Regulation of the Human Na/K-ATPase β1 Gene Promoter by Mineralocorticoid and Glucocorticoid Receptors*

(Received for publication, January 21, 1998, and in revised form, May 12, 1998)

Assia Derfoul‡, Noreen M. Robertson‡, Jerry B Lingrel§, David J. Hall‡, and Gerald Litwack‡§

From the ‡Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 and the §Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, Ohio 45267

Expression of the human Na/K-ATPase β1 subunit is regulated by a mineralocorticoid- and glucocorticoid-responsive elements. Here we identified an MR and GR responsive element, at positions −650 to −630, within the β1 gene promoter that is required for both MR and GR activation. Independent expression of MR and GR activated by aldosterone or triamcinolone acetonide (TA) leads to significant transactivation of the β1 promoter. Yet coexpression of both receptors activated by aldosterone plus TA or cortisol results in a much lower induction, indicating that coexpression of MR and GR is inhibitory. Gel shift mobility assay using an oligonucleotide including the 21-base pair MRE/GRE with whole cell extracts prepared from CV-1 cells overexpressing MR or GR showed specific MR and GR binding to this sequence. Additionally, antibodies to both MR and GR effectively supershifted the protein-DNA complexes, indicating that these receptors bound to the DNA sequence. Finally, the 21-base pair MRE/GRE was capable of activating transcription from a heterologous promoter in response to both aldosterone and TA. Together these data indicate that the 21-base pair sequence represents a true MRE/GRE and that optimal activation of the human Na/K-ATPase β1 promoter is controlled by mineralocorticoid and glucocorticoid hormones. It appears that an interaction of MR with GR on the β1 promoter effectively down-regulates transcription.

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 (for review, see Refs. 1 and 2). Na/K-ATPase is composed of two subunits, the α subunit (113 kDa) which mediates the catalytic activity, and the smaller glycosylated β subunit (35 kDa) whose exact function is unclear (3). It has been proposed that it may be involved in the localization of the enzyme to the plasma membrane, protein folding, or stabilization of the K⁺-bound form of the enzyme (4). Na/K-ATPase is encoded by a multigene family and isoforms were described for both the α and β1 subunits (5–7). Expression of these isoforms occurs in a tissue-specific manner (8). While the α1 and β1 isoforms are expressed in most tissues, α2 is predominant in skeletal muscle and can be detected in the brain and heart, whereas α3 is found in excitable tissues. The β2 and β3 isoforms are mostly found in neural tissues (4).

Aldosterone and glucocorticoid hormones regulate the expression of the α and β subunits of the Na/K-ATPase genes (9, 10). This regulation by steroid hormones was shown to occur in a tissue-specific manner. Aldosterone but not dexamethasone activates Na/K-ATPase gene expression in cardiac cells while the reverse is observed in colonic cells, suggesting that both mineralocorticoid and glucocorticoid receptors (MR and GR) are involved in the regulation of the enzyme and non-receptor factors might be involved in conferring mineralocorticoid versus glucocorticoid specificity (11). MR is highly homologous to the GR (12, 13) which enhances expression of target genes by binding to specific promoter DNA sequences. These sequences consist in imperfect inverted hexanucleotide repeats separated by 3 nucleotides (GGTTAC NNNTGTTC), known as the glucocorticoid response element (GRE) (for review, see Refs. 14 and 15). MR was shown to bind, in vitro, to GRE sequences (14, 16), but no distinct mineralocorticoid response element (MRE) has ever been identified in genes induced by aldosterone.

In the current study, we examined the promoter region of the human Na/K-ATPase β1 gene subunit, for the presence of putative glucocorticoid or mineralocorticoid response elements (GRE or MRE). We also analyzed the regulation of Na/K-ATPase β1 gene promoter by MR or GR or coexpression of both receptors. This included both transcriptional and DNA binding studies. Our results show that, both MR and GR are able to activate the Na/K-ATPase β1 gene promoter. Using 5' deletion constructs and electrophoretic mobility shift assays, we identified a functional GRE/MRE at position −650 of the Na/K-ATPase β1 gene promoter for both glucocorticoid and mineralocorticoid. In addition we show that co-transfection of both receptors inhibits transcription from the Na/K-ATPase β1 gene promoter.

EXPERIMENTAL PROCEDURES

Materials—All hormones were purchased from Sigma. RU38486 was a gift from Roussel Uclaf (Romainville, France). Stock solutions were prepared in ethanol. Stock solutions were at 10⁻³ M for aldosterone and TA and at 10⁻² M for cortisol, RU38846, and spironolactone. 1 µl of hormone solution was mixed with 10 ml of cell culture medium for each experiment.

