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Identification of a Mineralocorticoid/Glucocorticoid Response Element in the Human Na/K ATPase α1 Gene Promoter[1]

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Sodium–potassium ATPase (Na/K ATPase) is a major target of mineralocorticoids. Both aldosterone and glucocorticoids activate the human Na/K ATPase α1 and β1 genes transcriptionally. The mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) have been shown to bind the glucocorticoid response element (GRE); however, a specific element responsible for the activation of the MR is not known. Sequence analysis of the putative regulatory region of the Na/K ATPase α1 gene revealed the presence of a hormone response element that allows the MR to interact with it, at least as well as if not better than the GR. This response element is designated MRE/GRE. In this investigation, we demonstrated the MR and GR induced gene expression in COS-1 cells by cotransfecting with respective expression plasmids (RshMR and RshGR) along with a luciferase reporter. The synthetic MRE/GRE linked to a neutral promoter was activated by MR (6-fold); however, the GR induced a lower level of expression (3.8-fold), suggesting that the element may be preferably MR responsive. Mutations in the synthetic MRE/GRE could not induce the expression with MR, whereas GR had a small effect. Electrophoretic mobility shift analyses demonstrated a direct interaction of MR and GR with the MRE/GRE that was supershifted by an antiMR antibody and the complex was partially cleared by an antiGR antibody, respectively, whereas nonimmune serum had no effect. Footprinting analyses of the promoter region showed that a portion of the DNA containing this element is protected by recombinant MR and GR. Thus these data confirm that this MRE/GRE interacts with both MR and GR but interaction with receptors may be more MR-responsive than response elements previously described. © 1999 Academic Press

Key Words: **MR (NR3C2); GR (NR3C1); aldosterone; TA; transcription; promoter; transfection.**

Abbreviations used: MR, mineralocorticoid receptor (NR3C2); GR, glucocorticoid receptor (NR3C1); TA, triamcinolone acetonide; GRE, glucocorticoid response element; MRE/GRE (MRE), mineralocorticoid/glucocorticoid response element.

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Na/K ATPase is an integral membrane protein (1) responsible for the active transport of sodium and potassium across the plasma membrane in an ATP-dependent manner (2, 3). Na/K ATPase is composed of two subunits, the larger α subunit (113 kDa) which mediates the catalytic activity and the smaller glycosylated β subunit (35 kDa) whose exact function is unclear (4). It has been proposed that the β subunit may be involved in the localization of the enzyme to the plasma membrane, protein folding, and stabilization of the K^+-bound form of the enzyme (5). Na/K ATPase is encoded by a multigene family and isoforms were described for both the α (α1, α2, α3) and the β (β1, β2, β3) subunits (6). The mRNA for the α1 subunit is present in most tissues but is expressed at higher levels in kidney (7, 8). Aldosterone modulates cellular ion 'homeostasis' at least in part through the regulation of Na/K ATPase gene expression and may involve a direct interaction of MR with potential hormone responsive elements present in the promoter region of these genes. Aldosterone acts at the transcriptional level through its ligand inducible MR (8–11). However, the mechanisms of corticosteroid regulation of mammalian Na/K ATPase subunit gene expression as well as the significance of the potential hormone regulatory and the *cis*-acting elements in the 5′ flanking region of the human Na/K ATPase α1 gene have not been established.

The mineralocorticoid receptor (MR/NR3C2) (12) and glucocorticoid receptor (GR/NR3C1) (12) are members of the steroid/thyroid hormone receptor superfam-

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ily of ligand inducible transcription factors (13, 14). Other nuclear hormone receptors in this family include thyroid (TR/NR1A1, NR1A2) (12), retinoic acid (RARs/ NR1B1,2,3) and vitamin D receptors (NR1I1) (12). MR and GR exhibit a high degree of homology in the DNA binding domain (DBD) (94%) and ligand binding domain (LBD) but not in their N-terminal regions (15%). Both MR and GR, two classes of adrenal steroid hormones, differ greatly in their physiologic effects. They can elicit opposing *in vivo* effects on ion transport within a single tissue (15, 16), a single cell type (17), and within an individual (18), despite close structural homology and similarities *in vitro* (19). The latent MR and GR reside in the cytoplasm in a complex comprised by heat shock proteins (hsp90) and other stress family proteins (20). The binding of hormone induces critical conformational changes in steroid receptors (SRs), that cause them to dissociate from an inhibitory hormone receptor complex resulting in activation of the receptor. The activated MR and GR translocate to the nucleus where the receptors, as homodimers, bind to a specific palindromic DNA sequence known as a glucocorticoid response element (GRE) associated with target genes, and modulate their transcription (21). The MR has been shown to bind the glucocorticoid response element (GRE), (22, 23), however, a unique element responsible for specific activation by the MR has not been identified.

The mechanism by which nuclear receptors (NRs) regulate transcriptional initiation is currently under intensive investigation. It is thought that the ligand-activated receptor bound to enhancer elements may stabilize or promote the formation of the pre-initiation complex of basal transcription factors for the RNA polymerase on the promoter (22). These effects can be transmitted by a direct interaction between nuclear receptors and basal transcription factors, or by indirect interactions mediated by intermediary proteins called transcriptional coactivators. Nuclear hormone receptor (NRs) are conditional transcription factors that play important roles in various aspects of cell growth, development, and homeostasis by controlling expression of specific genes (20, 22). Transcriptional factors require indirect proteins such as transcriptional coactivators, steroid receptor coactivator-1 (SRC-1), the glucocorticoid receptor interacting protein 1 (GRIP1), transcriptional intermediary factor 2 (TIF2) and the androgen receptor associated protein (ARAP70) to mediate the stimulation of transcriptional initiation after binding to enhancer elements (20, 24, 25). Steroid receptors and class II nuclear receptors have two distinct transactivation domains: AF-1 located in the N-terminal activation domain (AD), and AF-2 located in the hormone binding domain (HBD) (26, 27). Several transcriptional coactivators of the C-terminus AF-2 of the nuclear receptors have been reported recently (28). While all these proteins interact specifically in a ligand dependent manner with the HBD of all the nuclear receptors, only SRC-1, GRIP1, TIF2 and ARAP70 have exhibited the ability to enhance transactivation of nuclear receptors (20).

