Transcriptome of a mouse kidney cortical collecting duct cell line: Effects of aldosterone and vasopressin

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Aldosterone and vasopressin are responsible for the final adjustment of sodium and water reabsorption in the kidney. In principal cells of the kidney cortical collecting duct (CCD), the integral response to aldosterone and the long-term functional effects of vasopressin depend on transcription. In this study, we analyzed the transcriptome of a highly differentiated mouse clonal CCD principal cell line (mpkCCD$_{cl4}$) and the changes in the transcriptome induced by aldosterone and vasopressin. Serial analysis of gene expression (SAGE) was performed on untreated cells and on cells treated with either aldosterone or vasopressin for 4 h. The transcriptomes in these three experimental conditions were determined by sequencing 169,721 transcript tags from the corresponding SAGE libraries. Limiting the analysis to tags that occurred twice or more in the data set, 14,654 different transcripts were identified, 3,642 of which do not match known mouse sequences. Statistical comparison (at $P < 0.05$ level) of the three SAGE libraries revealed 34 AITs (aldosterone-induced transcripts), 29 ARTs (aldosterone-repressed transcripts), 48 VITs (vasopressin-induced transcripts) and 11 VRTs (vasopressin-repressed transcripts). A selection of the differentially-expressed, hormone-specific transcripts (5 VITs, 2 AITs and 1 ART) has been validated in the mpkCCD$_{cl4}$ cell line either by Northern blot hybridization or reverse transcription–PCR. The hepatocyte nuclear transcription factor HNF-3-α (VIT39), the receptor activity modifying protein RAMP3 (VIT48), and the glucocorticoid-induced leucine zipper protein (GILZ) (AIT28) are candidate proteins playing a role in physiological responses of this cell line to vasopressin and aldosterone.

Aldosterone and vasopressin actions on the kidney are crucial for the maintenance of sodium and water balance, and for the control of blood volume and blood pressure. In the principal cell of the cortical collecting duct (CCD), aldosterone-dependent sodium transport is fully inhibited by actinomycin D, indicating that the steroid hormone acts mainly through transcriptional effects (1). In the same cell, vasopressin elicits a rapid (within minutes), actinomycin D-independent effect on water, chloride, and sodium reabsorption. However, up to 50% of the sodium transport response becomes actinomycin D-sensitive after longer exposure (4–24 h), thus suggesting that the peptide hormone mediates part of its effect through transcription (2, 3). Synergism between adrenal steroid hormones and vasopressin has been well documented *in vivo* and *in vitro* (4–8). In the amphibian cell model, synergistic effects on sodium transport are observed at supramaximal concentrations of each agonist, indicating that, albeit binding to different receptors, the two hormones have common cellular site(s) of interaction (2). The two main effector molecules that are involved in the amiloride-sensitive sodium transport in principal cells are the apical epithelial sodium channel (ENaC) and the basolateral ouabain-sensitive sodium pump (Na,K-ATPase). The two hormones regulate both sodium transporters by transcriptional and posttranscriptional mechanisms.

Aldosterone acts through a high-affinity (type 1) mineralocorticoid receptor (MR) and/or a low-affinity (type 2) glucocorticoid receptor (GR) (9). Aldosterone induces the translocation of the activated receptors from the cytoplasm to the nucleus, where they bind to the MR/GR-responsive elements within the promoter region of aldosterone-induced transcripts (AITs) and aldosterone-repressed transcripts (ARTs). ENaC α subunit and Na,K-ATPase α1 and β1 subunits have been identified as AITs, but their time course of induction (>3 h) indicates that the transcriptional activation of these genes is involved in the late phase of aldosterone action. The early activation (within 1 h) of ENaC and Na,K-ATPase by aldosterone depends on early induced or repressed genes. The transcription-dependent aldosterone-signaling pathway (between receptor and membrane transport effectors) remains poorly understood. Only two gene products (Sgk and K-Ras) have recently been shown to be part of the pathway, but upstream and downstream mediators have not been identified at the molecular level (10, 11).