Plasmids—Construction of the luciferase plasmids containing the human Na/K-ATPase β1 gene promoter (pHβ1-1141Luc) or various...
Regulation of Na/K-ATPase β1 Gene Expression by MR and GR

lengths of the 5′-flanking region (pHβ1–726Luc, pHβ1–554Luc, pHβ1–414Luc, pHβ1–456Luc, pHβ1–327Luc, and pHβ1–833Luc) was described previously (17). A pHβ1CAT promoter vector was constructed. A double-stranded synthetic oligonucleotide β1MRE and β1mutMRE containing either a wild type sequence of the human Na/K-ATPase gene promoter from –662 to –628 (GGTTTGGCAATTGCTGTGAGTGGTCCAGG) or a deletion at bases –650 to –645, were inserted into a BgII site, upstream of the SV40 promoter in the pCAT promoter vector (Promega, Madison, WI). The structures of these plasmids called pHβ1MRE CAT and pHβ1mutMRE CAT were confirmed by sequencing. Expression plasmids for the human mineralocorticoid receptor (RshMR) and the human glucocorticoid receptor (RshGR) were a generous gift from R. M. Evans. The GRE TKCAT plasmid was provided by G. Schutz. β-Galactosidase expression plasmid (Rev-β-gal) and 7ZβI were purchased from Promega (Madison, WI).

Cell culture and transfection—CV-1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.), penicillin at 100 units/ml, and streptomycin at 100 µg/ml. Cells were washed 3 times with phosphate-buffered saline and 8 × 10⁶ cells were plated overnight on 100-mm dishes in duplicates, in Dulbecco’s modified Eagle’s medium supplemented with charcoal-stripped, steroid-free fetal calf serum. Cells were transfected by the calcium phosphate method with 10 µg of pHβ1–1141Luc containing the human Na/K-ATPase β1 gene promoter or various lengths of the 5′-flanking region fused to a promoterless luciferase gene in pGL3 reporter plasmid (Promega). For CAT analysis, CV-1 cells were transfected with 10 µg of the expression plasmid for the human MR (RshMR) or 1 µg of the human GR (RshGR) or 1 µg of each receptor expression plasmid along with 5 µg of β-galactosidase expression plasmid (Rev-β-gal) and 7ZβI carrier DNA to a total of 20 µg. Cells were incubated with DNA precipitates for 18 h, washed 3 times with phosphate-buffered saline and refed with fresh medium supplemented with 100 nM aldosterone and 1 µM RU 38486 in MR transfections; 100 nM triamcinolone acetonide (TA) and 1 µM spironolactone in GR transfections and 1 µM cortisol when both MR and GR were co-transfected. Forty-eight hours later, cells were harvested and cell extracts were assayed for luciferase activity.

For CAT analysis, CV-1 cells were transfected with 10 µg of β1 MRE CAT or β1 mut MRE CAT construct and 1 µg of RshMR or RshGR, or 1 µg of each receptor, along with 5 µg of Rev-β-gal and 7ZβI carrier DNA to a total of 20 µg. Following 24 h of hormone treatment as above, cell extracts were prepared and processed for CAT enzyme-linked immunosorbent assay analysis (Boehringer Mannheim).

Luciferase and CAT values were all normalized to β-galactosidase activity as follows. For each experiment, cells were co-transfected with equal amounts of β-galactosidase reporter plasmid (Rev-β-gal) and each transfection was carried out in duplicate. β-Galactosidase activity was evaluated for all samples by measuring the absorbance of the o-nitrophenol at 420 nm, produced by transformation of the substate o-nitrophenyl-β-D-galactopyranoside, by the β-galactosidase enzyme contained in cell extracts. Differences in the β-galactosidase activity were expressed as a fraction (highest activity divided by the other values). CAT values were then normalized to β-galactosidase activity by multiplying the luciferase or CAT value by the corresponding β-galactosidase ratio for the same transfection.

COS-1 cells were grown and transfected as described above, with 7 µg of receptor expression vector RshMR or RshGR, and 3 µg of 7ZβI plasmid. When both MR and GR were co-transfected, carrier DNA was supplemented to a total of 20 µg. Hormone incubations were conducted for 24 h as above and cells were harvested and pellets stored at –80 °C for preparation of whole cell extracts for in vitro DNA binding analysis.

