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Regulation of Aquaporin-2 Gene Transcription by GATA-3

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To evaluate the functional role of GATA motifs in the 5′-flanking region of a kidney-specific AQP-2 water channel gene, we sought to isolate a GATA factor(s) expressed in collecting ducts and determined the role on the AQP-2 promoter. Two cDNAs encoding GATA factors were isolated from rat kidney, whose sequences were highly homologous with human GATA-2 and -3. Reverse-transcription PCR using dissected nephron segments revealed that rat GATA-3 but not GATA-2 was expressed in collecting ducts, thus indicating that GATA-3 could interact with GATA motifs in the AQP-2 promoter. Transactivation experiments utilizing the rat GATA-3 expression vector indicated that rat GATA-3 increased the AQP-2 promoter activity about fourfold. These results indicated that GATA motifs in the 5′-flanking region of the hAQP-2 gene were functional cis-elements and that GATA-3 in collecting ducts may be one of the important regulators of AQP-2 expression *invivo*.

AQP-2 is a kidney-specific water channel that plays a pivotal role in urinary concentrating mechanisms (1). The characterization of the promoter region of the AQP-2 gene is an initial step in elucidating the mechanism of tissue-specific expression of the AQP-2 gene. An approximately 400 bp 5′-flanking region of the human AQP-2 (hAQP-2) gene has been shown to have basal promoter activity in the outer medullary collecting duct (OMCD) cells (2). This region contained AP-1, AP-2, Sp-1, cyclic-AMP-responsive element (CRE), E-boxes and GATA motifs (3). Among these consensus sequences, three GATA motifs were clustered at about 150∼200 bp upstream of the transcription start site. Since the isolation of GATA-1 as a regulator of the stage-specific globin gene expression in cells of the erythroid lineage (4), several other GATA factors have been cloned as transcriptional regulators in non-erythroid cells (5-10).

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The accession number of rat GATA-3 is AB000217.

In this report we sought to determine the functional role of GATA motifs in the 5′-flanking region of the hAQP-2 gene. As a first step, we tried to isolate the cDNAs encoding GATA binding proteins that were present in collecting ducts, and then isolated two candidate GATA factors. The intrarenal localization of these GATA factors was determined by RT-PCR using microdissected nephron segments, and the ability of GATA-3 to transactivate the AQP-2 promoter was determined by co-transfection experiment.

METHODS

Cloning of cDNAs encoding GATA factors expressed in rat collecting ducts. Rat collecting ducts were microdissected and cDNA was synthesized with avian myeloblastosis virus reverse transcriptase as described previously (11,12). Two degenerate primers, 5′-CCGAATTC(A/G)TTNC(G/T)NGT(C/T)TG(A/G/T)ATNCC, 5′-CCGGATCCACNCCN(C/T)TNTGG(A/C)GN(A/C)G corresponding to the conserved regions of zinc-finger domains (95 amino acid residues from T at 303 to N at 397 in hGATA-2 (8)) of mammalian GATA-1 and -2 were used for polymerase chain reaction (PCR). PCR was performed in the following profile: 94°C for 1 min, 55°C for 1 min, 72°C for 3 min for 35 cycles. PCR products of expected size (∼300 nt.) were cloned and sequenced. Using this PCR clone as a probe, rat kidney cDNA library (12) was screened under high stringency.

Reverse transcription PCR along rat nephron. Microdissection of rat nephron segments and the synthesis of single strand cDNAs were as described previously (12,13). PCR primers for rGATA-2 were 5′-AGTCGGCAGCTGGCGCCAGG and 5′-CGGTCCAGGGGCTGTGGTGAT, which amplified a part of rGATA-2 cDNA corresponding to the region of human GATA-2 from nt. 26 to 632 (8). PCR primers for rGATA-3 were 5′-TTCTTTCCTCCCTAAACCCT (nt. 27-46) and 5′-GTGGATGGACGTCTTGGAGAA (nt. 560-580). According to the gene structure of mouse GATA-3 gene (14), sense and anti-sense PCR primers were designed not to be present in the same exon in order to discriminate the amplification from genomic DNA from that from reverse-transcribed cDNA. PCR was performed in the following profile: 94°C for 1 min, 62°C for 1 min, 72°C for 3 min, for 35 cycles. PCR samples were electrophoresed in 2% agarose gel, transferred to nylon membrane, and hybridized with ^{32}P-labeled probes, 5′-CGACAGCGCGTCCCAAGGCT (corresponding to nt. 132-153 in hGATA-2) for rGATA-2 and 5′-GGTAGAGGGAGGGCTGTC (nt. 148-165) for rGATA-3.