Modulation of genetic and cellular responses at the level of transcription by physiological or environmental stimuli may involve synergistic or antagonistic interactions of transcription factors such as nuclear factor I (NF-I) and Sp1 with target gene promoter sequences (29, 30). A functional synergy between estrogen receptor (ER) and Sp1 has been shown where protein-protein interaction was observed in an estrogen-induced transactivation pathway (31). Recently, we have demonstrated the upregulation of MR and GR expression by transcription factor Sp1 and the nuclear factor I (NF-I) (32). In an effort to understand the molecular mechanisms involved in MR mediated transcriptional regulation of Na/K ATPase α1, we identified an element in the promoter of the human Na/K ATPase α1 gene that is bound and activated preferentially by MR rather than GR. We further demonstrate specific binding of expressed MR (NR3C2) and GR (NR3C1) to this element by both electrophoretic mobility shift assays and DNase I footprinting analyses.

MATERIALS AND METHODS

Reagents and plasmids. All hormones were purchased from Sigma Chemical Company. RU38486 was a gift from Roussel Uclaf (Romainville, France). DNase I footprinting kit was purchased from Promega Inc. The human GR (NR3C1) and MR (NR3C2) expression plasmids and pTK-CAT were obtained from Dr. Ronald M. Evans (Salk Institute, La Jolla, California) and from the late Dr. Violet Daniel (Weizmann Institute of Science, Rehovot, Israel), respectively. Deletion constructs of the human Na/K ATPase α1 were a generous gift from Dr. Jerry B. Lingrel, University of Cincinnati, Cincinnati, Ohio.

Oligonucleotides used as probes and competitors. The oligonucleotides (MRE/GRE, GRE and mutant MRE/GREs) used in this study were commercially synthesized by Gibco-BRL Life Technologies. These were used for electrophoretic mobility shift assays (EMSA) and cloned into a tk-luciferase vector.

Wild type MRE/GRE: 5′AGATCTAG*T*C*A*C*A*GGAGGCACTCTGA*G*A*G*C*A*A3′)

Half site nucleotides are represented by asterisks. Mutant oligonucleotide sequences indicating mutated bases are underlined. Italicized nucleotides are consensus GRE half sites.

MRE/GRE-Mut 1: 5′ AGATCTGGGATGCGGAGGCACTCTGAGAGCAA3′

MRE/GRE-Mut 2: 5′ AGATCTAGTCACAGGAGGCACTCTGGCGATCA3′

MRE/GRE-Mut 3: 5′AGATCTCTGAGCCGGAGGCACTCTGCCATGAA3′

GRE31: 5′*TGTACA*GGA*TGTTCT*CTAGCGACTAGCTAGT*TGTACA*GGA*TGTTCT* 3′

A nonspecific oligonucleotide Sp1 sequence used in competition experiments is: 5′ GGGTAGAGGGCGGGGCGCACG3′.

MR and GR polyclonal antibodies. Polyclonal MR (αMR-raised against N-terminus and the DNA binding domain of the human MR) and GR (αGR- raised against the DNA binding domain of the human GR) antibodies (Abs) were generated by immunization of rabbits

with 50 μg of conjugated protein and peptide, respectively, each for four times. Abs titres were assessed by enzyme-linked immunosorbent assay (ELISA) and aliquots of serum were stored at −80°C. MR-specific Ab was also obtained from Affinity BioReagent Inc.

Cell culture and transfections. Monkey kidney cell lines, COS-1 and CV-1 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with charcoal-treated fetal bovine serum (10%). Transient transfection of COS-1 cells with various reporter genes was performed as described in standard protocols (33) and by SuperFect (Qiagen). Cells (2×10^5) were electroporated with 5 μg of luciferase reporter construct, 0.5 μg of MR or GR expression vectors and 1 μg of β-galactosidase plasmid. For luciferase assays whole cell extracts were prepared according to the manufacturer's instructions (Promega).

Spodoptera frugiperda. (*Sf9*) insect cells are derived from fall armyworm ovaries and obtained from the American Type Culture Collection (ATCC, Rockville, MD). These cells were cultured in Grace's insect cell culture media (Gibco-BRL) with 10% fetal bovine serum at 25°C. Cells were grown as monolayers in regular cell culture flasks and were routinely infected with $1–4 \times 10^8$ plaque forming units (pfu)/ml recombinant baculovirus at a density of 1.5–2×10^6 cells/ml and >95% viability (23, 34). Antibiotics such as gentamycin and Fungizone were added at a final concentration of 50 μg/ml and 2.5 μg/ml, respectively.

Cloning. The deletion constructs created from the promoter region of the human Na/K ATPase α1 in a CAT vector were subcloned into a pGL3-basic vector (Promega) at SacI and XhoI sites without altering their orientation. pGL3-TK was constructed by excising the TK promoter (168 bp) from the pTK-CAT vector and cloning into the pGL3-basic vector. MRE/GRE and its mutants were cloned at the BglII site of pGL3-TK. All constructs were confirmed by both restriction enzyme analyses and DNA sequencing.

Whole cell extract preparation. COS-1 whole cell extracts, expressing MR and GR were prepared as described by standard procedures. In brief, cells were harvested and homogenized by 20 strokes using a dounce homogenizer in ice-cold buffer containing 20 mM Tris Cl (pH 7.4), 600 mM KCl, 20% glycerol, 2 mM dithiothreitol (DTT), 5 mM phenylmethylsulphonylfluoride (PMSF), 5 μg/ml leupeptin and 5 μg/ml antipain. The homogenate was centrifuged at 100,000× g for 30 min at 4°C and protein concentration was estimated by Coomassie Plus protein assay reagent kit (PIERCE).

Western blot analyses. The expression of MR and GR was examined by Western blot analyses as described (35). Total protein (50–70 μg) from transfected and mock transfected, COS-1 cells was resolved on 8% SDS-PAGE and transfered electrophoretically (100 V constant for 1 h) to Hybond ECL nitrocellulose filter paper (Amersham Life Science). The filter paper was blocked overnight in 10% nonfat milk. Blots were incubated in 5% nonfat milk with indicated polyclonal primary antibodies (1:1000) for 1 h at room temperature. After stringent washing, they were incubated with secondary antibody (1:3000, Anti-rabbit Ig horseradish peroxidase antibody, Amersham Life Science) for an hour and developed by chemiluminiscent ECL as described (Amersham Inc., Arlington Heights, IL).