Preparation of whole cell extract—for luciferase and CAT assays, cell extracts were prepared from CV-1 cells as recommended by the manufacturer (Promega and Boehringer Mannheim). Pellets from transfected COS-1 cells were thawed on ice and homogenized by 20 strokes in ice-cold buffer containing 20 mM Tris-HCl (pH 7.4), 600 mM KCl, 20% glycerol, 2 mM diithiothreitol, 1 mM phenylmethylsulfonfluoride, 5 µg/ml leupeptin, and 5 µg/ml aprotinin, using a glass homogenizer. The homogenate was centrifuged (75,000 × g at 4 °C) for 30 min to obtain whole cell extract.

Electrophoretic mobility shift assay—a double-stranded oligonucleotide corresponding to the wild type human Na/K-ATPase β1 gene promoter from position –662 to –628 GGGTTTGGCAATTGCTGTGAGTGGTCCAGG, or including different deletions (β1 mutMRE 1 to β1 mutMRE 5), were synthesized (Fig. 5A). A 31 oligomer consisting of two GREs (TGTACAGAGTTTTTC), separated by 16 base pairs, was obtained by excising the GRE 31 TR-CAT plasmid using BamHI and HindIII digestion. GRE 31 and β1 wt MRE were filled using the Klenow fragment (Boehringer Mannheim) and labeled by incorporating [α-32P]dCTP (NEN Life Science Products Inc.) and were used as probes. Whole cell extract of cells overexpressing MR or GR or both MR and GR (3 to 6 µg) were incubated on ice for 10 min in 10 mM Tris-HCl (pH 7.5), 80 mM KCl, 10% glycerol, 1 mM diithiothreitol, 1 mM poly(dI-dC), in a total volume of 18 µl. An anti-GR polyclonal antibody (αGR), directed against the DNA-binding domain, or an anti-MR polyclonal antibody (αMR) recognizing the N terminus and the DNA-binding domain, as well as a preimmune serum were included in reaction mixtures where indicated. A 50–200-fold excess of unlabelled oligonucleotide was used in competition reactions. 0.1 ng of 32P-end labeled GRE 31 or β1 MRE was added to the reaction and incubation was continued for 10 min at room temperature. Reaction mixtures were applied to a 4% non-denaturing polyacrylamide gel and DNA-protein complexes were resolved by electrophoresis (250 mV at 4 °C), with buffer recirculation in 1× TAE (6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, 1 mM EDTA). The gel was dried under vacuum and autoradiographed at –80 °C.

RESULTS

Both Aldosterone and Glucocorticoid Induce Na/K-ATPase β1 Gene Promoter Activity—To analyze regulation of the Na/K-ATPase β1 promoter by mineralocorticoid and glucocorticoid, CV-1 cells were transiently transfected with a reporter plasmid containing the human β1 gene promoter, spanning nucleotides –1141 to +490, linked to the luciferase gene (pHβ1–1141Luc). An expression vector for the human MR (RshMR) or the human GR (RshGR) or both MR and GR were co-transfected in CV-1 cells. These cells are known to express very little if any endogenous MR and GR.

In MR experiments, treatment of the transfected cells with 100 nM aldosterone for 48 h, resulted in a 17-fold increase in the activation of the luciferase gene by the β1 full-length promoter (FLP) (Fig. 1). In cells transfected with GR, 100 nM TA induced a 23-fold increase in luciferase activity, indicating that GR is a somewhat weaker transactivator of the β1 gene promoter than MR (Fig. 1). Interestingly, co-transfection of both receptors, followed by treatment with 1 µM cortisol, which activates both MR and GR, induced only a 2-fold increase in activation (Fig. 1). These data indicate that together MR and GR have an inhibitory effect on activation of the human Na/K-ATPase β1 gene promoter.

To identify cis-acting elements involved in MR/GR induced activation, we carried out a detailed examination of the 5′-flanking region of the β1 gene subunit for the presence of GRE-like sequences. Three potential response elements were

