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Cistrome of the aldosterone-activated mineralocorticoid receptor in human renal cells

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ABSTRACT Aldosterone exerts its effects mainly by activating the mineralocorticoid receptor (MR), a transcription factor that regulates gene expression through complex and dynamic interactions with coregulators and transcriptional machinery, leading to fine-tuned control of vectorial ionic transport in the distal nephron. To identify genome-wide aldosterone-regulated MR targets in human renal cells, we set up a chromatin immunoprecipitation (ChIP) assay by using a specific anti-MR antibody in a differentiated human renal cell line expressing green fluorescent protein (GFP)-MR. This approach, coupled with high-throughput sequencing, allowed identification of 974 genomic MR targets. Computational analysis identified an MR response element (MRE) including single or multiple half-sites and palindromic motifs in which the AGTACAGxatGTtCt sequence was the most prevalent motif. Most genomic MR-binding sites (MBSs) are located >10 kb from the transcriptional start sites of target genes (84%). Specific aldosterone-induced recruitment of MR on the first most relevant genomic sequences was further validated by ChIP-quantitative (q)PCR and correlated with concomitant and positive aldosterone-activated transcriptional regulation of the corresponding gene, as assayed by RT-qPCR. It was notable that most MBSs lacked MREs but harbored DNA recognition motifs for other transcription factors (FOX, EGR1, AP1, PAX5) suggesting functional interaction. This work provides new insights into aldosterone MR-mediated renal signaling and opens relevant perspectives for mineralocorticoid-related pathophysiology.—Le Billan, F., Khan, J. A., Lamribet, K., Viengchareun, S., Bouligand, J., Fagart, J., Lombès, M. Cistrome of the aldosterone-activated mineralocorticoid receptor in human renal cells. *FASEB J.* 29, 000–000 (2015). www.fasebj.org

Key Words: chromatin immunoprecipitation sequencing • sodium • nuclear receptor • transcription factor

Abbreviations: αENaC, epithelial sodium channel, α-subunit; AP-1, activated protein 1; AR, androgen receptor; BSA, bovine serum albumin; CCD, convoluted collecting duct; ChIP, chromatin immunoprecipitation; ChIP-seq, sequencing of ChIP-enriched fragments; DBD, DNA-binding domain; EGR1, early growth response protein 1; *ERGIC1*, endoplasmic reticulum-

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ALDOSTERONE, THE MAIN MINERALOCORTICOID HORMONE in humans, plays a critical role in maintaining sodium homeostasis by regulating sodium reabsorption within the distal collecting duct of the nephron (1). This steroid hormone primarily exerts its genomic effects by binding to and activating the mineralocorticoid receptor (MR), a ligand-induced transcription factor belonging to the nuclear receptor superfamily (2). In the absence of hormone, MR has been shown to be located mainly in the cytoplasm, as a part of a heat shock protein (HSP)-containing heterooligomeric complex. Aldosterone binding to MR leads to a conformational change in the receptor, triggering its nuclear translocation. Thus, it has been proposed that the aldosterone-MR complex binds, as a dimer, to specific DNA sequences, so-called hormone response elements (HREs), located in the regulatory sequences of target genes. Subsequent recruitment of the basal transcriptional machinery, as well as several transcriptional coregulators, leads to fine-tuned regulation of gene transcription.

Altered mineralocorticoid signaling pathways have been documented in numerous human diseases, such as arterial hypertension and cardiovascular and kidney diseases. Thus, a better understanding of renal mineralocorticoid signaling requires identification of aldosterone-regulated genes and characterization of fine-tuned molecular mechanisms of gene transactivation. During the past decades, intensive research on MR target genes in several mineralocorticoid-sensitive tissues or cells has led many laboratories to develop various experimental approaches, including SAGE (3), microarrays (4), and transcriptomics (5). These approaches aided in the description of important aldosterone-regulated effectors, most notably those involved in sodium reabsorption in animal and cell models. For instance, the transcriptional regulation of classic MR target genes, such as sodium channel, non-voltage-gated 1

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alpha (*SCNN1A*), encoding for the α -subunit of the epithelial sodium channel (α ENaC), was first identified in the distal convoluted tubule (6) and was subsequently studied by measuring the activity of a promoter construct containing MR response elements (MREs) linked to a reporter gene (7). Afterward, SAGE was performed on aldosterone-treated cells from the distal convoluted tubule and identified new aldosterone target genes, such as serum glucocorticoid kinase 1 (*SGK1*) and glucocorticoid-induced leucine zipper (*GILZ*) (3, 8). More recently, Fakitsas *et al.* (4) identified 9 new aldosterone-activated genes, including ubiquitin-specific protease 2-45 (*USP2-45*), by using a DNA microarray approach on complementary DNAs obtained from mouse distal nephron segments. Another group identified and characterized the gene connector enhancer of kinase suppressor of ras 3 (*CNKSR3*), as an aldosterone target with renal activity, by using chromatin immunoprecipitation (ChIP) and reporter-coupled mutated promoter approaches (9). All these results were validated at the transcriptional level by quantitative (q) PCR and Northern blot analysis and at the protein level by Western blot analysis. These new approaches have decidedly improved understanding of the mechanistic aspects of the renal MR pathway and have integrated that knowledge into the understanding of the mechanisms of transepithelial electrolytic transport.

In an effort to undertake a more comprehensive assessment of MR genomic targets in the human kidney, we took advantage of the ChIP assay followed by deep high-throughput sequencing (ChIP-seq). For the first time, we applied ChIP-seq analysis to acute aldosterone exposure to identify the genome-wide MR-binding regions in a human aldosterone-sensitive renal cell line (10) using a recently generated anti-MR antibody (11). Thus, we set up an exhaustive genomic mapping of MR binding sites (MBSs) on approximately 1000 sequences, allowing extensive description of a consensus MRE motif. Furthermore, novel aldosterone target genes were identified correlating genomic MR occupancy and target gene transactivation. This first ChIP-Seq analysis on human cells and appropriate

experimental tools undoubtedly helped deepen our understanding of renal mineralocorticoid signaling and provided new insights into how MR participates in the hormonal control of the ion vectorial transport.