β-Galactosidase assays and protein estimations. β-Galactosidase assays were performed to normalize for the variations in transfection efficiencies (33). Briefly, equal amounts of protein from all samples were incubated at 37°C with 0.1 M sodium phosphate, 4 mg/ml ONPG (*o*-nitrophenyl β-galactopyranoside) and 100× Mg buffer in a volume of 300 μl (33). The reactions were stopped with either 1 M sodium carbonate or 1 M Tris solution and measured spectrophotometrically at 420 nm. β-Galactosidase values were used as an internal reference in transfection experiments and normalized against the luciferase numbers. Protein estimations were done by Coomassie Plus protein assay reagent kit (PIERCE) a modified Bradford colorimetric method. Readings were recorded at 595 nm using BSA (bovine serum albumin) as standard.

Electrophoretic mobility shift analyses (EMSA). Probes for EMSA were prepared by filling the ends of a synthetic MRE/GRE oligonucleotide with Klenow fragment in the presence of α-^{32}P dCTP (NEN Life Science Products Inc.) (33). Whole cell extracts containing equal amounts of protein (6–8 μg) were incubated in a binding buffer containing 25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 2 mM DTT, 50 mM NaCl and 0.5–1.0 μg of poly(dI · dC) for 15 min. To this, labeled MRE/GRE probe (60,000 CPM) was added and the mixture was incubated for another 20 min at room temperature. For supershift analyses the extracts were incubated with preimmune serum or GR or MR specific antibodies for 30 min on ice before adding the labeled probe. DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels using 0.5× TBE. The gels were dried and subjected to autoradiography. The specificity of the complexes was identified by supershifting the complexes with specific antibodies and cold competition experiments.

Nuclear extract preparation. Nuclear extracts were prepared as described earlier by the NP-40 lysis method. The *Sf9* cells, wild type and infected with recombinant baculovirus, after appropriate treatments (aldosterone for MR and TA for GR), were washed with 1× PBS and lysed in buffer A containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF by gentle pipetting with a micro tip. The cells were allowed to swell for 15 min on ice, after which 1/16th volume of buffer A of 10% NP-40 was added and vigorously vortexed for 10 seconds. The homogenates were centrifuged for 30 sec at 14 K in a microfuge at 4°C. The supernatant containing the cytoplasm fraction was discarded and the nuclear pellet was resuspended in appropriate volume of buffer B containing 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF and the tubes were vigorously rocked at 4°C for 15–30 min on a shaking platform. The nuclear extracts were centrifuged for 10 min at 4°C in a microfuge at 14 K and the supernatants were stored at −80°C after estimating the protein concentrations. These extracts were used in DNase I footprinting experiments.

DNase I footprinting analyses. Nuclear extracts from *Sf9* cells, overexpressing either with MR or GR were prepared after treating the cells with aldosterone and TA respectively as recommended by the manufacturer (Promega). These extracts (20 μg) were incubated in a reaction volume of 50 μl with binding buffer and probes (1–2 ng of promoter fragments DNA end labeled with γ-^{32}P ATP) for 15 min on ice followed by the addition of Ca/Mg solution (5 mM $MgCl_2$ and 5 mM $CaCl_2$ final concentrations) for 1 min at room temperature. DNase I (0.01–0.02 unit) was added to the mixture, which was then incubated for 2 min at room temperature. Samples were extracted once with phenol-chloroform-isoamylclcohol and precipitated with 2 volumes of ethanol. Pellets were resuspended in 80% formamide, 1 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue, and equal amounts of counts were subjected to 6% acrylamide/8M urea gel after denaturing the samples by boiling for 3 min. Gels were dried and subjected to autoradiography.

RESULTS

Western blot analyses of MR and GR. To assess the level of MR and GR expression in COS-1 cells, cells were transfected with or without receptor expression plasmids and whole cell extracts were prepared followed by Western blot as described in Materials and Methods with respective polyclonal antibodies. Endogenous level of MR in COS-1 cells was negligible whereas enhanced level of MR was observed clearly in transfected cells (Fig. 1A). Similarly, GR expression was also observed in those cell lines (Fig. 1B) but not in original COS-1 cells. The present study involves the

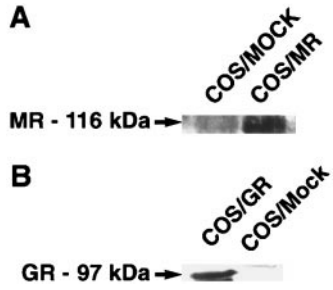


FIG. 1. Western blot analyses of MR and GR. Cells, as indicated were transfected with either MR or GR expression plasmids and prepared whole cell extracts as described under Materials and Methods. Extract (50–70 μg) was resolved on 8% SDS–polyacrylamide gel followed by Western blot with MR or GR polyclonal primary antibodies (1:1000 dilution) and goat anti-rabbit Ig horseradish peroxidase secondary antibody. Proteins were visualized with enhanced chemiluminiscence ECL kit. (A) MR and (B) GR proteins are marked with an arrow and molecular weights.

comparison of both MR and GR, COS-1 cells were used since these cells express little or no endogenous MR or GR as evidenced from Fig. 1 and also from previous reports (14, 36). Moreover, transfection of these cells with receptor expression plasmids having the same background yielded reasonable levels of expression (Fig. 1). We also performed Western blot analyses for MR and GR in *Sf9* insect cells after infecting the wild type cells with recombinant baculovirus and demonstrated the expression of MR and GR from their whole cell extracts as described previously (23, 34) (data not shown). Nuclear extracts of these cells after hormone induction were used in DNase I footprint analyses since the yields of protein were greater than in COS-1 cells.