FIG. 1. Activation of the Na/K-ATPase β1 gene full-length promoter by MR and GR. CV-1 cells were transfected by the calcium phosphate method with 10 µg of pHβ1–1141-Luc reporter plasmid, containing the human Na/K-ATPase β1 gene full-length promoter (–1141 to +490) (shaded box, FLP) and 1 µg of the expression plasmid for the human MR (RshMR) or 1 µg of the human GR (RshGR), or 1 µg of each receptor expression plasmid along with 5 µg of β-galactosidase expression plasmid (Rev-β-gal) and 7ZβI carrier DNA to a total of 20 µg. Shaded box, control. Cells were induced with 100 nM aldosterone and 1 µM RU 38486 in MR transfections, 100 nM TA and 1 µM spironolactone in GR transfections, and 1 µM cortisol when both MR and GR were co-transfected. Forty-eight hours later, cells were harvested and cell extracts were assayed for luciferase activity. Shown is an average of three experiments and all values are normalized to β-galactosidase activity.
FIG. 2. Sequence of the 5'-flanking region of the human Na/K-ATPase β1 gene and structure of β1 5' deletion constructs upstream of the luciferase gene. A, the sequence is numbered relative to the transcriptional start at position +1 (arrow). Half-sites of three potential MRE/GREs containing similar sequences to those found in GRE-binding sites, are underlined. B, the full-length β1 gene promoter and various lengths of the 5'-flanking region fused to a promoterless luciferase gene in pOLuc reporter plasmid are represented. The plasmids are named based on the end point of the β1 gene region in each 5' deletion luciferase construct. Three potential MRE/GREs are indicated with bars labeled with nucleotide position: −1048, −650, and 276.
identified at position −1048, −650, and −276 relative to the transcriptional start (Fig. 2A), containing consensus binding sequences for GR (GRE). The first two sequences contain two half-binding sites separated by 10 and 9 base pairs, respectively, whereas the last one consists of a single half-site (Fig. 2A). To determine the functionality of these potential response elements and their specificity for MR versus GR, luciferase plasmids containing various lengths of the 5′-flanking region of the human Na/K-ATPase β1 gene (Fig. 2B) were generated and transfected into CV-1 cells, along with the MR and/or GR expression plasmids.

In MR co-transfection experiments, plasmid with an end point at position −276 (726-Luc) induced an 8-fold increase in luciferase activity following treatment with 100 nM aldosterone (Fig. 3A). This activation consists of half of that induced by the full-length promoter construct (1141-Luc). Deletion of an additional 172-base pair in the 554-Luc plasmid resulted in a 4-fold reduction in transcriptional activation. Finally, deletion of the remaining sequence in the 83-Luc construct abolishes the hormonal response (Fig. 2A). These data suggest that the deleted intervals in the various constructs contained potential functional elements, including the GRE-like sequences, responsible for the activation of the β1 promoter by aldosterone.

Similar results were obtained in GR co-transfection experiments (Fig. 3B). Plasmids with end point at position −726 and −554 (726-Luc, 554-Luc) induced 10- and 5-fold increase in luciferase activity, respectively, following treatment with 100 nM TA (Fig. 3B). The construct with an end point at −83 (83-Luc) did not retain hormone inducibility (Fig. 3B). These results suggest that sequences deleted from these intervals, including the putative GREs, may constitute positive regulatory elements contributing to the activation of the β1 promoter by GR.

In addition, a luciferase plasmid containing the full-length promoter including an internal deletion (FLP*643–327) abolishing the sequence from position −643 through −327, was analyzed in the presence of MR or GR. In MR experiments, this construct induced a 2-fold increase in luciferase expression (Fig. 3A) and a 4-fold activation was obtained in GR experiments (Fig. 3B). This result reflects a significant decrease in promoter activity when compared with that of the full-length promoter (Fig. 1), suggesting that the deleted interval (−643 to −327) includes important positive cis-acting elements. Interestingly, deletion constructs such as 554-Luc, 456-Luc, and 327-Luc containing most of this interval (643–327), but not the GRE-like sequence at position −650, induced, similar to the FLP*643–327-Luc construct, 2–5-fold increases in luciferase expression but were not capable of re-establishing promoter activity to a higher level such as that of 726-Luc. Together these results indicate that the GRE/MRE sequence at position...
Fig. 4. Specificity of binding of β1 MRE to the human MR. A, a 32P-labeled double-stranded synthetic oligonucleotide β1 MRE (−662 to −628) was incubated with whole cell extracts from nontransfected COS-1 cells (lane 1) or cells overexpressing human MR and treated with 100
−650 plays an important role in conferring hormone responsiveness and that other elements including the GREs at position −1048 and −276 are necessary for optimal activation of β1 promoter by aldosterone and glucocorticoid.