Vasopressin activates the V2 vasopressin receptor (V2R), a member of the G protein-coupled receptor superfamily. V2R-stimulated cell cAMP content triggers transepithelial sodium, chloride, and water reabsorption by a two-phase mechanism (3, 12, 13): (*i*) during a short-term, nongenomic phase, vasopressin rapidly increases the number of functional ENaC and the density of aquaporin-2 molecules in the apical membrane of principal cells; and (*ii*) during the late, genomic phase, vasopressin participates to the long-term regulation of renal sodium, chloride, and water reabsorption through the cAMP-dependent transcriptional activation of a gene network that includes aquaporin-2 (14, 15), Na,K-ATPase, ENaC, and the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (3, 13). By analogy to aldosterone-dependent transcripts (AITs and ARTs), vasopressin-induced and vasopressin-repressed transcripts are referred to as VITs and VRTs, respectively. Again, the transcription-dependent vasopressin-signaling pathway (between receptor and membrane transporters) has not yet been defined at the molecular level.

Abbreviations: ADX, adrenalectomy; AIT, aldosterone-induced transcript; ART, aldosterone-repressed transcripts; CCD, cortical collecting duct; SAGE, serial analysis of gene expression; ENaC, epithelial sodium channel; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GILZ, glucocorticoid-induced leucine zipper protein; GR, glucocorticoid receptor; HNF-3-α, hepatocyte nuclear factor-3-alpha; Na,K-ATPase, basolateral ouabain-sensitive sodium pump; MR, mineralocorticoid receptor; RAMP3, receptor activity modifying protein 3; VIT, vasopressin-induced transcripts; VRT, vasopressin-repressed transcripts; V2R, V2 vasopressin receptor.

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Obviously, the identification of aldosterone- and vasopressin-dependent signaling pathways represents a major step for the molecular understanding of sodium and water reabsorption by principal cells. Functional synergism observed for both hormones indicates that these networks are probably composed of independent and overlapping sets of genes acting in a complementary fashion. Potentially, these networks include a set of candidate genes for salt-sensitive hypertension, salt-loosing syndromes, and dysfunction of the urinary concentrating mechanisms responsible for nephrogenic diabetes insipidus. Supporting this concept, it has been shown that mutations in MR (16, 17), V2R (18), ENaC (19), or aquaporin-2 (20) cause monogenic diseases with hypertension, severe salt or water loosing syndromes. Thus, the principal aims of this study were (*i*) to characterize the full transcriptome of a principal cell and (*ii*) to identify the changes in the transcriptome induced by aldosterone and vasopressin. To achieve these goals, we used the only powerful tool presently available, i.e., serial analysis of gene expression (SAGE), which allows a quantitative monitoring of all mRNA transcripts or transcriptome, of a cell (21). The transcriptome of the collecting duct, which is made of at least two cell types (principal and intercalated cells), has been recently reported (22). For methodological reasons (limitation in cell isolation and in the amount of mRNA that can be obtained), it is not yet feasible to analyze the transcriptome of the principal cells *per se*, directly isolated from a CCD. The use of a well characterized clonal cell line derived from the CCD can overcome this difficulty and should allow to delineate more precisely the transcriptome of this cell type (23). As a first step toward the characterization of the aldosterone- and vasopressin-dependent signaling pathways, we have analyzed the effects (4-h exposure) of the two hormones on the transcriptome of the principal cell. In this study, analyses of the transcriptomes from a differentiated mouse CCD principal clonal cell line, mpkCCD$_{cl4}$ (23), treated or not with aldosterone or vasopressin, permitted the identification of a new set of aldosterone- and vasopressin-dependent genes.