Functional analysis of regulatory regions of the human Na/K ATPase α1 promoter. To understand the regulation of the human Na/K ATPase α1 promoter activity by mineralocorticoid and glucocorticoid receptors, both COS-1 and CV-1 cells, were transiently cotransfected with a full length reporter construct VKL-1 (-744 to $+124$) linked to a luciferase gene, along with either the MR or GR expression plasmid and pSVβ-gal. Transfected cells were treated with either 100 nM aldosterone and 1 μM RU486 or 100 nM TA and 1 μM spironolactone for MR and GR respectively. Transfection experiments with either MR (16-fold) or GR (12-fold) in COS-1 cells (Fig. 2A), and in CV-1 cells (13.5-fold and 10-fold) (Fig. 2B) respectively, revealed that these receptors could activate this full length reporter VKL-1, a full length promoter cloned in a pGL3 basic vector (Fig. 2A). An empty basic vector, pGL3 basic did not show induction with either MR or GR as expected and served as a control. Fold inductions for MR and GR were calculated over the basic vector with respective receptors and all the luciferase values were normalized with β-gal for the variations in transfection efficiencies. We initially performed a dose response experiment with aldosterone and TA and determined that a concentration of either 100 nM aldosterone or TA gave optimal induction in these cells (data not shown). We observed that the extent of expression induced by MR (16-fold) was slightly higher than induction by GR (12-fold) in COS-1 cells (Fig. 2A). Similar results were obtained in CV-1 cells except slightly lower induction

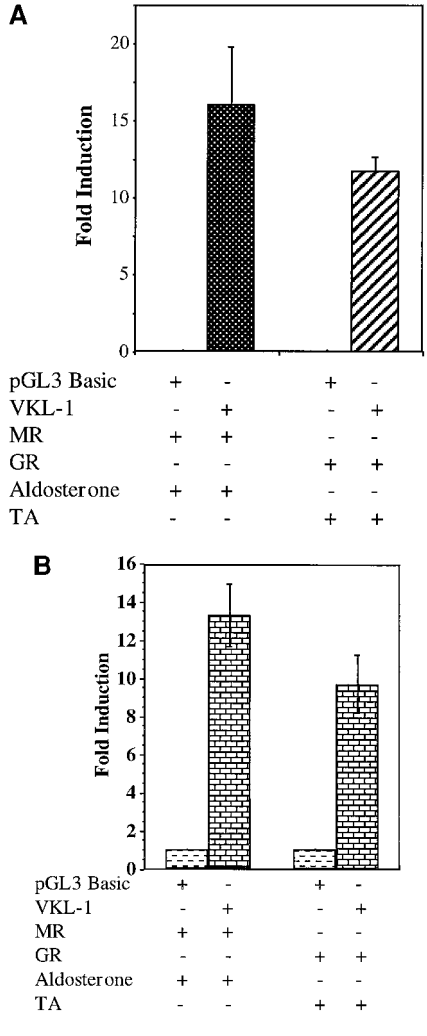
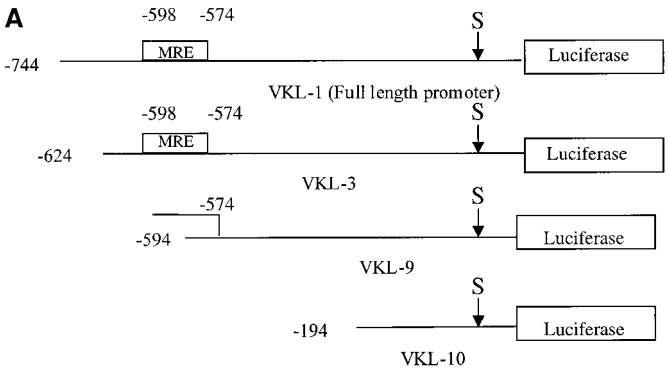


FIG. 2. MR and GR induced gene expression in (A) COS-1 cells and (B) CV-1 cells. Five micrograms of full length reporter construct of Na/K ATPase α1 (-744 to $+124$) and either 1 μg of MR expression plasmid (RshMR) or GR (RshGR) along with 1 μg of β-galactoside expression plasmid (pSVβ-gal) were transfected into COS-1/CV-1 cells by electroporation. Cells were induced with 100 nM aldosterone and 1 μM RU486 in MR transfections, 100 nM TA and 1 μM spironolactone in GR transfections for 24 h. Cells were harvested after 48 h and luciferase assays were carried out using equal amounts of protein (10–20 μg) from each sample (35). MR showed relatively more induction by aldosterone compared to GR for the same reporter construct. pGL3 Basic is a basic promoterless luciferase vector whereas VKL-1 (Na/K ATPase promoter cloned in pGL3 Basic vector) is a reporter construct. The results are the mean of five independent experiments each run in triplicate and all the values were normalized to β-galactosidase activity. The error bars indicate $\pm$SEM.

for both MR (13.5-fold) and GR (10-fold) (Fig. 2B). Moreover, we performed these experiments with multiple GR ligands (data not shown) since transcriptional response depends upon the activating ligand. These data indicate that aldosterone is a relatively stronger activator of the human Na/K ATPase α1 gene promoter when compared to the glucocorticoid receptor agonists, Dexamethasone and triamcinolone acetonide (TA), in either COS-1 or CV-1 cells, since the background of both expression plasmids and their expression levels are similar. In the absence of either aldosterone or TA no activity was observed (data not shown).

To identify the potential hormone response elements (HREs) including the putative MRE/GRE region and *cis*-acting elements involved in MR (NR3C2) or GR (NR3C1) induced transactivation, a full length reporter construct VKL-1, deletion constructs VKL-3, VKL-9 and VKL-10 (−624, −591 and −194 respectively) were generated by subcloning various lengths of the promoter fragments into a luciferase vector, pGL-3 basic. The schematic representation of the full length and the deletion constructs with their sizes is shown in Fig. 3A. These reporter constructs were transfected into COS-1 cells along with either the MR or GR expression plasmids (RshMR and RshGR) and pSVβ-gal. Cells were treated with either 100 nM aldosterone or 100 nM TA for 24 h and whole cell extracts were prepared and examined for luciferase activity using equal amounts of protein. Relative luciferase fold inductions for MR and GR with various reporter constructs after normalizing all the luciferase values with β-gal for the variations in transfection efficiencies are shown in Fig. 3B. We demonstrated that VKL-3 is induced 21-fold by MR and 19-fold by GR whereas VKL-9 is induced by 12 and 7-fold, respectively (Fig. 3B). Moreover, VKL-3 showed an enhanced induction with both MR and GR than VKL-1 which may be due to the deletion of possible inhibitory sequences present in VKL-1. Both VKL-1 and VKL-3 constructs have a putative MRE/GRE, whereas the VKL-9 construct has a deletion of 5 bases from half of the MRE/GRE sequence which resulted in a 2-fold reduction of the promoter activity. The smaller construct, VKL-10, in which the entire MRE/GRE was deleted, was not induced with MR, however, weak induction was observed in the presence of GR, that may be due to the presence of a half GRE element in close proximity to the start site. These data suggest that the functional element may reside in the larger constructs and that both aldosterone and TA, through their respective receptors, regulate the Na/K ATPase α1 gene promoter. Sequence analysis revealed the presence of a consensus half GRE binding site (−246) and an element between −574 to −598 bp (37) in which two half sites are separated by 12 bp that could serve as a mineralocorticoid/glucocorticoid responsive element but not the half GRE binding site (−246) since neither MR nor GR bound to