MATERIALS AND METHODS

Aldosterone, protease inhibitor cocktail (PIC), and Laemmli buffer were purchased from Sigma-Aldrich (Lyon, France). DMEM high glucose with L-glutamine medium, trypsin, G418, Fast SYBR Green Master Mix buffer, random primers, dNTP, and Superscript reverse polymerase were purchased from Life Technologies (Saint-Aubin, France). PBS and penicillin-streptomycin solution were purchased from GE Healthcare (Vélizy-Villacoublay, France). Western blot equipment was purchased from Bio-Rad (Marnes-la-Coquette, France).

Purified rabbit polyclonal 39N antibody was raised against the first amino acids (1–18) at the N terminus of the human MR (11). Rabbit anti-SRC1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), mouse anti-polymerase II antibody was from Millipore (Paris, France), and control unrelated antibody was from the HighCell# ChIP kit (Diagenode, Seraing, Belgium).

Cell Culture

Human kidney GFP-hMR cells or parental cells [HK-GFP-MR or HK (10, 12)] were routinely cultured at 37°C, in a humidified incubator gassed with 5% CO₂, seeded on Petri dishes (BP100) with 10 ml of DMEM (high-glucose medium with L-glutamine) containing 2.5% fetal bovine serum (FBS) (Biowest, Courtaboeuf, France), 100 U/ml penicillin, and 100 μ g/ml streptomycin. For the HK-GFP-MR cells, the medium was supplemented with geneticin (200 μ g/ml).

ChIP

Five days before the experiments, geneticin was removed from the cell medium. Twenty-four hours before hormonal treatment, complete medium was removed, HK-GFP-MR cells were washed twice with 5 ml PBS and incubated with steroid-free medium (dextran-coated charcoal medium), containing 2.5% dextran-coated charcoal-treated FBS. The next day, the cells were treated for 1 h at 37°C with 100 nM aldosterone or the same amount of vehicle (ethanol).

Treated cells were fixed with 1% paraformaldehyde (Electron Microscopy Sciences, Saint-Germain-en-Laye, France) for 10 min at room temperature. Paraformaldehyde fixation was quenched with 0.125 M glycine for 8 min. The cells were washed twice with ice-cold PBS. Cell lysis and chromatin shearing were performed with the HighCell# ChIP kit (Diagenode) buffers, according to the manufacturer's recommendations, with the following modifications: chromatin shearing was performed in 500 μ l shearing buffer S1+protease inhibitor mix, applying 2 runs of 10 cycles at high intensity (Bioruptor; Diagenode). Each cycle was composed of 30 s with effective application of ultrasounds (ON) and 30 s without (OFF). Between each run, heated water was replaced by ice-cold water and ice. Concentrations of sheared chromatin protein were measured, using the bicinchoninic acid assay (Interchim, Montluçon, France) on a spectrophotometer Victor3 (Perkin Elmer, Courtaboeuf, France), in parallel with a bovine serum albumin (BSA) standard range (Interchim).

Protein A-coated magnetic beads (25 μ l of the stock suspension) were washed 3 times in buffer C1 and resuspended in C1 buffer supplemented with 1% PIC (100 μ l final volume). Sheared chromatin (total amount of 1.3 mg protein) and 7 μ g of one of the tested antibodies were then added to the beads and the final volume was adjusted to 500 μ l with C1 buffer supplemented with

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Golgi intermediate compartment; *FAM65B*, family with sequence similarity 65, member B; FBS, fetal bovine serum; *FKBP5*, FK506 binding protein; FOX, forkhead box; GR, glucocorticoid receptors; GRE, glucocorticoid response element; HRE, hormone response element; HSP, heat shock protein; IGV, integrative genome viewer; *LINC00963*, long intergenic non-protein-coding RNA 963; MACS, model-based analysis for ChIP-seq; MBS, MR-binding site; MR, mineralocorticoid receptor; MRE, mineralocorticoid response elements; *NEATC2*, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2; *NFKB1A*, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; PAX5, paired box protein 5; *PER1*, period circadian protein homolog 1; PIC, protease inhibitor cocktail; PR, progesterone receptor; *PTPN1*, protein tyrosine phosphatase, non-receptor type 1; qPCR, quantitative PCR; RNA pol II, RNA polymerase II; *SCNN1A*, sodium channel, non-voltage-gated 1 alpha; *SERPINE1*, serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1; *SGK1*, serum glucocorticoid kinase 1; SRC1, steroid receptor coactivator 1; TBS-T, Tris-buffered saline-Tween; *TPRGL1*, tumor protein p63 regulated 1; TSS, transcription start site; *ZBTB16*, zinc finger and BTB domain containing 16; *ZMYND8* zinc finger, MYND-type containing 8

1% PIC and 0.1% BSA (final concentrations). Samples were incubated overnight at 4°C with a rotary incubator. Magnetic beads were then isolated and washed 3 times with C1 buffer and once with W1 buffer. DNA fragments from the immunoprecipitated chromatin were eluted with the DNA isolation buffer supplemented with 1% proteinase K for 15 min at 55°C and finally for 15 min at 100°C. Isolated DNA fragments were then sequenced (ChIP-Seq) or quantified by real-time qPCR.

cDNA libraries and ChIP sequencing

Library preparation and Illumina (San Diego, CA, USA) sequencing were performed at the Ecole Normale Supérieure Genomic Platform (ENS, Paris, France). Libraries were prepared by using NEXTflex ChIP-Seq Kit (Bioo Scientific, Saint Marcel, France), with 1 ng IP or input DNA. The libraries were multiplexed by 6 on 1 flow cell lane. A 50 bp read sequencing was performed on a HiSeq 1500 device (Illumina).

ChIP-seq bioinformatics analysis and data managing

The analyses were performed with the Eoulsan pipeline (13), including read filtering and mapping, alignment filtering, and formatting. Before mapping, poly-N read tails were trimmed, reads ≤ 11 bases were removed, and reads with quality mean ≤ 12 were discarded. Reads were then aligned against the Human Genome version 19 (hg19) annotation [Genome Reference Consortium; National Center for Biotechnology Information (NCBI), Bethesda, MD, USA] using Bowtie (ver. 0.12.9) (14). Alignments from reads matching more than once on the reference genome were removed by using the Java version of SAMtools (15). PCR duplicates were filtered out with the MarkDuplicates tool of the Picard suite (<http://broadinstitute.github.io/picard/>; Broad Institute, Cambridge, MA, USA) and the peak calling step was performed with Model-based Analysis for ChIP-seq (MACS; ver. 1.4.2) (16). The raw fastq files are available on the GEO repository (www.ncbi.nlm.nih.gov/geo/; NCBI; accession number in progress).