Methods

Cell Culture. The mpkCCD$_{cl4}$ is a clonal cell line derived from the CCD of a transgenic mouse (simian virus 40 large T antigen under the control of the simian virus 40 enhancer/L-type pyruvate kinase promoter) (23). This clone was selected for its high Na^+ transport, its high transmural resistance, and its responsiveness to aldosterone and vasopressin. Cells of passages 20–35 were used for all experiments. Cells were routinely grown in plastic tissue culture flasks in a modified DMEM/Ham's F-12 medium (Life Technologies, Basel, Switzerland) supplemented with hormones as described (23). For electrophysiological studies and mRNA extraction, the cells were subsequently seeded and grown on collagen-coated Transwell filters (Costar) until they reached high transepithelial potential. Filters were then placed in a low-hormone, serum-free, and epidermal growth factor-free medium for 5 days, and then in hormone-free, serum-free, and epidermal growth factor-free medium for 18 h before experiment (23). The transepithelial potential difference (PD, mV) and short-circuit current (I_{sc}, $\mu A/cm^2$) were monitored on the monolayers cultured on filters. Measurements were performed in a modified Ussing chamber connected to an automatic voltage-clamp apparatus. Transepithelial electrical resistance ($\Omega \times cm^2$) was calculated from the transepithelial potential difference and I_{sc}.

mRNA Extraction and Generation of SAGE Libraries. Confluent cells grown on 44.2 cm^2 collagen-coated Transwell filters that have developed a high transepithelial resistance ($>3{,}000\ \Omega \times cm^2$) were used for mRNA preparation. Total RNAs were extracted from untreated mpkCCD$_{cl4}$ cells (control condition) and cells treated either with 10^{-6} M aldosterone (Aldo4h) or 10^{-8} M vasopressin (Vaso4h) for 4 h. These two hormone concentrations were chosen because they yielded maximum I_{sc} (23, 24). The high (10^{-6} M) aldosterone concentration used should occupy both type 1 (MR) and type 2 (GR) receptors. In principal cells, type 2 (GR) is not occupied by cortisol, converted to inactive cortisone by the receptor-protective enzyme 11-beta HSD2, and occupancy of both MR and GR by aldosterone is required to induce maximal sodium transport response. Our screen is thus intended to identify the complete set of genes involved in the full aldosterone sodium transport response. The mRNAs of the control and of the Aldo4h and Vaso4h conditions were prepared in two independent sets of confluent cells grown on filters having identical electrical properties. Poly(A) mRNAs were selected through hybridization to oligo-$(dT)_{25}$ covalently bound to magnetic beads (Dynal, Norway). Control, Aldo4h, and Vaso4h SAGE libraries were prepared from 5 μg of the corresponding poly(A) mRNAs by using the SADE protocol (21, 22). A major modification of this protocol consisted in the use of *Sau*3AI (instead *Nla*III) as the anchoring enzyme. A total of 10,000 sequencing reactions were performed by Genoscope (Evry, France).

SAGE Data Analysis. Sequence files were analyzed by using SAGE300 software (21). Tags corresponding to linker sequences were discarded and those originating from duplicate ditags were counted only once. For tag identification, the tag list of each library was matched against a mouse tag database extracted by SAGE software from GenBank release 118. For an expressed sequence tag (EST) match to be considered correct, we checked that the EST displayed the most 3′ *Sau*3AI site of the cDNA. This was achieved by the analysis of the corresponding contiguous sequences of TIGR database (http://www.tigr.org). Assessment of significant differences between two SAGE libraries was made by Monte Carlo simulation analysis and validated by a Z-test used for comparison of SAGE libraries of different sizes (25). The tags, the numbers of which were significantly different between all three libraries (i.e., Aldo4h vs. control and Vaso4h, and Vaso4h vs. control and Aldo4h) were considered as tags corresponding to the transcripts specifically regulated by aldosterone and vasopressin, respectively. A P value of 0.05 or less was considered significant.