MRE/GRE Sequence: 598- AGTCACAGGAGGCACTCTGAGAGCA -574

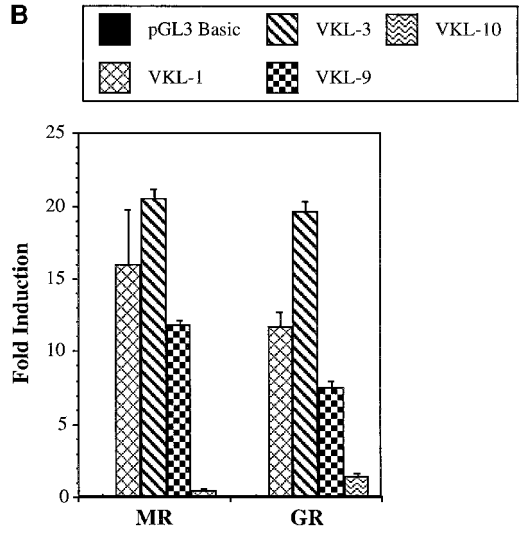


FIG. 3. (A) Schematic representation of the Na/K ATPase α1 gene promoter fragments. These consisted of the 5′ promoter and 124 bp of the first exon linked upstream of a promoterless luciferase gene in pGL-3 basic vector. Length of the fragment is shown with respect to the transcription start site (S). (B) MR and GR induced gene expression of various deletion constructs in COS-1 cells. Five micrograms of each reporter deletion construct of Na/K ATPase α1 and 1 μg of MR expression plasmid (RshMR) or GR (RshGR) along with 1 μg of β-galactoside expression plasmid (pSVβ-gal) were transfected into COS-1 cells either by electroporation or by SuperFect. Cell treatments and luciferase assays were exactly the same as in Fig. 1. MR showed relatively more induction by aldosterone compared to GR for all the reporter constructs. The results are the mean of five independent experiments each run in triplicate and all the values were normalized to β-galactosidase activity. The error bars indicate ±SEM.

this element in EMSA (data not shown). These data suggest that the potential MRE/GRE at position −574 may play a role in conferring aldosterone responsiveness to the α1 promoter.

Corticosteroid induction of mineralocorticoid/glucocorticoid response element (MRE/GRE). To examine whether the sequence extending from −574 to −598 bp constitutes a functional MRE/GRE, a double-stranded synthetic oligonucleotide corresponding to the putative

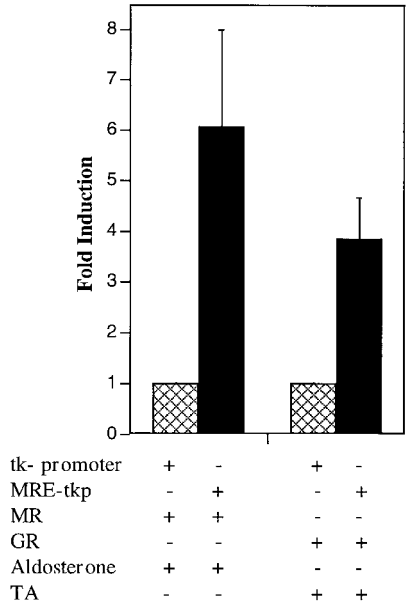


FIG. 4. MR and GR dependent transcriptional induction of MRE/GRE in COS-1 cells. Synthetic element MRE/GRE from Na/K ATPase (−574 to −598) driven by the tk-promoter-induced luciferase reporter gene in COS-1 cells by aldosterone and TA. Reporters (5 μg), MR (1 μg), GR (1 μg), and 1 μg of pSVβ-gal plasmid were transfected and luciferase assays were carried out as described earlier. Treatments were exactly the same as in the previous figure. The results are the mean of five independent experiments each run in triplicate and normalized with β-galactosidase activity. The error bars indicate ±SEM.

MRE/GRE sequence (5′ AGATCTAGTCACAGGAGGCACTCTGAGAGCAA 3′) was cloned at the BglII site of a neutral thymidine kinase (tk) promoter in the luciferase reporter gene. As shown in Fig. 4, cells transfected with a reporter construct bearing MRE/GRE along with the MR demonstrated a 6-fold induction, whereas cells transfected with GR were induced 3.8-fold. MRE/GRE exhibited lower activity in the transactivation assays which may be due to deletion of multiple downstream and upstream sequences and limited availability of basal transcription factors that are required for binding and transcriptional activation. Moreover, luciferase activation was observed both for MR and GR suggesting that this element MRE/GRE is responsive to both aldosterone and TA. Since MR activation of the reporter construct MRE-59-tk-Luc was found to be slightly higher than GR, the sequence extending from −574 to −598 bp of the human Na/K ATPase α1 promoter may be preferentially induced by aldosterone. To determine the statistical significance of these data, we performed a paired t-test analysis for five sets of normalized luciferase values with β-gal and found a p-value of 0.05. This suggests that this element MRE/GRE may prefer the MR to the GR.