Sequencing data were analyzed with the MACS software (17), available on the online platform Nebula (Curie Institute, Paris). MACS aligned all the read sequences on the reference hg19. This alignment allowed comparison between the DNA fragments obtained from aldosterone-treated and vehicle samples, permitting selection of the most significant MR genomic fixation sites (peaks). A peak was considered significant when it differed from the background signal with $P \geq 10^{-5}$. For each site, MACS calculated a score that corresponds to $-10\log(P)$. MACS classified the significant sites on a spreadsheet containing related information [chromosome, position, score, name of the nearest gene, distance from transcription start site (TSS), and type region]. The Integrative Genomic Viewer software (IGV) was used to visualize the DNA fragment alignment on hg19 and to select the DNA sequence of the significant sites. Identification of the MRE within significant ChIP-seq peaks was performed using Jaspar (<http://jaspar.genereg.net/>) (18) and FIMO (<http://meme-suite.org/tools/fimo>) software. The resemblance between the identified MRE was monitored by using the Weblogo website (<http://weblogo.berkeley.edu/logo.cgi>; University of California, Berkeley, Berkeley, CA, USA) (19). Identification of half-MRE and other motifs was performed using the MEME suite web server (<http://meme-suite.org/tools/meme>).

Primer design for genomic amplification and RT-qPCR assays

Primer pairs encompassing significant MBSs were designed by using NCBI's software Primer BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>; NCBI). Amplicon length was kept

between 80 and 130 bp. Primers were from Eurogentec (www.eurogentec.com), purified by the selective precipitation optimized process (SePOP), desalted, and delivered at 100 μ M concentration in water.

qPCR

ChIP-resulting DNA samples were amplified by real-time qPCR, by using the QuantStudio 6 Flex System (Life Technologies), with a 5 μ l DNA sample, 6 μ l fluorescent buffer Fast SYBR Green Master Mix, and 0.5 μ l forward and reverse primers (300 nM final) (primer sequences are provided in Supplemental Table S1). After 10 min preincubation at 95°C, 40 cycles were run. Each cycle was composed of 15 s denaturation phase at 95°C, followed by 1 min hybridization and amplification phase at 60°C. Each DNA sample was analyzed in triplicate. All results are expressed as a percentage of input (Percent Input Method, ChIP analysis; Life Technologies) and expressed as means $\pm$ SEM of 3 independent experiments.

RT- and real-time-qPCR

Treated HK-GFP-MR cells were washed twice with ice-cold PBS. Total RNA was extracted by the guanidium isothiocyanate +phenol-chloroform method, with the TRI reagent (Molecular Research Center, Inc., Euromedex, Mundolsheim, France), according to the manufacturer's recommendations. The RNA pellet was dissolved in 20 μ l water and quantified with a Nanodrop spectrophotometer (Thermo Scientific, Villebon, France). Total RNA (1 μ g) was incubated for 10 min at 37°C with 0.5 μ l DNase I (Biolabs, Evry, France) in a total volume of 10 μ l to remove potential genomic DNA, followed by a 10 min incubation at 75°C with 1 μ l 25 mM EDTA (Life Technologies). For each sample, 9 μ l RT mix was incubated in the presence of Superscript reverse transcriptase (1 μ l), random primers (2 μ l), 10 mM dNTP (0.8 μ l), 10 $\times$ RT buffer (2 μ l), and water (3.2 μ l). After enzymatic activation for 10 min at 25°C, RT was performed for 2 h at 37°C, and the transcriptase was rapidly denatured at 85°C. Real-time qPCR was performed on the cDNA, as described above. Specific primer sequences are provided in Supplemental Table S2. A 10th dilution standard range was run in parallel to quantify cDNA [attomole amount = $f(C_t \text{ value})$]. All samples were quantified in duplicate. Relative expression in a given sample was normalized to the internal reference rRNA 18S values, where the control condition values are arbitrarily set at 1. The results are expressed as means $\pm$ SEM.

Protein quantification and Western blot analysis

Total protein extracts were prepared from treated HK-GFP-MR cells, using the protein extraction buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 30 mM sodium pyrophosphate, and 50 mM NaF], extemporaneously supplemented with 1% Triton X-100 (Sigma-Aldrich), 1% PIC 100 $\times$, and 0.1% SDS. Samples were kept at 4°C for 30 min and then centrifuged at 16,000 g for 20 min at 4°C. The supernatant was resuspended in water, and the protein concentrations were quantified with the bicinchoninic acid assay.

MR immunoprecipitation was performed from 500 μ g total proteins with anti-MR 39N antibody (7 μ g), 0.1% BSA, 1% PIC 100 $\times$, 100 μ l washed protein-A-coated magnetic beads (from 25 μ l of the stock solution; HighCell# ChIP kit; Diagenode) and C1 buffer in a total volume of 500 μ l. After an overnight incubation at 4°C, the supernatants were removed, and the immunoprecipitated proteins were eluted from magnetic beads after addition of 4 $\times$ Laemmli buffer and incubation at 95°C for 5 min. Fifty

micrograms of the proteins was processed in parallel runs for immunoblot analysis and considered to be input samples.

The samples were electrophoresed in a Trans-Blot SD tank (Bio-Rad), first into a concentration gel for 20 min at 120 V, and then into a 7.5% separation gel for 40 min at 200 V. The proteins were then transferred on a membrane using a semi-dry method, for 45 min at 18 V, at room temperature. Membranes were blocked for 1 h at room temperature with 5% nonfat milk in Tris-buffered saline-Tween (TBS-T) and incubated at 4°C overnight with 1:1,000 anti-MR 39N and 1:10,000 anti- α -tubulin (Sigma-Aldrich) for loading controls. The membranes were rinsed 3 times with TBS-T and incubated 1 h at room temperature with the DyLight Fluor secondary antibodies Rabbit 680 and Mouse 800 (1:15,000; Thermo Scientific). After 3 washes, the membranes were analyzed with Odyssey Fc (Li-cor Biosciences, Lincoln, NE, USA).