Plasmid construction. The plasmids, pAQP2-504CAT, pAQP2-364CAT and pAQP2-224CAT, shown in Fig. 1, were constructed as described previously (2). To introduce the point mutations to three GATA sites, three primers were synthesized and used for *in vitro*

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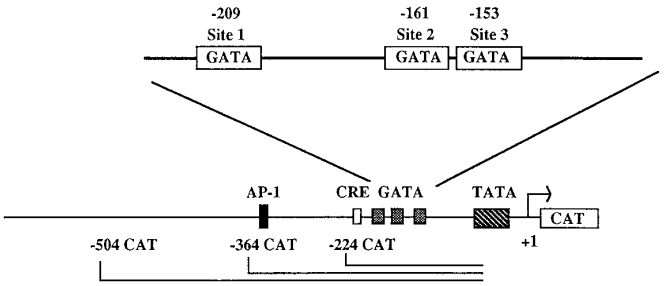

FIG. 1. Schematic representation of 5′-flanking region of hAQP-2 gene. Transcription start site (3) is shown as +1.

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                    10          20          30          40
clone1    MEVAPEQPRW MAHPAVLNAH DPDSHHPGLA HNYMEPAQLL
          :::::::::: :::::::::: :::::::::: :::::::  :
hGATA2    MEVAPEQPGW MAHPAVLNAH DPDSHHPGLA HNYMEPAHVL

                    10          20          30          40
clone2    MEVTTDQPRW VSHHHPAVLN GQHPDTHHPG LGHSYMDPAQ
          :::: ::::: :::::::::: :::::::::: : ::::: ::
hGATA3    MEVTADQPRW VSHHHPAVLN GQHPDTHHPG LSHSYMDAAQ
          :::::::::: :::::::::: :::::::::: : :::: :
mGATA3    MEVTADQPRW VSHHHPAVLN GQHPDTHHPG LGHSYMEAQY
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FIG. 2. Amino acid sequences of clones 1 and 2 and their alignment with hGATA-2, hGATA-3, and mGATA-3. Colons indicate an identical amino acid residue between GATA factors.

mutagenesis system purchased from Promega (Altered Site *in vitro* Mutagenesis Kit). The primers for mutations were 5′-CCCACAGGC-CTAT**TA**CCCTATCTTGGC for -153GATA, 5′-CCTATCACCCTA-T**T**TTGGCCTTCACAG for -161GATA, and 5′-TTAATGAGCTCC-**AA**ATAAGACTGACG for -209GATA. GATA motifs are underlined and the mutations are shown in bold.

Cell culture and transfection of DNA. Mouse outer medullary collecting duct (OMCD) cells, prepared as described previously (15), were a generous gift from Dr. H. Endou (Kyorin Univ., Tokyo, Japan). About 5×10^7 cells growing in exponential phase were harvested and resuspended in K-PBS buffer (30.8 mM NaCl, 120.7 mM KCl, 1.46 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 10 mM $MgCl_2$) containing plasmid DNA (30 μg of the CAT reporter gene constructs containing various fragments of 5′-flanking region of the hAQP-2 gene; and, for assessing transfection efficiency, 5 μg of SV40-βGAL, a plasmid containing the β-galactosidase under the control of the SV40 early promoter). Electroporation was performed in a 0.4 mm width cuvette under the condition of 360 volts at a setting of 960 μF. Forty eight hours after transfection, the cells were harvested for CAT assay or β-galactosidase assay. Data were expressed as means±SD (n=3).