Mutational analysis of the MRE/GRE element. We further examined the effects of mutations created in the functional wild type element on transcriptional activation. Mutations were created in either the first half site, the second half site or by changing both half sites of this element. All three mutated elements (Fig. 5A) were cloned under the same heterologous neutral tk-promoter and transfected into COS-1 cells along with either MR or GR. Luciferase assays were performed after treating the cells with aldosterone or TA for 24 h. None of the mutated constructs showed activity with MR (Fig. 5B) whereas moderate expression was observed with GR when the mutation was created in the first (mut 1) or second (mut 2) half site, however, only basal level of activity was observed when both half

A

MRE/GRE

5 ' AGATCTAG*T*C*A*C*A* GGAGGCACTCTGA*G*A*G*C*A*A 3'

3' ATC*A*G*T*G*T* CCTCCGTGAGAC T*C*T*C*G*T* TTCTAGA 5'

MRE/GRE-mut1

5' AGATCTGGGATGCGGAGGCACTCTG*AGAGCA*A3'

3'ACCCTACGCCTCCGTGAGAC*TCTCGT* TCTAGA5'

MRE/GRE-mut2

5' AGATCTA*GTCACA*GGAGGCACTCTGGCGATCA3'

3'A*TCAGTGT* CCTCCGTGAGACCGCTAGTTCTAGA5'

MRE/GRE-mut3

5'AGATCTCTGAGCCGGAGGCACTCTGCCATGAA3'

3'AGACTCGGCCTCCGTGAGACGGTACTTCTAGA5'

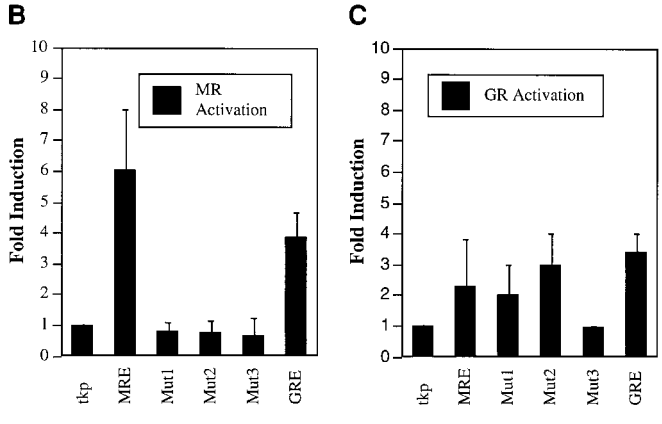


FIG. 5. Mutational analysis of the mineralocorticoid/glucocorticoid response element. (A) Wild type and mutated MRE/GRE oligonucleotides used in cloning under tk-promoter. MRE/GRE represents wild type element and half sites are marked with asterisks. MRE/GRE-mut 1, MRE/GRE-mut 2, and MRE/GRE-mut 3 are mutant versions of MRE/GRE and bases are underlined where the mutations were created. COS-1 cells were transfected with 5 μg of luciferase reporter plasmid containing either a wild type sequence of the Na/K ATPase α1 gene promoter from −574 to −598 or the mutated sequence created in one or both half sites upstream of tk-promoter along with either 1 μg of (B) MR (RshMR); or (C) GR (RshGR) expression plasmids and 1 μg of pSVβ-gal plasmid. Cell treatment and luciferase assays were exactly the same as in the previous figures. The results are the mean of three independent experiments each run in triplicate and normalized with β-galactosidase activity. The error bars indicate ±SEM.