Statistical analysis

Experimental data are presented as the means $\pm$ SEM. Statistical analyses were performed with Prism 5 software (GraphPad Software, La Jolla, CA, USA), with the nonparametric Mann Whitney *t* test. Differences reaching $P < 0.05$ were considered significant, as shown in detail in the figures.

RESULTS

Pilot MR and H3K4me3 ChIP experiments

To ensure appropriate experimental conditions before ChIP-seq analysis, we conducted pilot ChIP experiments. We used the HK-GFP-MR cells, a human renal cell line originating from the distal convoluted tubule that was originally stably transfected with an expression vector encoding for the fusion protein GFP-hMR (10). This cell line expresses both endogenous MR and GFP-MR fusion protein that can be immunoprecipitated with the anti-MR antibody 39N (11) (Supplemental Fig. S1A, B). Moreover, as illustrated by a transactivation assay with a reporter plasmid encoding the luciferase gene placed under the control of a promoter containing 2 glucocorticoid response elements (GREs), or by the measurement of the aldosterone-induced expression of the *SCNNIA* gene, an endogenous and classic mineralocorticoid target gene, which encodes for α ENaC, the GFP-MR fusion protein is transcriptionally active (Supplemental Fig. S1C, D). After cross-linking, lysis, and sonication, HK-GFP-MR cell samples were immunoprecipitated with the anti-MR 39N, anti-H3K4me3, or negative control IgG antibody. The trimethylation of histone [3 H]lysine 4 is known to mark active transcription sites, enabling the recruitment of transcription factors. Isolated DNA was quantified by qPCR, with primers encompassing the well-documented MRE in the *SCNNIA* promoter (−289 bp from the TSS) or a nearby control region (+1068 bp from the TSS). Aldosterone treatment induced a 15-fold increase in MR recruitment on the MRE, as compared to that induced by the vehicle control (Fig. 1). Moreover, MR enrichment was not observed in the control region after aldosterone exposure, clearly demonstrating that MR recruitment was locus specific. The strong H3K4me3 footprint enrichment on the MRE and control sequences indicated that the regions were transcriptionally active. Taken together, these findings clearly

Figure 1. MR recruitment on promoter region of the *SCNNIA* gene. ChIP assays on HK-GFP-MR cells, which were incubated with ethanol (V) or 100 nM aldosterone (A) for 1 h, fixed by paraformaldehyde, and lysed, and the chromatin was sheared. The samples were then immunoprecipitated with the anti-MR 39N antibody, an antibody directed against the trimethylated lysine 4 of the [3 H] histone (H3K4me3) or control rabbit IgG (C). After elution, the DNA was quantified by qPCR, using primers for the genomic fragment encompassing the MRE corresponding to the MBS located in the promoter region of *SCNNIA* or a control negative region located at +1068 bp from the *SCNNIA* TSS (Control). Results are expressed as the percentage of input before immunoprecipitation (means $\pm$ SEM). * $P < 0.05$ vs. vehicle.

demonstrate that our experimental tools were suitable to identify genomic MR targets in human renal cells.

MR ChIP-seq analysis

To explore the whole-genome target genes of MR in HK-GFP-MR cells, we performed a ChIP-seq analysis with 100 nM aldosterone- or vehicle-treated cells. The bioinformatic analysis of sequencing data, using the MACS software, identified 974 peaks as putative MBSs. For each locus, MACS calculates a score that correlates directly to the probability that the number of reads in the aldosterone-treated condition is significantly higher than in the vehicle conditions [$\text{score} = -10 \cdot \log(P)$]. A locus was regarded as a putative MBS when its score was higher than 50 (*i.e.*, $P \geq 10^{-5}$). These peaks were annotated to flanking genes on the hg19 annotation of the human genome (GRCh37) by MACS, because the software had not been updated to include the latest version of the reference human genome GRCh38. Sequencing data were submitted to the Integrative Genomics Viewer (IGV; <http://www.broadinstitute.org/igv/>; Broad Institute) software, which aligns immunoprecipitated and sequenced fragments on hg19. The sequencing profiles of 2 MBSs, located on the *SCNNIA* gene promoter and on an intron of the *ZBTB16* gene, showed, respectively, 1 and 2 peaks only in the aldosterone-treated condition (Fig. 2A). Adding that there is a peak in the H3K4me3-immunoprecipitated sample, corresponding to an open reading frame, these peaks correspond to MBSs. It is important to note that the binding sites are found throughout the genome. Of the 974 identified MBSs, 84% were more

Figure 2. Distribution of ChIP-Seq reads across the human genome. A) Snapshot showing IGV software windows of the loci corresponding to the *SCNN1A* and *ZBTB16* genes in the hg19 annotation of the human genome. ChIP-seq reads were aligned to the whole human sequence. For both *SCNN1A* and *ZBTB16*, the chromosome annotation (name, arm, and loci) are shown, with
(continued on next page)

than 10 kb from the nearest TSS, with almost half of them more than 100 kb from the TSS (Fig. 2B). The remaining 16% present a Gaussian distribution centered on the TSS position (Fig. 2C). Among the 974 identified MBSs, almost half were intragenic: 39% within introns, 2% in exon regions, and 2% within exon–intron junctions (Fig. 2D). For the others, 39% were intergenic and between 30 and 2000 kb from the TSS, 11% within enhancers (2–30 kb from the TSS), 4% within promoter regions (>2 kb from the TSS), and 3% more than 5 kb downstream of the TSS.