Adrenalectomy (ADX), Microdissection of CCDs and Reverse Transcription (RT)-PCR Analysis. Males C57BL/6J mice (RCC Ltd., Füllinsdorf, Switzerland) were adrenalectomized 4 days before the experiments under pentobarbital anesthesia. During the postoperative period, the animals had free access to a high-sodium tap water (0.9% NaCl) and a normal pelleted mouse diet, with a sodium content of 3 g/kg. Four days after ADX, the animals received an i.p. injection of aldosterone (1 μg/g) and were killed 1 or 2 h later. Untreated ADX animals were used as control. For isolation of CCDs, the left kidney was perfused with Hanks' solution containing 0.15% collagenase (Roche). Microdissection and RNA extraction were performed as described (26). Semiquantitative RT-PCR analysis was carried out on RNA amounts corresponding to 1 mm of CCD. GILZ and internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were amplified in a same reaction tube. GILZ and GAPDH downstream primers (5′-TTCCGGGGTTTCCGGGGTTTC-3′ and 5′-GTGGGTGCAGCGAACTTTATT-3′, respectively) were present during RT and the corresponding upstream primers were added for PCR amplification (5′-GTGAGAGAGGAG-GTGGAG-3′ and 5′-GACGTGCCGCCTGGAGAAA-3′, respectively). Conditions of RT and PCR were identical to what has been described (26). To keep the proportionality between the amount of mRNA in the sample and the DNA product, a radiolabeled deoxynucleotide was included in the assay. This allowed to reduce the number of PCR cycles and to detect the PCR product at a detection threshold significantly lower than the sensitivity of ethidium-bromide staining. The amplified PCR fragments were electrophoresed through a 3% agarose slab gel

in TBE. The gel was fixed in 10% acetic acid, dried, and exposed to MolecularImager screen (Bio-Rad).

Results and Discussion

Construction of SAGE Libraries. We used the recently developed highly differentiated mpkCCD$_{cl4}$ cell line derived from the mouse collecting duct principal cells. This cell line was demonstrated to exhibit several characteristic properties of principal cells, including (*i*) a high responsiveness to aldosterone and vasopressin (23, 24), (*ii*) expression of MR and GR as well as V2R (23), (*iii*) full inhibition of aldosterone-stimulated sodium transport and partial inhibition of long-term vasopressin-stimulated sodium transport by actinomycin D (ref. 23 and M.F. and D.F., unpublished data), and (*iv*) expression of a number of proteins specific of principal cells (the present study).

To prepare the SAGE libraries, the mRNAs were extracted from the mpkCCD$_{cl4}$ cells treated with 10^{-6} M aldosterone (Aldo4h library), 10^{-8} M vasopressin (Vaso4h library), or vehicle (control library) for 4 h. The chosen hormonal concentrations were demonstrated to produce a maximal stimulation of sodium transport in the mpkCCD (23) (see *Methods*). The 4-h incubation time point was selected for the following reasons. First, according to the current model, the aldosterone response on sodium reabsorption consists of (*i*) an early phase (lasting up to 4 h) during which the sodium reabsorption is increased by the activation of preexisting transport proteins (ENaC, Na,K-ATPase) via transcriptional and/or translational activation of a set of so-called early genes, and (*ii*) the late response, starting 3–4 h after stimulation with the hormone and characterized by an accumulation of additional transport proteins and other elements of the sodium transporting machinery. Therefore, the 4-h time point represents a point of transition between the early and the late responses in which the early and the late sets of genes are potentially coexisting. In favor of this hypothesis, the mRNAs accumulation kinetics of several recently identified early genes reveal (i.e., Sgk) that the mRNA levels remain significantly induced at least 6 h after hormone stimulation (10, 11). Second, the major transcriptional effects of vasopressin presumably occur after several hours of hormonal treatment as it was demonstrated for ENaC, CFTR, AQP2, and Na,K-ATPase (3, 13, 14). Third, a long exposure to the hormones (24 h or more) may induce significant morphological changes in the principal cell and, therefore, regulate the transcriptional rate of a number of genes indirectly related to hormone action (27).

