC119/ATP1A1 was incubated with MDCK nuclear extract without competitors (lane 1). DNA fragments from rat serine dehydratase (lanes 2 and 8), human thymidylate synthase (lanes 3 and 9), mouse mitochondrial malate dehydrogenase (lanes 4 and 10), human ATP synthase β subunit (lanes 5 and 11), human NF-IL6 (lanes 6 and 12), ATP1A1 (−49 to +52; lanes 7 and 13), a *Hin*dIII-*Eco*RI fragment of pUC119/ATP1A1 (lane 14; positive control), and a *Hin*dIII-*Eco*RI fragment of pUC119 (lane 15; negative control) of 68 fmol (lanes 2 to 7) or 680 fmol (lanes 8 to 15) were added to the reaction mixture as competitors. (B) Sequences showing similarities to the ATP1A1 promoter (line 1). Line 2, rat serine dehydratase, reverse sequence; line 3, human thymidylate synthase; line 4, mouse mitochondrial malate dehydrogenase; line 5, human ATP synthase β subunit; line 6, human NF-IL6; line 7, ATP1A1 from −49 to +52. The tentative minimal consensus sequences required for formation of the C1, C2 (line 8), and C3 (line 9) complexes are shown. The protected region is shown. The interfered-with G residues shown in Fig. 5 are marked by triangles (▲, C1 and C2 of MDCK and B103; △, C3 of MDCK). The nucleotides identical to those of ATP1A1 are shaded. Positions of the 5′-most nucleotides relative to their transcription start sites are noted.

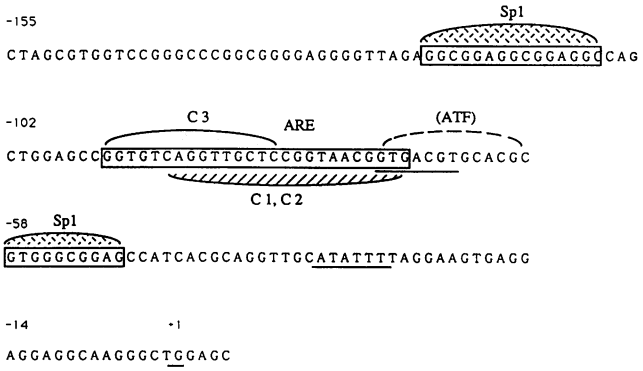

FIG. 7. Summary of the ATP1A1 promoter elements. The *cis*-acting elements that regulate the expression of ATP1A1 identified in this study are boxed. The binding factors are indicated as filled arcs. The TATA-like box, ATF/CRE consensus sequence, and transcription initiation site (+1) are underlined.

There are at least two Sp1 binding sites (from −56 to −51 [the proximal element] and from −120 to −103 [the distal element] which possibly binds multiple Sp1 elements) in the ATP1A1 promoter region. Sp1 is a well-characterized factor that is ubiquitously expressed and involved in the transcription of gene expression in all cell types (2). The existence of an Sp1-binding element(s) in the promoter region is one of the common features of housekeeping genes (19). Since ATP1A1 is one of the housekeeping genes, it is reasonable to suppose that Sp1 acts as a positive regulator of transcription. The 5′ deletion mutation analysis indicated that the distal Sp1-binding element enhanced the transcription level (luciferase activity) of ATP1A1 about twofold (pA1U-155LF versus pA1U-102LF; Fig. 1), while analysis of internal deletion mutations (pA1LS6LF versus pA1U-61LF; Fig. 3) indicated that the enhancement was at least fivefold. There are three possible interpretations of this discrepancy. First, the region from −102 to −61 compensated for the enhancing activity by the distal Sp1-binding element. Second, the region from −102 to −61 inhibited the enhancing activity of the distal Sp1-binding element. Third, the two Sp1-binding elements may bind closely enough to each other in pA1LD6LF to function cooperatively. Internal deletion mutation of the proximal Sp1-binding element (pA1ID1LF) decreased transcription about fivefold less, regardless of the presence or absence of the distal elements (from −155 to −58) (Fig. 2). Together, these observations indicate that the proximal Sp1-binding element enhances the transcription of ATP1A1 independently of other distal elements.

The regulation of transcription through the region between −102 and −61 seems to be very intricate. The results of the 5′ deletion mutation analysis clearly indicated the existence of a positive regulatory element (three- to eightfold activation) between −102 and −61 (Fig. 1), while the transcription levels of the internal deletion mutations of ATP1A1, from −87 to −61 (pA1ID2LF) and from −87 to −73 (pA1ID3LF), were higher than that of pA1U-102LF (Fig. 2). These observations indicate that the region from −102 to −61 and the distal Sp1 element function in a mutually compensating manner or, since the distal Sp1-binding element had become close to the proximal one in these mutations, the two Sp1-binding elements of the promoter could activate the promoter more efficiently. The analysis of linker substitution mutations of ATP1A1 indicates that this region is composed of multiple elements which are functionally different in various cell types (Fig. 3). In fact, we detected multiple binding factors in the nuclear extracts of MDCK and B103 cells.