MR binding motif analysis and definition of MRE

To evaluate the consensus motif on which the MR binds, we used FIMO software on our results of ChIP-seq analysis. We found 251 identified palindromic and half-MREs among the MBSs. More precisely, only 7.4% (70/974 peaks) of the MBSs exhibited a palindromic MRE that was close ($P < 0.0001$) to the consensus classic palindromic GRE, a 15 bp motif composed of 2 inverted hexanucleotides (half-sites) separated by 3 bp, commonly recognized by MR, GR, androgen receptor (AR), and progesterone receptor (PR) (AGAACA_{nnn}TGTTCT; Fig. 3A; Table 1). To our surprise, only 1 MBS, related to the *FKBP5* gene, exhibited 2 well-conserved palindromic MREs. It is interesting that palindromic MRE distribution throughout the whole genome was similar to that of the MBSs, in that 94% of them were located more than 10 kb away from the nearest TSS. Moreover, among all identified MREs, 58.6% (147) consisted of single conserved half-MREs (AGAACA; 120/147; 81%) or more (2, 3, or 4-half sites). Some MBSs (16/251; 6.3%) even exhibited a combination of a palindromic and at least 1 half-MRE (Table 1. A detailed analysis of the identified MRE on high-, medium-, and low-score MBSs was performed with the Jaspar software (18). In each group, base variability was analyzed with the Weblogo and compared with the MRE (Fig. 3B). The highest scores (>200) were associated with an MRE motif close to the consensus response element, with a notable variability on bases 3 and 13 (AGxACA_{nnn}TG_xTxCT). Peak scores >80 were associated with relatively well-conserved MRE motifs, keeping a palindromic dyad in which the bases G2, A4, C5, and A6 (and T10, G11, T12, and C14 by symmetry) seemed to be the most important for MR binding and occupancy (xGxACA_{nnn}TG_xTxCx). In contrast, peaks with the lowest scores (~50) were often associated with very degenerated MREs in which the bases C5 and G11 remained essential. The recently solved crystal structure of a dimer of the MR DNA-binding domain (DBD), bound to the palindromic sequence AGAACA_{nnn}TGTTCT, revealed the sequence-specific contacts between the MR DBD and the DNA bases (20). Consistent with our definition of a consensus MRE and, as depicted in Fig. 3C, D, on 1 half-site, Lys⁶²⁴ contacts guanine² (fG2) from the forward oligonucleotide and Val⁶²⁵ and Arg⁶²⁹ contact thymine¹² (rT12) and guanine¹¹ (rG11)

from the reverse oligonucleotide, respectively. In sharp contrast, the bases at positions 1, 3, and 6 (numbered in the forward oligonucleotide) do not establish any contact with the side chains of the MR DBD (Fig. 3C, D).

To determine the occurrence of other motifs in the MBSs, we used the MEME suite software. Four motifs were found to correspond to DNA binding recognition sites of 4 family members of transcription factors, including early growth response protein 1 (EGR1), forkhead box (FOX), paired box protein 5 (PAX5), and activated protein 1 (AP-1) (Fig. 4A). Their number and percentage are reported in a Venn diagram (Fig. 4B). Only 4.5% (54/974) displayed a combination of an MRE with another motif, suggesting potential cooperation between these transcription factors and MR. Moreover, most of the MBSs (723 peaks; 74.2%) did not exhibit any MRE, indicating that MR did not directly bind to DNA but rather may have interacted with other transcription factors within multimeric transcriptional complexes that remain to be further characterized.

Validation of genomic MBSs

To confirm the results of ChIP-seq, we focused on the first 15 peaks with the highest scores (Table 2). They were associated with 13 genes. Two of them, *ZBTB16* and *FKBP5*, contained 2 MBSs. All identified genes were scattered throughout the genome. Peaks are located upstream, downstream, or within a gene, or in the intergenic region (94–223 kb from their TSSs). Four of the genes have already been associated with mineralocorticoid signaling: zinc finger and BTB domain containing 16 (*ZBTB16*), FK506 binding protein (*FKBP5*), *SCNN1A*, and period circadian clock 1 (*PER1*). So far, the 9 others have not been identified as playing any significant role in renal mineralocorticoid signaling: endoplasmic reticulum-Golgi intermediate compartment 1 (*ERGIC1*); family with sequence similarity 65, member B (*FAM65B*); long intergenic non-protein coding RNA 963 (*LINC00963*); nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 (*NFATC2*); nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (*NFKBIA*); protein tyrosine phosphatase, non-receptor type 1 (*PTPN1*); serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1); member 1 (*SERPINE1*); tumor protein p63 regulated 1 (*TPRG1*); and zinc finger, MYND-type containing 8 (*ZMYND8*). It was thus essential to validate ChIP-seq data analysis. We first evaluated the aldosterone-induced recruitment of MR on the sites corresponding to these 13 genes by performing additional ChIP experiments on HK-GFP-MR cells. To quantify DNA fragment enrichment after MR immunoprecipitation, we performed real-time PCR with specific genomic primer pairs surrounding the identified MREs on each site (Supplemental Table S1). The results are expressed as the percentage of input, according to the formula in which the C_t value of

a focus on the gene with its intron and exon organization and the aligned ChIP-seq reads for the vehicle- or aldosterone-treated samples immunoprecipitated by the anti-MR 39N antibody and the anti-H3K4me3-immunoprecipitated sample. Distribution of the reads by their distance from (B) the TSS and (C) a zoom on the zone at less than 10 kb from the TSS. D) Distribution of the reads according to locus type.

Figure 3. Characterization of the MRE. *A*) Identification of an MRE and a half-MRE within the MBS by FIMO software, and their distribution alone or in combination (Table 1). *B*) Distribution profile of the scores [equal to $-10\log(P)$] allotted by the MACS software to the identified MBSS. MREs were identified with Jaspas software on MBSS, according to 3 grades: high (≤ 200), intermediate (≥ 80), and low (~ 50). *C*) The crystal structure of a dimer of MR DBD bound to the canonical MRE (Protein Database identification number: 4TNT) (20). Forward (blue) and reverse (gold) MR DBD monomers are depicted. Only critical residues of the DBD interacting helix are shown with the nitrogen atoms in blue. The DBD is schematically represented with the nucleotides in red, blue, yellow, and green for the adenine, cytosine, guanine, and thymine bases, respectively. Dashed lines: interactions between the amino acids of the MR DBD and the DNA bases. The figure was generated using Open PyMol, ver. 0.93. *D*) Schematic representation of the key contacts between the residues from the DNA interacting helix of the MR DBD dimer with the nucleotide of the MRE. Bases are numbered from the 5' to the 3' end. Arrows: specific contacts between the MR residues and the DNA bases.

each immunoprecipitated sample was normalized to its input C_t value. Of interest, a significant increase in MR enrichment ($P < 0.05$) was detected for all sites when the aldosterone-treated conditions were compared to the vehicle-treated conditions (Fig. 5). These results validate aldosterone-induced MR occupancy on all 13 binding sites identified by ChIP-seq (Table 2). We also found that MR recruitment was significantly enhanced by aldosterone stimulation for *PIK3R1*, a genomic target that lacks classic MREs (Supplemental Fig. S2), a finding that validates the discovery of genomic MR targets by ChIP-seq analysis.