Transcriptome of mpkCCD$_{cl4}$ Cells. We identified the transcriptomes of the mpkCCD$_{cl4}$ cells in the control, Aldo4h, and Vaso4h conditions by sequencing a total of 169,721 SAGE tags (39,091 tags, 62,796 tags, and 67,834 tags, respectively). Considering the large number of sequenced tags, the number of genes expressed in this cell line was evaluated by considering tags present at more than one copy in all three libraries taken together. The 141,589 retained tags corresponded to 14,654 different transcripts. GenBank match analysis (GenBank release 118, nonredundant sequences) revealed that 9,151 of the 14,654 retained tags do not match characterized mouse genes or cDNAs. When considering EST databases, the number of orphan tags drops to 3,642. As expected, the most abundant transcripts corresponded to genes involved in protein synthesis (i.e., ribosomal proteins, elongation factor 1-α), oxidative metabolism (i.e., cytochrome C oxidase subunits), and cytoskeleton (i.e., cytokeratines, beta- and gamma-actin). Analysis of the transcripts for genes relevant for water and sodium transport in principal cells revealed the presence of α, β, and γ subunits of ENaC, aquaporin-2, and the α1, β1 (two spliced forms), and β3 subunits of Na,K-ATPase. The full set of data obtained in this study is available at http://www-dsv.cea.fr/thema/get/sade.html.

Identification of AIT/ARTs and VIT/VRTs. The major aim of this study was to identify sets of genes that are specifically regulated under aldosterone and/or vasopressin action. For this aim, the assessment of significant differences among the control, Aldo4h, and Vaso4h libraries was performed by using the Monte Carlo simulation analysis. This analysis was further validated by a Z-test used for comparison of SAGE libraries of different sizes (25). SAGE tag abundance was considered as significantly different when the estimated P value was lower than 0.05 as compared with the two other libraries. Such analysis allowed the identification of 63 and 59 tags, specifically up- or down-regulated in the Aldo4h and Vaso4h libraries, respectively (see our table at http://www-dsv.cea.fr/thema/get/sade.html). Among the 63 aldosterone-regulated transcripts, 34 were identified as AITs and 29, as ARTs. For the vasopressin-regulated transcripts, 48 were identified as VITs and 11, as VRTs. Analysis of the differentially regulated transcripts using the nonredundant and EST domains of GenBank revealed that 33 aldosterone-regulated transcripts and 36 vasopressin-regulated transcripts matched known mouse genes (see our table at http://www-dsv.cea.fr/thema/get/sade.html). The remaining tags either matched orphan ESTs (15 and 17, respectively) or had no match in the GenBank (15 and 6, respectively). Comparison of the aldosterone- and vasopressin-regulated sets of genes shows that a dozen AITs and VITs are identical, and potentially correspond to the genes responsible for the known morphological adaptation of principal cell to the sodium load induced by both hormones (27). Among the regulated transcripts matching known genes, only a small fraction was previously characterized as genes transcriptionally regulated by steroids or cAMP. For example, expression of the glucocorticoid-induced leucine zipper protein (GILZ) was found to be significantly increased by dexamethasone in thymocytes and peripheral T cells (28). However, expression and function of this protein in tight epithelia have not been investigated. Similarly, despite a known cAMP-dependent regulation of cytokeratin 18 in several systems, its role in the context of vasopressin action in the principal cell remains unexplored (29).

Example of VITs Validation. To further validate the SAGE data, Northern blot hybridization was performed on a selection of five VITs. In this selection, two VITs matched known genes, i.e., hepatocyte nuclear factor-3-alpha (HNF-3-α, VIT38) and receptor activity modifying protein 3 (RAMP3, VIT48), and three VITs corresponded to orphan ESTs (VIT23, VIT31, VIT32). As shown in Fig. 1, Northern blot hybridization confirmed the vasopressin-stimulated expression of mRNAs corresponding to the selected tags. The levels of mRNAs induction at 4 h varied from 1.8 (VIT23) to more than 10 (RAMP3 and VIT31) and corresponded well to the difference in tag copy number from control and Vaso4h libraries (see our table at http://www-dsv.cea.fr/thema/get/sade.html). Except for VIT32, a 24-h treatment with vasopressin led to a down-regulation of the VITs mRNA expression as compared with the 4-h vasopressin treatment (Fig. 1). Thus, these Northern blot analyses confirm that the sampling of our SAGE data are representative and permitted to identify the five tested VITs as novel vasopressin-regulated genes.