Co-transfection of both MR and GR, followed by treatment with 1 µM cortisol, resulted in a 2–3-fold increase in luciferase activity with the variant deletion constructs (Fig. 3C). The inhibition of promoter activity was similar to that obtained with the full-length promoter (Fig. 3C) suggesting that MR and GR are inhibitory to each other when they are coexpressed. To examine the ability of cortisol to activate the β1 promoter via MR and GR, CV-1 cells were co-transfected with the full-length promoter (−1141–Luc) or various deletion constructs in the presence of MR or GR and cells were treated with 100 nM cortisol. These treatments induced significant luciferase activation by the full-length promoter for both MR and GR (data not shown). This activation was lower than that induced by the more specific ligands aldosterone and TA when used in MR and GR experiments, respectively.

To examine whether the inhibition of promoter activity observed in the presence of both receptors and cortisol is ligand specific, CV-1 cells were co-transfected as above in the presence of both MR and GR and cells were treated with 100 nM aldosterone plus 100 nM TA. In these conditions, the full-length promoter induced a 5-fold increase in luciferase gene expression, while 1–3-fold increases were observed with the different deletion constructs (Fig. 3D). These data indicate that activation by the β1 promoter, in the presence of both MR and GR and their specific ligands, aldosterone and TA, is 2-fold higher than that induced in the same conditions by cortisol. However, these results confirm that coexpression of MR and GR inhibits gene expression.

Mineralocorticoid Receptor Binds to the Na/K-ATPase β1 MRE/GRE (−650 to −630) in Vitro—Based on our observation of a 4-fold reduction in hormone inducibility of the β1 promoter upon deletion of the putative binding site at position −650 in the presence of MR but not with GR, it appears to function as a mineralocorticoid response element. To examine DNA-binding properties of MR to the β1 promoter −650 to −630, human MR was transfected into COS-1 cells and whole cell extract was prepared following 2 h treatment with 100 nM aldosterone. Electrophoretic mobility shift assay was performed using 3 µg of whole cell extracts and the β1 MRE as a probe. Unlike the wild type oligonucleotide (β1 wt MRE), none of the mutated sequences were able to compete effectively for DNA binding, when a 200-fold excess of cold mutated oligonucleotide (β1 mut MRE) was included in gel shift reactions (Fig. 5B), suggesting that optimal binding of MR requires both half-sites and the NF1 site, in total spanning at least 28 base pairs.

Protein-DNA Complexes Contain Both MR and GR When Coexpressed—To examine protein binding to the β1 MRE (−662 to −628) in the presence of both receptors, human MR, GR, or both MR and GR were transfected into COS-1 cells. Cell extracts were prepared following 2 h induction with 100 nM TA or 1 µM cortisol, respectively. Electrophoretic mobility shift assay was performed using 3 µg of whole cell extracts and the β1 MRE as a probe. In extracts of cells overexpressing GR, two classes of DNA-protein complexes were formed on the β1 MRE in vitro, similar to that seen for MR (Fig. 6). Protein binding to the β1 MRE was eliminated when a GR antibody was included in the gel shift reaction, and was unaffected in the presence of a nonspecific serum. In the presence of both receptors, extracts from cells transfected with both MR and GR (3 µg), or in vitro mixtures of equal amounts of protein extracts prepared from cells overexpressing one of the two receptors (3 µg total), show no difference in DNA binding pattern, when compared with the binding of receptors expressed separately. Addition of MR antibody (α-MR) or GR antibody (α-GR) to reaction mixtures resulted in band shifting and elimination of DNA binding, respectively, indicating the presence of both MR and GR in the protein-DNA complexes formed in vitro, when both receptors are present (Fig. 6).