TABLE 1. Distribution of palindromic MREs and half-MREs, alone or in combination

	Total	Sites (n)			
		1	2	3	4
Palindromic MRE	70	69	1	–	–
Half-MRE	147	120	22	4	1
Combination	16	15	1	–	–

The MRE, as identified by FIMO software, is shown in Fig. 3A.

Correlation between genomic MR occupancy and transcriptional activation

To clarify whether the enhancement of MR binding on gene regulatory sequences leads to a subsequent transcriptional regulation of the corresponding genes, we determined mRNA levels of the corresponding gene after aldosterone treatment of human renal cells. Given that MR-induced gene transactivation is a dynamic process that depends not only on the complex interaction between molecular partners but also on the stability of newly synthesized nascent transcripts, we performed a time-dependent stimulation of HK-GFP-MR cells with 10 nM aldosterone. Total RNAs were extracted, reverse transcribed, and quantified by real-time PCR with specific primer pairs (Supplemental Table S1). Data analysis enabled us to classify genes into 4 categories, according to their mineralocorticoid responsiveness, consisting of early, intermediate and late target genes or nonregulated genes (Fig. 6 and Table 3). Among those, steady-state mRNA levels of the *PER1* gene significantly increased ($P < 0.05$) as early as 30 min after aldosterone stimulation, in comparison to the vehicle

Figure 4. Identification of alternative response elements. *A*) Identification of other transcription factor response elements by the MEME suite web server and (*B*) association with a palindromic MRE or another half-MRE.

condition. Accordingly, *PER1* a major actor in the endogenous intracellular gene clock, was classified as an early target gene of MR. Likewise, mRNA levels of *FKBP5*, *SCNN1A*, *SERPINE1*, *NFATC2*, *NFKB1A*, and *FAM65B* were significantly increased after 4 h of aldosterone treatment and thus were classified as intermediate MR-regulated target genes. Finally, *PTPN1*, *TPRG1*, *ERGIC1*, *ZBTB16*, and *ZMYND8* were considered to be late regulated genes, because their expression levels were significantly increased after 24 h of treatment ($P < 0.05$). With respect to *LINC00963*, its expression was not significantly modified after aldosterone exposure. Thus, although MR was found to bind to a sequence located in the intergenic region at

112,000 bp from the TSS of the nearest gene (*i.e.*, *LINC00963*), it is very likely that MR occupancy on this specific motif affects transcription of another unidentified gene located within a region >100 kb downstream or upstream.

Composition of MR-mediated preinitiation complexes

MR recruitment on MRE in response to aldosterone treatment is one of the first and most critical steps in the ongoing transcription process of target genes. Recruitment of the preinitiation complex is an extremely

TABLE 2. Characteristics of the first 15 genomic MR binding motifs

Gene	Chromosome	Localization	Distance TSS	Score	Locus type	MR response element
<i>ZBTB16</i>	11	114,033,640	+102,353	497	Consensus GRE	<u>AGAACA</u> nnnTGTCT
		114,030,889	+99,602	415	Intron	<u>AGAACA</u> cacTGTACC
<i>FKBP5</i>	6	35,695,858	+502	427	Intron	GGTACAgtatGTACA
		35,699,815	−5,043	264	Intron	GGCACAttgTGTTC
<i>SCNN1A</i>	12	6,485,465	−1,075	424	Promoter	GGCACAggaTGTCT
<i>PER1</i>	17	8,057,797	−2,044	383	Enhancer	<u>AGAACA</u> gaaTGTCT
<i>NFKB1A</i>	14	35,873,155	+805	271	Intron	<u>AGAACA</u> tgaTGTTC
<i>TPRG1</i>	3	188,665,933	−223,829	262	Intergenic	AGTACAgggCGTTCC
<i>PTPN1</i>	20	49,032,357	−94,533	243	Intergenic	AGGACTgtaAGGCC
<i>NFATC2</i>	20	49,948,239	+211,019	235	Intergenic	GGGACAtaaTGTGCT
<i>FAM65B</i>	6	25,006,801	−95,606	227	Intergenic	<u>AGAAC</u> GgcgCGTCCT
<i>ERGIC1</i>	5	172,296,284	+35,062	223	Intron	<u>AGAACA</u> gagAGTTCC
<i>LINC00963</i>	9	132,195,193	+111,899	216	Intergenic	<u>AGGACA</u> gcgTGTGCT
<i>ZMYND8</i>	20	45,963,033	+22,441	200	Intron	CTAACAcacTGTTC
<i>SERPINE1</i>	7	100,755,724	−14,654	197	Enhancer	AGTACAgacAGTTCC
					Consensus MRE	AGnACAnnnTGTnCC/T

The MR-binding motifs identified by ChIP-seq are summarized. The sequence localization is according to the hg19 annotation of the human genome (GRCh37). Proposed MRE sequences are identified by Jaspar. Nucleotides in uppercase letters correspond to the nucleotide bases of the 2 half-sites and those underlined correspond to the ones that are identical to the corresponding nucleotide of the canonical consensus GRE.

Figure 5. MR recruitment on regulatory regions of identified target genes. HK-GFP-MR cells, treated for 1 h with vehicle (V) or 100 nM aldosterone (A), were fixed by paraformaldehyde and lysed. After they were sheared, chromatin extracts were immunoprecipitated by the anti-MR 39N antibody or by the control IgG antibody (C). Eluted DNAs were quantified by real-time qPCR, with primer pairs encompassing MBSs (Supplemental Table S1). Results are expressed as percentage of input, and are means $\pm$ SEM of 3 independent experiments performed in triplicate. * $P < 0.05$ or ** $P < 0.01$ vs. the vehicle condition.

intricate mechanism, in which hundreds of molecular partners are associated in a sequential, cyclic, combinatorial manner (21). Combining analysis of MR-binding, MR coactivator, and RNA polymerase II (RNA pol II) occupancy aided us in evaluating the functional consequences of aldosterone treatment on preinitiation complex assembly in the context of ongoing transcription associated with modifications of local chromatin landscape and remodeling and histone posttranscriptional signatures. As a first approach, we sought to determine the ability of MR to promote the recruitment of the SRC1 coactivator and of RNA Pol II. New ChIP experiments were performed on chromatin extracts from HK-GFP-MR cells treated for 1 h with 100 nM aldosterone or vehicle or 1 μ M of the mineralocorticoid antagonist, spironolactone. ChIP was subsequently performed with anti-MR 39N, anti-SRC1, or anti-RNA Pol II antibodies. MR, SRC1, and RNA pol II occupancies were evaluated on the *SCNN1A* promoter by quantitative genomic PCR. As illustrated in **Fig. 7**, aldosterone treatment led to significant recruitment of MR but also of SRC1 and RNA pol II, as compared with the vehicle-treated condition ($P < 0.05$). Of interest, under similar experimental conditions, although the MR antagonist promoted both MR and SRC1 recruitment on the

SCNN1A promoter ($P < 0.05$), spironolactone did not induce substantial RNA pol II recruitment, which may account for the repression of transcription initiation in response to this antagonist.