Identification of HNF-3-α and RAMP3 as vasopressin-induced genes reveals possible new interesting signaling links activated by the hormone. HNF-3-α belongs to a large family of transcriptional factors [HNF-3/fork head homologue (HFH)], which shares homology in their winged helix DNA binding domain. Members of the HNF-3/HFH family have been shown to be involved in cell fate determination during embryonic formation, in organogenesis, and in cell-type-specific gene expression (30). Interestingly, putative DNA binding sites have been identified in the promoter regions of several sodium transporters (Na,K-ATPase, Na/H exchanger) or of the MR

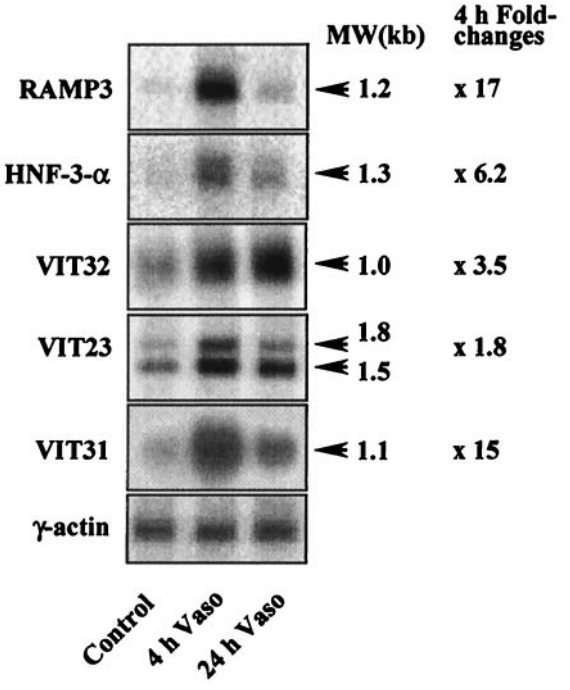

Fig. 1. Northern blot analyses of selected vasopressin-regulated transcripts. Selected VITs [RAMP3 (VIT48); HNF-3-α (VIT38); VIT23, VIT31 and VIT32] were validated on the Northern blots prepared with mRNAs extracted from the $mpkCCD_{cl4}$ cells grown on filters and treated with vehicle (Control) or 10^{-8} M vasopressin (Vaso) for 4 or 24 h. Blots were hybridized with the corresponding VIT probes, stripped, and rehybridized with a gamma-actin probe. The blots were quantified by using a MolecularImager (Bio-Rad), and the levels of mRNAs induction were expressed as fold-changes of corresponding Control values.