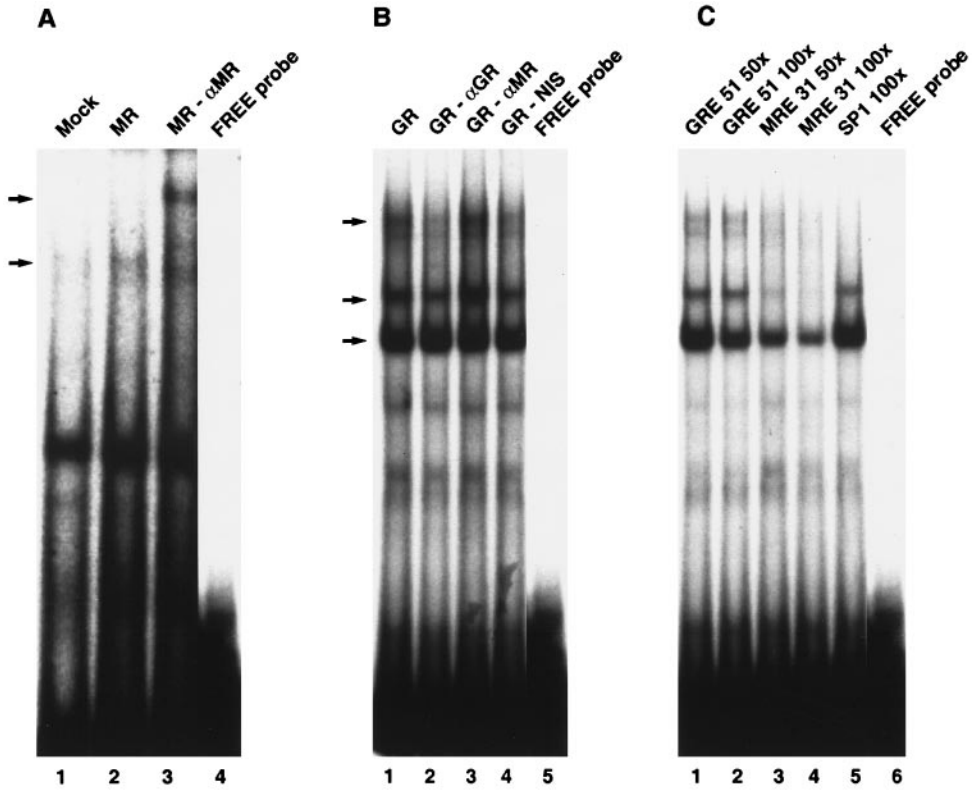


FIG. 6. Electrophoretic mobility shift assay (EMSA). MR (5 μg) or GR (5 μg) was transfected separately into COS-1 cells and treated with aldosterone and TA (100 nM), respectively, for 24 h. Whole cell extracts (WCE) were prepared after 48 h of transfection and EMSA were carried out with 8 μg of WCE and a ^{32}P-labeled MRE/GRE element on a 6% polyacrylamide/0.5× TBE gels as described in Materials and Methods. (A) Lane 1, Mock transfected COS cell extract; lane 2, MR; lane 3, MR with antiMR antibody (raised against N-terminus and the DNA binding domain of the human MR) (36); lane 4, free probe MRE/GRE. MR and supershifted complex are marked with arrows. (B) Lane 1, GR; lane 2, GR with antiGR antibody (raised against the DNA binding domain of the human GR) (36); lane 3, GR with antiMR antibody; lane 4, nonimmune serum; lane 5, free probe. GR complex is shown with 3 arrows of which the top complex is cleared fully with GR antibody and the rest of the two partially. (C) Lanes 1 and 2, competition with the GRE 31 element; lanes 3 and 4, cold competition with MRE/GRE; lane 5, a nonspecific Sp1 oligonucleotide; lane 6, free probe MRE/GRE as indicated. Lane 4 in Fig. 6A and lane 5 in Fig. 6B, were originated from a different EMSA gel. The data are representative of 3 independent experiments.

sites were mutated (Fig. 5C, mut 3). All the luciferase values were normalized with β-gal for the variations in transfection efficiencies.

Binding of overexpressed MR and GR to MRE/GRE. To confirm that regulation of the α1 promoter by aldosterone involves binding of the MR to the promoter, electrophoretic mobility assay (EMSA) was performed using a ^{32}P-labeled synthetic MRE/GRE element and the overexpressed proteins containing either MR or GR in COS-1 cells as described in Materials and Methods. As shown in Fig. 6A, lane 1 represents the binding pattern of mock transfected extract. The MR bound specifically to the MRE/GRE in lane 2. Pre-incubation with an anti-MR antibody supershifted the complex (Fig. 6A, lane 3) indicating the presence of MR in the protein-DNA complex whereas non-immune serum had no effect on this complex (data not shown). A major non specific complex could be seen in all lanes (lanes 1–3) which is not marked with an arrow. To determine the binding pattern of GR to the same element similar EMSA was performed with whole cell extract containing GR. Unlike MR, the GR gave three complexes with MRE/GRE element (Fig. 6B, lane 1) which may be probably due to homo- or heterodimerization. The specificity of this complex was determined by incubating the extract with an antiGR antibody where the top complex was cleared and the second partially suggesting that the complex is GR specific. It may be noted that the MR complex was supershifted whereas GR complex was cleared by respective antibodies. However, an anti-MR antibody and non-immune serum had no effect on this complex (Fig. 6B, lanes 4 and 5). We further tested the specificity of complex by cold oligonucleotide competition experiments. Binding of this complex was competed in a concentration-dependent manner upon pre-incubation of the whole cell extracts with the unlabeled GRE and MRE/GRE oligonucleotide as indicated (Fig. 6C, lanes 1, 2 and 3, 4 respectively), whereas Sp1 (lane 5), a non-specific oligonucleotide (except the top complex), and mutant oligo-

nucleotides (Fig. 5A) had no effect on this complex (data not shown). However, GRE 31 was a very weak competitor compared to MR due to the sequence analogy (compare Fig. 6B, lane 1 and Fig. 6C, lanes 1 and 2). These data suggest that MR and GR bind directly to the α1 promoter and regulate the expression of the α1 gene. These results also correlated with gene expression analyses in previous experiments (Fig. 4).

Identification of a mineralocorticoid/glucocorticoid response element in human Na/K ATPase α1 promoter by DNase I footprinting. In order to further confirm the identified *cis*-acting element in the promoter of the human Na/K ATPase α1, DNase I footprinting assays were performed using the entire promoter between -744 to $+124$ after making two pieces ((A) -504 to $+124$ and (B) -744 to -504)) separately as a probe. As shown in the Fig. 7B there were large protected regions for both MR and GR on one of the two DNA fragments which contains the above identified MRE/GRE (B) (lanes 3 and 4), whereas no protection was observed in the absence of the functional element on proximal fragment (A) by the Sf9 nuclear proteins (Fig. 7A). As an experimental control nuclear proteins from Sf9 wild type cells did not protect the DNA binding against DNase I treatment (lanes 2). Another control was also run parallel where the sample does not contain nuclear proteins (lane 1). This confirms the binding of both MR or GR to the *cis*-acting element MRE/GRE as observed in the case of electrophoretic mobility shift assay (EMSA).

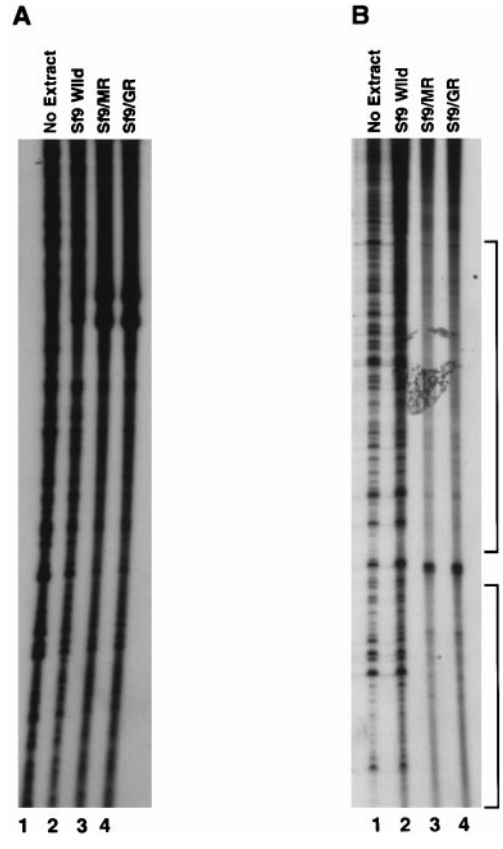


FIG. 7. DNase I footprinting analysis of the human Na/K ATPase α1 promoter with overexpressed Sf9 nuclear proteins by MR and/or GR. Footprinting reactions were performed as described under Materials and Methods. Reactions were carried out in the absence or presence of nuclear extracts (20 μg) of wild type Sf9, cells overexpressed with the MR or GR after treating the cells with aldosterone (100 nM) and TA (100 nM) respectively. probes were prepared by end-labeling the purified fragments with [γ-^{32}P]ATP and the samples were run on a denaturing 6% polyacrylamide/8 M urea gel. (A) Proximal promoter region (-504 to $+124$); (B) distal promoter region containing the identified element (-744 to -504). Lanes 1, without nuclear extract, lanes 2, Sf9 wild type extract; lanes 3, overexpressed MR; and lanes 4, overexpressed GR. Brackets indicates the footprinted regions and the nucleotides protected by both MR and GR. The data are representative of 3 independent experiments.