RESULTS

Cloning of rat GATA factors from rat kidney cDNA library. Reverse transcription PCR using microdissected collecting ducts as a starting material yielded PCR product of expected size (~300 nt.). Sequencing the PCR products confirmed the existence of mRNA encoding a GATA factor(s) in rat collecting ducts. Since the amino acid sequences within zinc-finger domain of GATA factors were highly homologous among several previously identified GATA factors, we could not determine whether the PCR clone was part of a cDNA encoding a new GATA factor or one of the previously identified ones. Accordingly, we screened the rat kidney cDNA library using the PCR clone as a probe. Four independent clones were isolated and subcloned. Restriction enzyme analysis revealed that three clones were part of the same cDNA. We picked the longest one (3.0 kb insert), and designated it as clone 1. Another clone which had 2.8 kb insert showed a different pattern of restriction enzyme analysis, and we designated it as clone 2. Sequencing of both clones revealed that clone 1 and 2 had over ~95% amino acid identity with human GATA-2 and -3, respectively. Fig. 2 shows partial amino acid sequences of clone 1 and 2 from the first methionine, and their alignment with those of human GATA-2, and human and mouse GATA-3. Although amino terminal portion of each GATA factor, e.g. human GATA-2 and human GATA-3, had little sequence similarity (8,16), the species difference within each GATA factor was small (5,8,16-18). As shown in Fig. 2, clone 1 and 2 were almost identical to hGATA-2 and hGATA-3, respectively. Therefore, we concluded that the clones we isolated were rat homologues of human or mouse GATA-2 and -3.

Identification of the site of expression of rGATA-2 and -3 along rat nephron segments by RT-PCR. Since the whole kidney cDNA library was screened to isolate GATA factors, the clones we isolated may not have been expressed in the collecting ducts. To identify the sites of expression in rat nephron segments, we performed reverse transcription PCR analysis using dissected nephron segments. As shown in Fig. 3, rGATA-2 was mainly expressed in glomeruli, and rGATA-3 was expressed in glomeruli and cortical and medullary collecting ducts. Based on this observation, we concluded that the GATA factor that could interact with hAQP-2 promoter was GATA-3.

Transactivation of hAQP-2 promoter by rGATA-3. We next determined the function of rGATA-3 in the promoter activity of hAQP-2 gene. CAT plasmids driven by various hAQP-2 promoter regions (30 μg) were co-transfected with pSV-rGATA3 or pSV-vector

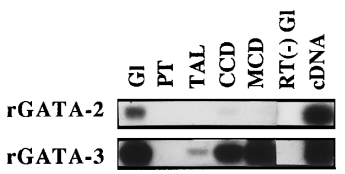

FIG. 3. rGATA-2 and rGATA-3 expression along rat nephron segments. GL, glomerulus; PT, proximal tubule; TAL, thick ascending limb of Henle's loop; CCD, cortical collecting duct; MCD, medullary collecting duct. Ten glomeruli and 2 mm tubules were dissected out from collagenase-treated rat kidney, reverse-transcribed, and then used for PCR for rGATA-2 and rGATA-3. GATA-2 and -3 cDNA indicate the positive control for PCR amplification of each GATA factor. RT(−) indicates the sample without reverse-transcriptase.

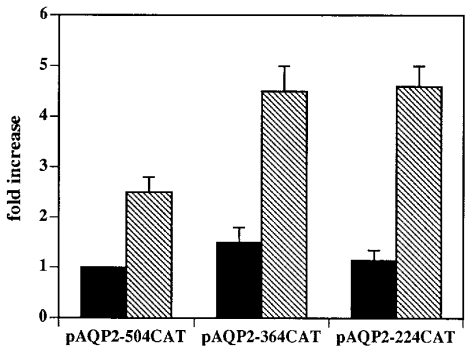

FIG. 4. Transactivation of hAQP-2 promoter by rGATA-3. 15 μg of pSV-SPORT1 vector only (closed column) or pSV-GATA3 (hatched column) was co-transfected with CAT plasmid (30 μg) driven by various hAQP-2 5′-flanking regions into the OMCD cells. pSV-βGAL (5 μg) was also transfected to assess transfection efficiency. CAT activity corrected by β-GAL activity in pAQP2-504CAT was expressed as 1. Data are means±SD (n=3).

alone (5-15 μg). pSV-rGATA3 was constructed by subcloning SalI and NotI cut fragment of clone 2 into pSV-SPORT1 (Life Technologies, Inc.) that had SV40 early promoter. We verified by sequencing and *in vitro* translation analysis that clone 2 contained a whole open reading frame (data not shown). As shown in Fig. 4, rGATA-3 transactivated the AQP-2 promoter activity 3- to 4-fold in different AQP2-CAT constructs. The magnitude of transactivation was dependent on the amount of pSV-rGATA3 plasmids up to 10 μg and almost saturated at 15 μg (data not shown).