Despite the absence of modifications in the binding pattern, aldosterone (lanes 2–7). Competition analysis of the of the β1 MRE binding (arrow) was assessed by incubating cell extracts 10 min prior to the addition of the probe with a 50- or 200-fold excess cold β1 MRE (lanes 3 and 4) or with a 50- or 200-fold excess cold GRE 31 (lanes 6 and 7). All lanes received 6 µg of protein. B, a 32P-labeled GRE 31 oligonucleotide containing two GRE-binding sites separated by 16 base pairs was incubated with whole cell extracts from COS-1 cells overexpressing human MR, treated with 100 nM aldosterone. Prior to the addition of the probe, a 50-, 100-, or 200-fold excess of unlabeled β1 MRE oligonucleotide was added to the gel shift reaction (lanes 2–4, respectively). The star symbol (*) indicates nonspecific binding and arrows indicate specific competed binding. C, a polyclonal antibody raised against the N terminus and the DNA-binding domain of human MR, αMR (lane 2), or a preimmune nonspecific serum (lane 3) was incubated with whole cell extracts from COS-1 cells overexpressing human MR and treated with 100 nM aldosterone, 10 min prior to the addition of the β1 MRE probe. Lane 1 contained a buffer control and lane 4 the free probe (FP). The arrow indicates the antibody mediated shift.
we observed in a reproducible manner, a reduction in the DNA binding of coexpressed receptors when compared with the binding of receptors expressed separately. Densitometric analysis of DNA binding, indicated as upper and lower bands, revealed 50–60% reduction in protein-DNA binding of coexpressed MR and GR for both bands, when compared with the binding of MR or GR expressed individually (Fig. 6, B and C, MR/GR bars). Interestingly, in vitro MR+GR mixtures show 35% reduction in binding corresponding to the lower band but no changes in the upper band, when compared with GR (Fig. 6C, MR+GR bars). However, little or no reduction in the binding corresponding to both bands was observed for in vitro mixtures of MR and GR when compared with the MR binding (Fig. 6B, MR+GR bars). These results suggest that MR/GR heterodimers formed in vivo bind to the β1 MRE with lower affinity in comparison with MR/GR or GR homodimers. In addition, differences in the reduction of the upper and lower bands observed between in vivo coexpressed MR/GR and in vitro mixtures of MR+GR may reflect variations in the formation and equilibrium of the MR/GR heterodimer under different conditions.

Mineralocorticoid and Glucocorticoid Activation of the Putative β1 MRE/GRE and Its Mutant Upstream of the SV40 Promoter—To determine whether the sequence extending from −650 to −628 of the Na/K-ATPase β1 gene relative to the transcriptional start constituted a functional mineralocorticoid and glucocorticoid regulatory element, a double-stranded oligonucleotide corresponding to this sequence was inserted into a BglII site, upstream of the SV40 promoter in the CAT promoter vector (pHB1MRE CAT). A mutated oligonucleotide was cloned, also, into the site. CV-1 cells were transfected with either β1MRE CAT construct or β1mutMRE CAT along with MR, GR, or both MR and GR and treated with 100 nM aldosterone or TA or 1 μM cortisol, respectively. In MR experiments, CAT activity was stimulated 3-fold in CV-1 cells transfected with MR and pHβ1MRE CAT. No induction was obtained with MR and the mutated MRE containing a deletion in the left consensus half-site (Fig. 7A). Following treatment with 100 nM TA, GR stimulated a 2-fold induction of CAT activity (Fig. 7B). However, coexpression of both MR and GR in the presence of 1 μM cortisol resulted in no induction of the CAT gene expression (Fig. 7B). These results demonstrate that the sequence from −662 to −628 of the Na/K-ATPase β1 gene contains a potential MRE/GRE that is sufficient for mineralocorticoid and glucocorticoid responsiveness in a heterologous promoter when MR and GR are expressed independently and that coexpression of both MR and GR leads to inhibition of transcription.

**DISCUSSION**

The mineralocorticoid receptor mediates aldosterone-induced regulation of sodium transport. It is well established that the α and β isoforms of the Na/K-ATPase gene family are regulated by corticosteroid hormones in a tissue-specific manner, at both the mRNA and protein levels (9–11), however, the molecular mechanism of this regulation and its tissue specificity is still to be elucidated. In the current study, we investigated the transactivation of the Na/K-ATPase β1 gene promoter by the mineralocorticoid and glucocorticoid receptors. To locate DNA sequences required for hormone transactivation,

---

**Fig. 5.** Effect of 5′ and 3′ deletions in the β1 MRE on DNA binding competition to the human MR. A, the wild type sequence of the human Na/K ATPase β1 gene subunit from −662 to −628, denoted β1 wt MRE contains consensus sequences of GREs organized in two-half sites separated by 9-base pair and a binding sequence for NF1. Deletions were introduced at one of the half GRE-like sequences or at the NF-binding site (β1 mutMRE1, β1 mutMRE 2, β1 mutMRE 3), or at one of the half GRE-like sequences and at the NF-binding site simultaneously (β1 mutMRE 4, β1 mutMRE 5). B, a 32P-labeled wild type β1 MRE was incubated with 6 μg of whole cell extracts from COS-1 cells overexpressing human MR treated with 100 nM aldosterone. Lane 1 did not include any competitor. Lane 2 whole cell extracts incubated with a 200-fold excess of unlabeled wild type β1 MRE 10 min before the addition of the probe. Lanes 3–7 received a 200-fold of one of the five deleted MREs (β1 mutMRE1 to β1 mutMRE5).
we carried out a detailed analysis of the promoter and used 5′ deletions of the flanking region of the β1 gene fused to the luciferase reporter gene.