DISCUSSION

In the present study, we report the first whole-genome identification and characterization of specific binding sites for the human MR on the chromatin of the human renal cell. The availability of a human renal MR-overexpressing cell type (HK-GFP-MR) and a specific and suitable anti-MR antibody (39N) enabled us to perform a ChIP-seq analysis for MR. This approach aided us in identifying 974 aldosterone-specific MBSs, only 7% of which contained a classic palindromic MRE. The aldosterone-induced recruitment of MR was further validated onto the first 15 highest scored peaks together, which correlated with the time-dependent, mineralocorticoid-specific activation of the corresponding gene transcription.

Identifying genome-wide MR targets is of major importance in providing a better understanding of renal mineralocorticoid signaling. The bioinformatic analyses of our

Figure 6. Aldosterone-induced transcriptional activation of identified MR target genes. HK-GFP-MR cells were treated at 3 time points (from 30 min to 24 h) with vehicle (V) or 10 nM aldosterone (A) and lysed, and total RNAs were isolated. After RT, the
(continued on next page)

Figure 7. Ligand specificity of MR recruitment together with coactivator SRC1 and RNA pol II occupancy on the *SCNNIA* gene promoter sequence. HK-GFP-MR cells, treated for 1 h with vehicle (V), 100 nM aldosterone (A), or 1 μ M spironolactone (S), were fixed and lysed. Chromatin extracts were sheared before being immunoprecipitated by a negative control antibody (C) or antibodies against MR, SRC1, or RNA pol II. Eluted and purified DNA fragments were quantified by real-time qPCR, using primers amplifying the genomic fragment of the *SCNNIA* promoter (Supplementary Table S1). Data are expressed as the percentage of input, and are means $\pm$ SEM of 3 independent experiments performed in triplicate. * $P < 0.05$ vs. vehicle and aldosterone, respectively.

ChIP-seq data, by comparing reads in aldosterone- vs. vehicle-stimulated conditions, enabled us to identify approximately 1000 MR binding regions. We focused our attention and performed subsequent characterizations on the first 15 identified MBSs, related to 13 different genes spanning the whole genome and having the highest score (≥ 200) of aldosterone-induced MR recruitment. The specific MR recruitment was further confirmed by additional ChIP qPCR experiments under 1 h aldosterone vs. vehicle exposure, indicating that the ChIP-seq approach was a robust method for identifying the genome-wide mapping of MR target genes. It is notable that the 6 highest scored MBSs were associated with 4 target genes, already defined as important players in the renal MR signaling pathway: *SCNNIA*, *FKBP5*, *ZBTB16*, and *PER1*. *SCNNIA* encodes the α -subunit of the well-characterized epithelial sodium channel (α ENaC), which plays a major role in transepithelial sodium reabsorption (22). *FKBP5* encodes an HSP90 cochaperone protein that participates in the stability of the cytosolic MR (23). *ZBTB16* encodes a transcriptional repressor of the β and γ subunits of ENaC (24), whereas *PER1*, belonging to the circadian clock gene family, is known to regulate the rhythmicity of renal sodium reabsorption through transcriptional activation of several sodium transporters, including ENaC (25–27). The 9 other genes (*NFATC2*, *FAM65B*, *NFKBIA*, *TPRG1*, *ERGIC1*, *ZMYND8*, *PTPN1*, *SERPINE1*, and *LINC00963*) have not been reported so far to play any functional or mechanistic role in human renal mineralocorticoid signaling. Of note, such MBSs have not been identified in the recently published ChIP-seq performed on a murine convoluted collecting duct (CCD) cell model expressing a 3xFLAG-tagged human MR (28). Despite the implication of these genes in various biologic processes, such as cell differentiation [*FAM65B* (29)], mobility [*NFATC2* (30)], and antiapoptotic actions [*TPRG1* (31)], their function in sodium-transporting polarized epithelial cells remains to be demonstrated.

We were able to show that MR binding to genomic sequences was associated with transcriptional activation of aldosterone-responsive genes whose expression was differentially stimulated in a time-dependent manner. The 13 target genes were classified into 3 categories of mineralocorticoid-responsive genes, according to their early (<1 h) (*PER1*), intermediate (4 h) (*FAM65B*, *FKBP5*, *NFATC2*, *NFKBIA*, *SCNNIA*, and *SERPINE1*), or late (24 h) (*ERGIC1*, *PTPN1*, *TPRG1*, *ZBTB16*, and *ZMYND8*) stimulation by aldosterone exposure. The time-dependent responsiveness is one of the main features of the transcriptional gene activation, consistent with the short- or long-term effects of hormonal signaling, and is well supported by the transcriptional clock defined as the cyclic, sequential, and combinatorial assembly of a transcriptionally productive complex onto a regulatory sequence (21). It has to be pointed out that, among the 13 investigated genes, *LINC00963* was unexpectedly not regulated by MR, even though a high-score peak was identified in a desert region, located 111 kb downstream from the nearest TSS of this gene, and the highly significant aldosterone-induced MR recruitment was validated on this MBS by ChIP qPCR. The massive MR recruitment at this site unambiguously demonstrated the validity of this MBS but clearly indicated that it is likely to be involved in the transcriptional regulation of another target gene, very likely located far away in the genome, as suggested by Hou and Corces (32). Even though such a regulated gene is presently unknown, the use of alternate methodology, such as the chromatin interaction analysis by the paired-end tag sequencing approach (ChIA-PET) (33) may actually facilitate its identification.