DISCUSSION

Aldosterone is a mineralocorticoid hormone that plays a major role in regulating sodium and potassium and modulates cellular ion 'homeostasis'. It is involved in the control of blood pressure and is implicated in some pathological disorders. Aldosterone exerts its effects by acting through a ligand-activated transcription factor, the MR (NR3C2) (12) at least in part through the regulation of Na/K ATPase gene expression by acting at the transcriptional level. Tissue specific and other transcriptional factors may interact with the MR (NR3C2) to modulate this regulatory response (8). Na/K ATPase is an integral membrane protein responsible for the transport of sodium and potassium across the plasma membrane in an ATP dependent manner (3). We and others have demonstrated that the human Na/K ATPase is transcriptionally regulated by aldosterone (38) and may involve a direct interaction of MR (NR3C2) with potential hormone response elements present in the promoter region of these genes (36). We demonstrated that the Na/K ATPase gene expression was induced by aldosterone in 293 kidney cells utilizing nuclear run-off transcription assays (11). We have shown previously that induction of gene expression by aldosterone appears to be at the level of transcription that does not require *de novo* synthesis of an intermediary protein since the transcriptional up-regulation was not global (11). The molecular mechanism of the transcriptional regulation by MR remains unclear. Recently, we have demonstrated that either MR or GR was able to induce the human Na/K ATPase β1 gene promoter, however, in the presence of both MR and GR an inhibitory effect was observed (36).

In the present investigation, we carried out transactivation studies of the Na/K ATPase α1 gene promoter by both mineralocorticoid and glucocorticoid receptors in COS-1 and CV-1 cells, since these cells express little or no endogenous receptors. We detected an increased induction of gene expression from the full length promoter after 100 nM aldosterone or 100 nM TA treat-

ment respectively. In addition, we observed a slight, yet statistically significant, increase in MR induced gene expression from both CV-1 (13.5-fold vs 10-fold) and COS-1 cells (16-fold vs 12-fold). To identify DNA sequences required for hormone activation we performed promoter analysis using 5′ deletions fused upstream to a luciferase gene. The complete nucleotide sequence of the Na/K ATPase α1 gene and the unidirectional deletions created in it allowed us to identify potential hormone response elements. We identified a consensus half GRE binding site at −246 bp and a completely functional MRE/GRE between −574 to −598 bp where the two half sites are separated by 12 bp that was mineralocorticoid/glucocorticoid responsive. Moreover, each half site exhibits a sequence mismatch with a consensus GRE. The functionality of this element was tested by cloning it upstream of a heterologous neutral tk-promoter under the luciferase gene in pGL3 vector. We demonstrate that both aldosterone and TA transactivate the gene expression from the identified MRE/GRE in COS-1 cells (6 and 3.8-fold respectively) where the MR is preferred to the GR. Thus these data confirm that this MRE/GRE may be more MR-responsive than response elements previously described. These results were correlated with the earlier results obtained with the deletion constructs where induction was observed in the presence of this element. Interestingly, when we created mutations in either half site the transactivation was completely inhibited to basal level with MR, which suggest an important role for this sequence in response to hormones, whereas little effect was observed in the presence of GR using the same set of mutants. These observations also fulfill the requirement of two half sites, to which both MR and GR bind as homo- or heterodimers to act as a functional MRE/GRE. The presence of multiple elements either in a full length promoter or cloned in a tandem repeat under a heterologous promoter has been described and an increase in the transcriptional activity by MR was observed recently (39). It has also been reported in the literature that multiple EREs (Estrogen Response Elements) upstream of a promoter responded in a linear fashion to estrogen (40). However, little effect was observed in the promoter of Na/K ATPase α1 where we found half GREs in addition to the identified functional element.

To examine whether there is any direct interaction of MRE/GRE sequence with either MR or GR we overexpressed these receptors in COS-1 cells and performed EMSA. We demonstrate that the activated human MR (NR3C2) (12) and GR (NR3C1) (12) were specifically bound to MRE/GRE. Importantly, the bound complex was supershifted using an antiMR antibody but not with an antiGR antibody and formed a single complex with MR upon protein-DNA interaction. However, three protein DNA complexes were formed with GR to the same element. Formation of multiple DNA-protein complexes under *in vivo* conditions have been reported earlier both for MR and GR suggesting the existance of homo- or hetero dimers (41–43). The possibility that other cellular factors may be required for the formation of the complex with the MR (NR3C2) and GR (NR3C1) cannot be ruled out. Transcriptional regulation by glucocorticoids is well established when compared to mineralocorticoids and composite GRE binding sites are necessary to bind receptor as well as other factors (44, 45). For example, the composite GRE was bound selectively *in vitro* both by GR and by c-Fos and c-Jun, components of the phorbol ester-activated AP-1 transcription factor (44). However, it is not clearly understood whether the MR also has similar requirements. These observations were further confirmed by DNase I footprinting analyses where we detected protection when MR or GR containing extracts were used but not with wild type cell extract or no extract. Thus, these data indicate that a direct interaction of the Na/K ATPase α1 gene promoter both with MR and GR involved in gene regulation by corticosteroid hormones aldosterone and a synthetic glucocorticoid analog TA.

In conclusion, regulation of Na/K ATPase gene expression involves a direct interaction of MR and GR despite their physiological differences and tissue specific expression. It has been shown previously that steroid hormones regulate Na/K ATPase gene expression purely in tissue specific manner (7, 8). Moreover, tissue specific factors may also be involved in conferring mineralocorticoid specificity. Further studies on the interaction of the MR with other transcription factors, coactivators such as SRC-1, GRIP1, RIP-140 and corepressors like NcoR and SMRT should permit elucidation of the mechanisms by which the MR (NR3C2) regulates transcription of aldosterone target genes (20, 24, 25, 28, 46, 47).

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