This approach allowed us to identify three potential sequences containing consensus binding sequences for GR (GRE) at positions −2104, −2650, and −2276, relative to the transcriptional start. Two of the sequences contained two half-binding sites, whereas the last one consisted of a single half-site at position −2276. Using 5′ deletions of the promoter region, MR and GR activation was shown with deletion constructs containing the MRE/GREs at position −650 and −276, suggesting that they are responsive both to aldosterone and the glucocorticoid TA. This activation was 50% lower than that induced by the full-length promoter construct indicating that the potential MRE at position −1048 may be functional. Importantly, 80–90% of promoter activation via MR and GR were lost with a deletion construct containing the full-length promoter including an internal deletion abolishing the left half-site of the MRE/GRE at position −650. This suggests an important role for this sequence in hormone responsiveness. Although a 3-fold activation was observed with constructs containing the single half-binding site at position −276, luciferase gene activation decreased dramatically when compared with that of constructs including the full site sequences at positions −1048 and −650. This observation is consistent with the current hypothesis for requirement of two copies of half-sites, to which GR and MR bind as homodimers, to generate a functional GRE. Presence of multiple GREs in the promoter of genes regulated by GR has been described and the transcriptional increase observed in our system when three or two of these sequences are present is in agreement with the known role played by multiple GREs in the amplification of the steroid hormone signal by synergy (18).

Functionality of one of these potential MRE/GREs as minimal sequences conferring mineralocorticoid and/or glucocorti-
gene promoter and vice versa. Inhibition of GR induced transcriptional activity by MR has been reported in a different regulatory context supporting the idea that MR-GR heterodimers are less efficient activators than GR-GR homodimers (19, 23). It has been proposed that a region within the N terminus of MR disrupts GR self-synergy in MR-GR heterodimers. In our system, inhibition of GR induced transcriptional activity of the β1 gene promoter by MR may represent a mechanism for differential regulation of the Na/K-ATPase by glucocorticoid and mineralocorticoid in different tissue contexts.

To determine whether activation of the Na/K-ATPase β1 gene promoter by mineralocorticoid involves a direct interaction between MR and the putative cis-elements identified in the flanking region, we examined the ability of the potential MRE/GRE sequence at position −650 to bind MR in mobility shift assays. We demonstrate that activated human MR binds specifically to the palindromic β1MRE (−662 to −628) and two protein-DNA complexes were formed. Affinity of β1 MRE for MR was similar to that of a double consensus GRE oligomer. The observation of a supershift in the bands when a polyclonal antibody against human MR (α-MR) is included, demonstrates that the latter complexes indeed contained MR. Formation of multiple protein-DNA complexes in vitro has been previously reported for MR and GR and reflect existence of monomeric and dimeric forms of MR bound to the DNA (20). Using whole cell extracts prepared from COS-1 cells overexpressing GR or both MR and GR, induced with 100 nM α-agonist or 1 μM cortisol, respectively, immuno-binding assays show, as expected, that GR binds to the Na/K-ATPase β1 MRE and further demonstrate the presence of both MR and GR in protein-DNA complexes formed in vitro. These data indicate that a direct interaction of the β1 gene promoter with MR and GR is involved in the regulation of the Na/K-ATPase gene by mineralocorticoids and glucocorticoids.

The specificity of mineralocorticoid hormone action remains unclear (24). Despite the fact that some physiological studies show differences in the effect mediated by MR and GR, few hypothesis for specific mineralocorticoid regulation have been established by molecular studies. Steroid hormones were shown to regulate the expression of Na/K-ATPase genes in a tissue-specific manner. Aldosterone but not the glucocorticoid dexamethasone induces Na/K-ATPase gene expression in cardiac cells, while the reverse is observed in colonic cells (11, 25), suggesting that both hormone receptors are involved in the regulation of the enzyme and that non-receptor factors might be involved in conferring mineralocorticoid versus glucocorticoid specificity. Tissue-specific factors may include other transcription factors. It is known that steroid receptors can bind to composite GREs containing binding sites both for the receptor and for other transcription factors. Differential regulation by MR and GR was shown at pIFG, a composite AP1/GRE site (26, 27). In the present study, we identified a putative NF1-binding site at position −657, upstream of the β1 MRE, indicating that NF1 may play a role in the regulation of Na/K-ATPase gene by MR and GR. Tissue-specific factors may also include members of the recently described family of steroid receptor coactivators and corepressors such as SRC1-a and GRIP1, N-COR and SMRT, which might be involved in conferring mineralocorticoid versus glucocorticoid specificity and their roles remain to be evaluated in the regulation of the Na/K-ATPase β1 gene promoter (28–30).