Role of three GATA motifs. To prove that GATA-3 transactivate AQP-2 promoter by specifically interacting with the GATA motifs in the 5′-flanking region of the AQP-2 gene, and next, to determine which GATA motif is most important, we generated the CAT constructs in which GATA site was mutated singly or simultaneously. Fig. 5 demonstrates that the mutations of all three GATA motifs abolished the transactivation by GATA-3, indicating that the transactivation by GATA-3 was mediated by these GATA motifs. Then, we tested the effect of single mutation at site 1, 2 or 3 on the transactivation by GATA-3. Transactivation was abolished by the mutations at either site 1 or 2 in the same manner that it was by mutations at all three sites, whereas the mutation at site 3 had no effect (Fig. 5). These results indicated that the transactivation by GATA-3 needed the two GATA sites, namely both site 1 and 2.

DISCUSSION

Cloning of GATA factors from kidney cDNA library and the identification of the sites of expression of these clones revealed that GATA factors were expressed constitutively in different nephron segments. Although the existence of GATA-2 and -3 in chicken kidney was previously identified by Yamamoto et al.(5), the role of GATA factors in kidney has not been identified. In the present study, we identified that GATA-3 is constitutively expressed in collecting ducts and that it transactivates AQP-2 promoter. The AQP-2 promoter is the first gene identified in kidney that was targeted by GATA factor. Recently, an increasing number of promoters involved in the tissue-specific expression have been found to be regulated by GATA factors (4,6,7, 9,10). In the case of gastric H^+/K^+-ATPase gene, the gastric epithelium-specific GATA factors (GATA-GT1 and -GT2) may have an important role in gastric epithelium-specific expression of H^+/K^+-ATPase (7). AQP-2 expression is also restricted to kidney, especially in collecting ducts (1). The existence of three GATA motifs in the 5′-flanking sequence led us to speculate that the GATA factors in kidney may be involved in the kidney-specific expression of AQP-2. Screening and sequencing so far conducted has not led to the identification of a kidney-specific GATA factor. However, we were able to confirm that GATA-3 was expressed specifically in collecting ducts among nephron segments other than glomeruli and that it could transactivate the AQP-2 promoter. Recently, Labastie et al. reported that GATA-3 is expressed in collecting duct and mesangial cells in developing human kidney (19); a result which is consistent with our identification of rat GATA-3 localization in adult kidney.

In the transactivation experiments, we found that the mutation of either one of the two GATA sites significantly reduced the transactivation by GATA-3. There were other reports in which multiple GATA motifs were present in the regulatory region of promoter (10,20). In the case of erythroid Kruppel-like factor

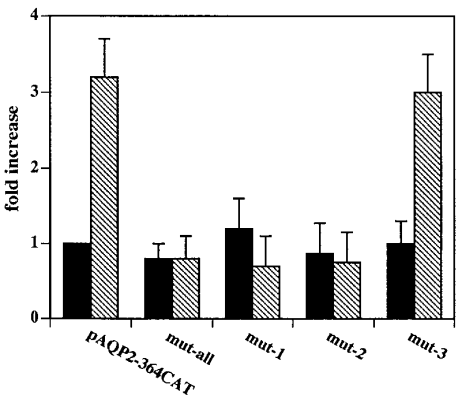

FIG. 5. Effect of point mutations in three GATA sites on the transactivation of hAQP-2 promoter by GATA-3. 30 μg of pAQP2-364CAT or mutant constructs and 15 μg of pSV-rGATA3 were used for transfection. Closed column, pSV-SPORT1; hatched column, pSV-rGATA3. Data are means±SD (n=3). Mut-1, 2, and 3 indicate constructs with the point mutation in each GATA site, respectively. Mut-all had the mutations in all three GATA sites.

gene (20), one GATA-like motif and two GATA motifs were present in the 5′-flanking region of the gene. Among them, only one site was responsible for the transactivation by GATA-1 (20). Rat BNP promoter also had two GATA motifs (10), but the single mutation did not significantly affect the activation by GATA-4, suggesting that either of the GATA sites was sufficient for maximal transactivation. In AQP-2, two binding sites (site 1 and site 2) were necessary for full activation. The exact mechanism(s) why two binding sites are required for full activation by GATA-3 remains to be elucidated.

In summary, hAQP-2 promoter contains two functional GATA sites, and GATA-3 may regulate the hAQP-2 expression in collecting duct *invivo* through these GATA sites.

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