The first 4 genes, displaying the highest MBS score (*ZBTB16*, *FKBP5*, *SCNNIA*, and *PER1*), have been defined as MR target genes. As opposed to the results of Ueda *et al.* (28), our ChIP-seq data unexpectedly did not identify any MBSs on the *SGK1*, another classic and well-characterized

cDNAs were quantified by real-time qPCR (primer pairs in Supplemental Table S2). Relative gene expression values were normalized to that of 18S rRNA and expressed as fold induction compared with vehicle, arbitrarily set at 1. Data are means $\pm$ SEM of 3 independent experiments performed in duplicate. NS, no significant difference; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. vehicle.

TABLE 3. Summarized relationship between genomic MR targets identified by ChIP-Seq and transcriptional activation of novel aldosterone-regulated genes

Gene	Locus type	Distance from TSS	Recruitment (fold enrichment)	Transactivation (fold induction)
Early <i>PER1</i>	Enhancer	−2,044	19	3.5
Intermediary <i>FAM65B</i>	Intergenic	−95,606	9	1.3
<i>FKBP5</i>	Intron	+502	39	1.6
	Intron	−5,043	ND	
<i>NFATC2</i>	Intergenic	+211,019	15	1.6
<i>NFKBIA</i>	Intron	+805	3	1.2
<i>SCNN1A</i>	Promoter	−1,075	15	2.1
<i>SERPINE1</i>	Enhancer	−14,654	18	2.2
Late <i>ERGIC1</i>	Intron	+35,062	12	1.2
<i>PTPN1</i>	Intergenic	−94,533	4	1.3
<i>TPRG1</i>	Intergenic	−223,829	12	1.3
<i>ZBTB16</i>	Intron	+102,353	22	1.6
	Intron	+99,602	ND	
<i>ZMYND8</i>	Intron	+22,441	18	1.4
Unregulated <i>LINC00963</i>	Intergenic	+111,899	18	Unregulated

Recruitment is the fold induction of MR occupancy expressed as a percentage of the input ratio of the aldosterone *vs.* the vehicle sample. Transactivation is the aldosterone-induced transcriptional activation of the corresponding gene expressed as the relative gene expression ratio of the aldosterone *vs.* the vehicle sample.

renal mineralocorticoid target (34). However, we were able to demonstrate significant MR recruitment onto the well-characterized SGK1-containing MBS by ChIP-qPCR analysis and to show that 1 h aldosterone exposure stimulated SGK1 expression in HK-GFP-MR cells, as measured by RT-qPCR (data not shown). Such discrepant results clearly indicate that the ChIP-seq approach has its own intrinsic limitations, depending on the experimental conditions used, and strongly suggest the imperative for developing alternative and confirmatory methods in the search of genomic MR-specific binding sequences and the functional consequences of such MR recruitment. Identification and characterization of transcription factor binding sites are obviously highly dependent on the experimental conditions—notably, the cell type, the antibody and its recognized epitope, and the endogenous nature of the studied DNA-binding protein.

Studies have already established a canonical HRE for the oxo-steroid receptors, referred as to GREs: AGAA-CAnnnTGGTTCT. Among the 974 MBSs identified in the present study, one quarter contained a well-conserved canonical MRE or a half-MRE (70 palindromic and 147 half-MRE motifs). However, sequence degeneration markedly increased as the peak scoring of the MBS decreased, the main conserved bases in the half-sites being xGxACx.

With respect to the MR binding motifs identified by our ChIP-seq analysis, it was somehow surprising to find that only ~15% of the MREs were located 10 kb from the TSS. This finding was in accordance with what was reported for genomic binding sites of PRs (35) and glucocorticoid receptors (GRs) (36). Unlike MR, most AR binding sites were adjacent to their target gene TSSs (37, 38). Most MBSs are located outside the 10 kb distance from the TSS, including 9 of the 13 genes we investigated. These findings

have led us to propose that the MR may directly bind a consensus MRE in a very restricted number of promoter regions (4%) (either 1 or 2 MRE or half-sites), whereas it more frequently interacts with a large number of other genomic motifs including not only the MRE but also the response elements of other transcriptional factors with which it may interact through a tethering mechanism, as previously described for other steroid receptors (39). This hypothesis is further supported by our MEME suite motif analysis that highlighted the occurrence of 4 other binding sites, notably the EGR1, FOX, PAX5, and AP-1 recognition sites, suggesting cooperative interplay between specific transcription factors and MR, even far away from the TSS. Identification of such alternative MR binding genomic motifs, sometimes in association with a full or half-MRE, is consistent with the combinatory and dynamic assembly of the preinitiation complex (40). This transcriptional clock described by Gannon's group (21) relied on results obtained by ChIP-qPCR, in which ER α binding to DNA directs ordered recruitment of coactivators, such as SRC1, sequentially followed by RNA pol II assembly, finally leading to competent and productive transcriptional complexes. Experiments investigating MR recruitment onto the *SCNN1A* promoter demonstrated a coordinate recruitment of SRC1 and of RNA pol II with MR after 1 h of aldosterone stimulation. In contrast, the MR antagonist, spironolactone, impaired the preinitiation complex assembly, because it allowed SRC1 recruitment, yet prevented RNA pol II binding to the transcriptional complex, providing an initial view of MR's regulatory mechanisms by antagonism. This finding, based on ChIP technology, differs from those obtained with glutathione S-transferase pull-down or mammalian 2-hybrid assays, using specific regions of MR or SRC1 rather than native full-length proteins, which have

shown a lack of MR-SRC1 interaction after spironolactone exposure (41, 42). A more thorough investigation of the preinitiation complex assembly should aid in identifying gene-specific MR partners and in evaluating the influence and the dynamics of coregulator recruitment governing transcriptional regulation.

In summary, the present study provides a first genome-wide mapping of the MR in human renal cells. By combining genomic, bioinformatic, and transcript analyses, we were able to define specific MREs and to identify novel MR target genes on which we correlated genomic MR recruitment and transcriptional activation. Our results constitute a first step toward a better understanding of the complex transcriptional regulation exerted by MR, including functional and dynamic interplay with other transcription factors. This research should contribute to future investigations exploring the pathophysiological role of MR. **FJ**

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