Acknowledgments—We express our appreciation to Dr. Gwen Gilinger and Michelle Croyle for their help with this work.
REFERENCES
1. Verrey, F., Beron, J., and Spindler, B. (1996) Mineral. Electrolyte Metab. 22, 279–292
2. Ewart, H. S., and Klip, A. (1995) Am. J. Physiol. C295–C311
3. Horisberger, J. D., Leman, V., Kraehenbuhl, J. P., and Rosier, B. C. (1991) Annu. Rev. Physiol. 53, 565–584
4. Lingrel, J. B, Van Huysse, J., O’Brien, W., Jewell-Motz, E., and Schultheis, P. (1994) Rev. Physiol. Biochem. 17, 198–200
5. Sweadner, K. J. (1989) Biochim. Biophys. Acta 988, 185–220
6. Lane, L. K., Shull, M. M., Whitmer, K. R., and Lingrel, J. B (1989) Genomics 5, 445–453
7. Lingrel, J. B (1992) J. Bioenerg. Biomembr. 24, 263–270
8. Munzer, J. S., Daly, S. E., Jewell-Motz, E. A., Lingrel, J. B, and Blostein, R. (1994) J. Biol. Chem. 269, 16668–16676
9. Whorwood, C. B., Ricketts, M. L., and Stewart, P. M. (1994) Endocrinology 135, 901–910
10. Whorwood, C. B., and Stewart, P. M. (1995) J. Mol. Endocrinol. 15, 93–103
11. Ikeda, U., Hyman, R., Smith, T. W., and Medford, R. M. (1991) J. Biol. Chem. 266, 12058–12066
12. Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E., and Evans, R. M. (1987) Science 237, 268–275
13. Evans, R. M., and Arriza, J. L. (1988) Neuron 3, 1105–1112
14. Tsai, M.-J., and O’Malley, B. W. (1994) Annu. Rev. Biochem. 63, 451–486
15. Weigel, N. L. (1996) Biochem. J. 319, 657–667
16. Alnemri, E. S., Maksymowycz, A. B., Robertson, N. M., and Litwack, G. (1991) J. Biol. Chem. 266, 18072–18081
17. Feng, J., Orlowski, J., and Lingrel, J. B (1993) Nucleic Acids Res. 21, 2619–2626
18. Jantien, H. M., Stahlle, U., Glass, B., Stewart, F., Schmid, W., Boshart, M., Mikesicke, R., and Schutz, G. (1987) Cell 49, 39–48
19. Liu, W., Wang, J., Sauter, N. K., and Pearce, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12480–12484
20. Liu, W., Wang, J., Yu, G., and Pearce, D. (1996) Mol. Endocrinol. 10, 1399–1406
21. Tsai, S. Y., Carlstedt-Duke, J., Weigel, N. L., Dahlman, K., Gustafsson, J. A., Tsai, M. J., and O’Malley, B. W. (1988) Cell 55, 361–369
22. Trapp, T., Rupprecht, R., Castren, M., Reul, J. M., and Holshoer, F. (1994) Neuron 13, 1457–1462
23. Bamberger, C. M., Bamberger, A. M., Wald, M., Chrousos, G. P., and Schulte, H. M. (1997) J. Steroid Biochem. Mol. Biol. 60, 43–50
24. Funder, J. W. (1990) Trends Endocrinol. Metabol. 1, 145–148
25. Orlowski, J., and Lingrel, J. B (1990) J. Biol. Chem. 265, 3462–3470
26. Diamond, M. I., Miner, J. N., Yoshinaga, S. K., and Yamamoto, K. R. (1990) Science 249, 1266–1272
27. Pearce, D., and Yamamoto, K. R. (1993) Science 259, 1161–1165
28. Onate, S. A., Tsai, S. Y., Tsai, M. J., and O’Malley, B. W. (1995) Science 270, 1354–1357
29. Hong, H., Kohli, K., Garabedian, M. J., and Staluppi, M. R. (1997) Mol. Cell. Biol. 17, 2735–2744
30. Harwitz, K. B., Jackson, T. A., Bain, J. K., Richer, J. K., Takimoto, G. S., and Tung, L. (1996) Mol. Endocrinol. 10, 1167–1177

Regulation of Na/K-ATPase β1 Gene Expression by MR and GR 20711