We named the region from −94 to −69, which was identified by DNase I footprinting and methylation interference experiments (Fig. 5), the ATP1A1 regulatory element (ARE) (Fig. 7). The ARE is highly conserved among the rat

(43), horse (13), and human (35) ATP1A1 (the only difference is the change of thymine at −76 to cytosine in horse ATP1A1). This finding suggests that the ARE is a common element for transcription regulation of mammalian ATP1A1. No other Na,K-ATPase subunit genes have sequence elements similar to that of the ARE (16, 18, 21, 34). The active transcription factor (ATF/CREB) consensus binding sequence (22, 36) is found from −71 to −65 (GTGACGT), but no binding factor was found in a gel retardation assay using a DNA fragment from −102 to −58 (Fig. 4) as a probe. Since the flanking region of the probe might be too short to detect ATF/CREB binding, we tested the binding of bacterially expressed TREB36 (ATF1) and CREB1 (44) to the DNA fragment from −102 to −42. TREB36 bound to the fragment but CREB1 did not (data not shown), suggesting that some members of the ATF/CREB family could bind to their consensus sequences on ATP1A1 (Fig. 7).

Three complexes (C1, C2, and C3) were formed at the ARE in the MDCK nuclear extract. The binding regions and the guanine residues which were in contact with the binding factors in C1 and C2 complex formation were almost the same, while those of C3 were different (Fig. 4 to 6). Two complexes (C1 and C2) were formed in the B103 nuclear extract. The binding regions and guanine residues required for C1 and C2 formation were the same as those of the MDCK nuclear extract except that the DNase I-hypersensitive site was observed only in the complexes of the B103 nuclear extract (Fig. 5A). Three complexes, which had the same competitive properties for DNA binding in the gel retardation assay as did the MDCK nuclear extract, were observed in L6 and HeLa nuclear extracts, although the relative content of these complexes was different from that of the MDCK nuclear extract (data not shown). These observations indicate that the ARE interacts with both common and specific factors in different cell lines. This finding suggests that although ATP1A1 is ubiquitously expressed in all animal cells, its transcription is supported by different set of factors in each cell type. It has been reported that the transcription factors binding to the mouse HTF9 housekeeping promoter are different among cell types (39). Efficient Sp1 binding to the promoter was observed in cultured fibroblast nuclear extract but not in liver nuclear extract, whereas CCAAT box-binding protein was found in liver extract but not in cultured fibroblast extract (39). Dissection of the dihydrofolate reductase gene promoter has yielded characterization of several factor-binding sites, whose locations sometimes overlap and which therefore are likely to interact with alternative factors in vivo (37).

Why must the ARE interact with a number of distinct factors in a cell-type-specific manner? One possible explanation is that ATP1A1 is a housekeeping gene, and so the promoter must respond to various *trans*-acting factors that accompany cell specialization. Some transcription factors are expressed in a cell type-specific manner and are involved in regulating the specialized function of the cell. For example, the Oct-2 protein is B cell specific (25, 30, 40) and Myo-D1 is muscle cell specific (6). Even in the case of ubiquitously expressed factor such as Sp1, expression varies greatly in different cell types and at different developmental stages (29). In the case of ATP1A1, the relative amounts of gel retardation complexes C1, C2, and C3 were different among nuclear extracts of MDCK, B103, L6, and HeLa cells. This finding itself reflects a difference in the content of multiple factors forming these complexes in these cells. Since Na, K-ATPase is an essential enzyme for all types of animal cells, Sp1 elements, an ATF/CRE element, and the ARE may be needed for ubiquitous expression of ATP1A1 in any cell type. Different combinations of the factors might be functionally equivalent, at least for supporting the basal level of transcription. Although a number of different factors bind to the ARE, it is not clear whether they all interact with the DNA at the same time. It is possible that different sets of transcription factors bind to the sequence element to modulate transcription under different cellular conditions.

The minimal sequence required for formation of the C1 and C2 complexes on the ARE is GGTTGCNNNGG, and that required for C3 formation might be GGNGNCNGGT TGC (Fig. 6B). The mouse mitochondrial malate dehydrogenase and human NF-IL6 genes have the GGTTGCNN NGG sequence in their 5′-flanking regions (Fig. 6). In the NF-IL6 promoter, a gel retardation complex was observed in nuclear extract of hepatic cells (Hep3B and normal liver), independent of lipopolysaccharide stimulation (1a). The ARE consensus sequence of the C1 and C2 complexes is also found from −1754 to −1744 in the 5′-flanking region of rat pyruvate kinase (5). The ATP1A1, malate dehydrogenase, and pyruvate kinase genes are involved in cellular energy metabolism, suggesting that the core sequence is a common regulatory element responsive to the state of energy metabolism.

Results of analyses of the promoter activity of ATP1A1 in vivo and those of analyses of the binding factors of ATP1A1 in vitro indicated that ubiquitous expression of the housekeeping promoter of ATP1A1 was regulated by at least three positive elements (Fig. 7): two Sp1-binding elements (from −120 to −106 and from −58 to −49), which act in all cell lines tested (MDCK, B103, L6, 3Y1, and HepG2), and the ARE (from −94 to −69), the multiple-factor-binding element. The ARE acts in a cell type-specific manner. The pA1LS2LF construct showed promoter activity almost the same as that of pA1U-102LF except in B103 cells (Fig. 3). The gel retardation assay clearly indicated that ARE-binding factors did not bind to the region from −102 to −61 of pA1LS2LF (Fig. 4). No other factors were found to bind to the fragment from −102 to −61 containing LS2 mutations (data not shown). These findings cannot be explained if the binding factors act simply as positive regulatory factors. The potential positive or negative transcription activities of the binding factors found in this investigation, as well as interactions between these factors with each other, with Sp1, with ATF/CREB, or with the basic transcription factors, remain to be explored with use of purified ARE-binding factors in an vitro transcription system to attain a more detailed understanding of the regulation of ATP1A1 expression.

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