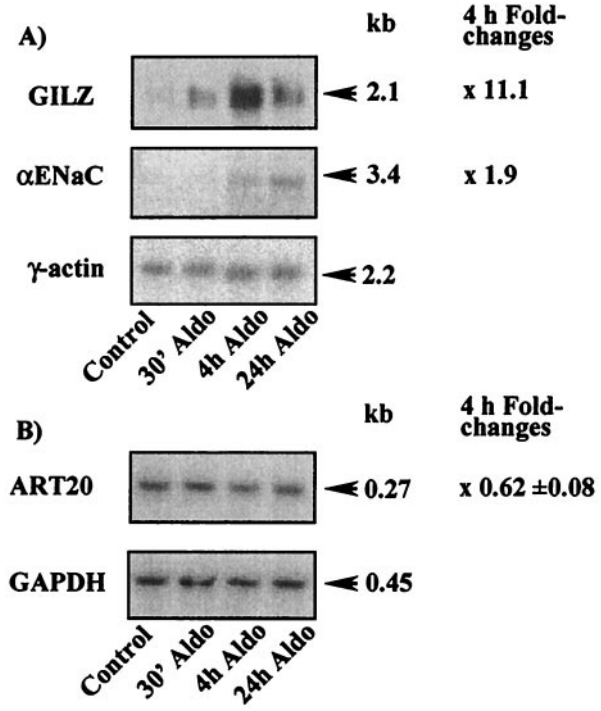

Fig. 2. Northern blot and RT-PCR analyses of selected aldosterone-regulated transcripts. (*A*) Selected AITs (GILZ and αENaC) were validated by using mRNAs prepared from $mpkCCD_{cl4}$ treated with vehicle (Control) or 10^{-6} M of aldosterone for 30 min, 4 h, or 24 h. Blots were hybridized with the corresponding AITs probes, stripped, and rehybridized with a gamma-actin probe. The blots were quantified with a MolecularImager (Bio-Rad), and the mRNAs stimulation levels were expressed as the fold-changes of the corresponding Control values. (*B*) ART20 repression was validated by semiquantitative RT-PCR analysis performed on the same mRNAs as those used for Northern hybridization. For RT-PCR amplification of ART20, the primers, available from the authors upon request, were selected from the TIGR database contiguous sequence TC75211 corresponding to the ART20 SAGE tag. Coamplification of the same quantity of mRNA with GAPDH specific primers was used as an internal control for mRNA extraction and PCR amplification. The RT-PCR products were quantified with a MolecularImager (Bio-Rad), and the mRNAs stimulation levels were expressed as the fold-changes as compared with the corresponding GAPDH values. Aldosterone-induced change is expressed as means ± SEM from three separate experiments.

(30). The HNF-3-α binding site was also identified in the promoter region of a novel, kidney-specific, cell adhesion molecule, cadherin 16 (or Ksp-cadherin), which is highly expressed in the CCD (31). RAMP3 belongs to a recently identified receptor activity modifying protein (RAMP) family of molecular chaperones responsible for the proper glycosylation and transport of the calcitonin receptor-like receptor (CRLR) to the plasma membrane. Association of CRLR with three known members of the RAMP family significantly modulates the affinity of this receptor for the five members of the calcitonin family, e.g., calcitonin, amylin, two calcitonin-gene-related peptides (CGRP1 and CGRP2), and adrenomedullin (32). Coexpression of CRLR with RAMP3 generates a specific adrenomedullin receptor. Because, adrenomedullin may act as a natriuretic hormone, the stimulation of RAMP3 expression by vasopressin raises the possibility of a feedback mechanism for vasopressin-stimulated sodium reabsorption in CCD principal cell.

Example of Validation for AITs and ARTs. The validation of SAGE data for the aldosterone-regulated transcripts was performed on two AITs that matched the known genes αENaC and GILZ genes, and one ART (ART20) that corresponded to orphan EST. mRNAs used for Northern blot hybridization or RT-PCR analyses were extracted from the $mpkCCD_{cl4}$ cells treated with aldosterone for 30 min, 4 h, or 24 h. The occurrence of αENaC tag in the three libraries does not display statistical significance ($P = 0.08$). Therefore, it does not fall in the category of AITs defined above ($P < 0.05$). However, its abundance in the three libraries (0 copy in control, 5 copies in Aldo4h, and 0 copy in Vaso4h) is consistent with the validation by Northern blotting (1.9-fold increase, Fig. 2*A*). Thus, analysis of candidates with a confidence level lower than the selected one (0.05) could also be considered for further screening. The semiquantitative RT-PCR analysis was performed for the ART20 because of a low signal obtained under Northern blot hybridization (data not shown). From this analysis, ART20 was confirmed as an aldosterone-repressed transcript, with a mean decrease in the mRNA content of 40% (Fig. 2*B*). This last result demonstrates that SAGE can be used for the identification of down-regulated genes. One of the most significant changes on the mRNA level under aldosterone treatment was observed for GILZ mRNA. The stimulatory effect of aldosterone is rapid because a threefold increase in the GILZ mRNA content was observed already 30 min after hormone addition (Fig. 2*A*). Aldosterone further stimulated GILZ mRNA expression by 11-fold increase after 4 h, and by 7-fold after 24 h. To date, only a similar rapid and high induction of gene expression caused by aldosterone in mammalian epithelia has been reported for the recently identified serum and glucocorticoid-regulated kinase (sgk-kinase) shown to significantly regulate the ENaC activity (11, 33, 34). GILZ belongs to the TSC-22/DIP family of proteins that were initially proposed to function as transcription factors (28). In contradiction to this

attribution, the members of this protein family do not contain the canonical DNA-binding domain and the TSC-22 protein has been shown to be localized in cytosol but not in nucleus (35). In addition, analysis of GILZ protein sequence reveals the presence of a consensus–S-X-V C-terminal sequence, known to interact with a broad range of PDZ-binding domains containing proteins responsible for protein clustering to the plasma membrane. Furthermore, this -(S/T)-X-V C-terminal consensus sequence has been found in many ion channels, transporters, and membrane receptors (36). To our knowledge, this consensus sequence was never identified in proteins with confirmed transcription factor function. Thus, the exact role of GILZ and other members of TSC-22/DIP family in CCD principal cells remains to be determined. One of the limitations of our SAGE analysis for the identification of AITs/ARTs and VITs/VRTs is evidenced by the lack of sgk-kinase in the list of the selected AITs. The SAGE tag corresponding to sgk-kinase was weakly represented (0 copy in control, 3 copies in Aldo4h, and 0 copy in Vaso4h). This low number of tags was also observed for most of the members of the kinase cascade presumably related to sgk-kinase function. Accordingly, the number of tags for K-ras, RAF, MEK, ERK, and other kinases varied from 1 to 3 copies in the three libraries. More SAGE tags will be sequenced to provide the statistical confidence levels sufficient to identify the variations in the low abundance genes.

To check that the genes identified in the mpkCCD$_{cl4}$ cells are also regulated *in vivo*, we analyzed GILZ mRNA levels in CCDs microdissected from ADX mice. Four days after ADX, the mice were i.p. injected with 10 μg/kg of body weight of aldosterone. This experiment was performed on groups of four animals that were killed either 1 or 2 h after aldosterone injection. CCDs were microdissected out and the RT-PCR amplification was performed on the extracted mRNAs with primers specific for GILZ sequence (see *Methods*). As shown in Fig. 3, aldosterone rapidly and significantly stimulated the mean level of GILZ mRNA expression in isolated CCDs by 2.1-fold and 4.7-fold after 1 h and 2 h of aldosterone administration, respectively. This experiment

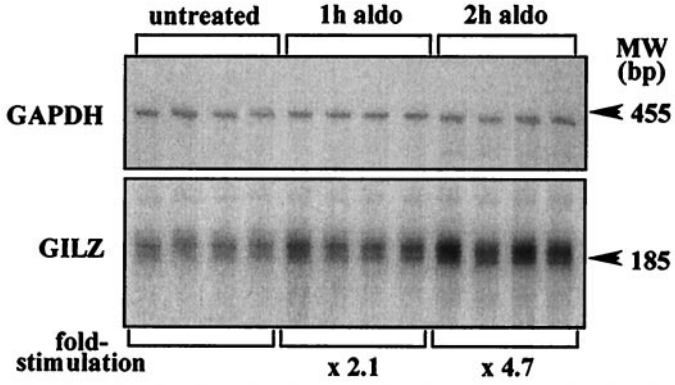

Fig. 3. RT-PCR analysis of GILZ mRNA expression in the CCD of the mouse kidney. CCDs were microdissected out from the kidneys of the ADX mice (n = 4) i.p. injected with 10 μg/kg of aldosterone and killed 1 or 2 h after injection. Untreated ADX mice (n = 4) were used as controls. RT-PCR was performed on the mRNA amounts corresponding to 1 mm of CCDs. Coamplified GAPDH mRNA was used as an internal control for mRNA extraction and PCR amplification.

strongly suggests thus that GILZ is a potential target for aldosterone regulation in the principal cell *in vivo*.

In conclusion, we have identified new sets of aldosterone- and vasopressin-regulated genes. Examining to which extent these genes contribute to the effects of aldosterone and vasopressin on sodium and/or water balance is a promising field for future works. Our study also provides a molecular insight on the transcriptome of a highly differentiated mouse clonal CCD principal cell line (mpkCCD$_{cl4}$).

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