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TTF-1, a Homeodomain-containing Transcription Factor, Participates in the Control of Body Fluid Homeostasis by Regulating Angiotensinogen Gene Transcription in the Rat Subfornical Organ*[S]

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In recent years, it has become increasingly evident that angiotensins synthesized in the brain contribute to regulating body fluid homeostasis. Although angiotensinogen, the unique angiotensin precursor, is produced in the brain, the factors that regulate its gene expression remain unknown. We recently found that TTF-1, a homeodomain-containing transcription factor essential for the development of the fetal diencephalon, is postnatally expressed in discrete areas of the hypothalamus. We now report that the subfornical organ, an important site of angiotensinogen synthesis, is an extrahypothalamic site of TTF-1 expression. Double *in situ* hybridization histochemistry demonstrated the presence of TTF-1 mRNA in angiotensinogen-producing cells of the rat subfornical organ. RNase protection assays showed that TTF-1 and angiotensinogen mRNA levels are simultaneously increased in the subfornical organ by water deprivation. The angiotensinogen promoter contains seven presumptive TTF-1 binding motifs, four of which are recognized by the TTF-1 homeodomain. In the C6 glioma cell line, TTF-1 transactivates the angiotensinogen promoter in a dose-dependent manner. This transactivation is abolished by deletion of the TTF-1 binding motif at −125. Intracranial administration of an antisense TTF-1 oligodeoxynucleotide decreased angiotensinogen mRNA in the subfornical organ and dramatically reduced the animal's water intake while increasing urine excretion. Moreover, plasma arginine vasopressin content was decreased by the same treatment. These results demonstrate a novel role for TTF-1 in the regulation of body fluid homeostasis, exerted via the transactivational control of angiotensinogen synthesis in the subfornical organ.

The control of body fluid volume and blood pressure is a critical homeostatic process required for normalcy of the internal environment and for the preservation of life. Many studies have demonstrated that the renin-angiotensin system (RAS)[1] plays an important role in the regulation of fluid volume balance, blood pressure, and other related biological responses through the generation of angiotensin II (ANG II) (1). The existence of a brain RAS in addition to the peripheral RAS has also been established (2, 3). All components of the peripheral RAS have been found in the brain, most notably in the subfornical organ (SFO). The SFO is a circumventricular organ (CVO) of the lamina terminalis located at the top of the dorsal third ventricle (4). It is composed of fenestrated capillary loops in addition to glial cells, but in contrast to other CVOs such as the median eminence of the hypothalamus, it also contains neuronal cell bodies (1, 5). The SFO of rats contains high concentrations of angiotensinogen (AoGen) (6), the angiotensin precursor; renin-like activity (that converts AoGen to ANG I) (7); angiotensin-converting enzyme (which converts ANG I to ANG II) (8); the peptide ANG II (9); and AT_1-type ANG II receptors (3). Thus, the SFO is not only a target for circulating ANG II, which exerts a potent dipsogenic effect, but also represents a local site of ANG II production, believed to be important in the regulation of body fluid homeostasis (1).

Given the importance of AoGen in the pathogenesis of ANG II-dependent hypertension (10), it is critically important to acquire a more complete understanding of the mechanisms regulating central AoGen gene expression. The genomic structure of the AoGen gene, including its 5′-flanking region, has been characterized (11–13), and recently AoGen-activating elements in the proximal promoter region of the AoGen gene were found to be essential for the regulation of AoGen gene expression in the liver (14) and for the AoGen-dependent con-

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[1] The abbreviations used are: RAS, renin-angiotensin system; ANG, angiotensin; AoGen, angiotensinogen; AS, antisense; AVP, arginine vasopressin; C/EBP, CCAAT-box/enhancer-binding protein; CVO, circumventricular organ; ODN, oligodeoxynucleotide; PVA, anterior thalamic paraventricular nucleus; SCR, scrambled; SFO, subfornical organ; TTF-1HD, TTF-1 homeodomain; GFAP, glial fibrillary acidic protein; RPA, RNase protection assay; SON, supraoptic nucleus; EMSA, electrophoretic mobility shift assay.

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trol of blood pressure by both the liver (15) and brain (16). Nevertheless, the identity of the binding protein(s) recognized by these AoGen-activating elements remains unknown.

Here we report that the homeodomain-containing transcription factor, TTF-1, regulates AoGen gene transcription in the SFO. TTF-1 was first found in the thyroid gland (17, 18) and then shown to also be expressed in the embryonic diencephalon and lung (19). Recently, we and others reported that TTF-1 remains expressed in discrete regions of the postnatal rat brain (20, 21). In the hypothalamus, TTF-1 is present in sets of neurons containing luteinizing hormone-releasing hormone, enkephalin, and pituitary adenylate cyclase-activating polypeptide (20, 22), in addition to ependymoglial cells lining the third ventricle and astrocytic tanycytes of the median eminence, where it is coexpressed with the epidermal growth factor-related receptor ErbB2 (20). We also showed that TTF-1 binds to specific recognition motifs present in each one of these promoters to regulate transcriptional activity (20, 22).

In the present report, we identify the SFO as an unexpected site of TTF-1 expression in the brain. Because the SFO is a prominent site of AoGen synthesis, we sought to determine whether there is a functional, hierarchical relationship between TTF-1 and AoGen. The results of this study demonstrate that both mRNAs are indeed expressed by the same cells of the SFO, that TTF-1 regulates AoGen transcription via binding to a specific binding motif in the 5′-flanking region of the AoGen gene, and that inhibition of TTF-1 synthesis by antisense oligodeoxynucleotide (ODN) administration decreases AoGen synthesis and leads to a decrease in water intake and an increase in urine excretion.

EXPERIMENTAL PROCEDURES

Animals and Experimental Design—This study was conducted in accordance with the University of Ulsan Regulations for the care and use of experimental animals. Two-month-old male Sprague-Dawley rats (body weight 250–300 g; Daehan Animal Breeding Company, Chungwon, Korea) were used. Upon arrival, they were housed in a room with a conditioned photoperiod (12 h of light/12 h of darkness, lights on from 7:00 a.m. to 7:00 p.m.) and temperature (23–25 °C) and allowed *ad libitum* access to tap water and pelleted rat chow. For water deprivation experiments, drinking water was withdrawn for 48 h starting at 10:00 a.m. with food available *ad libitum*. The animals were sacrificed by decapitation, and their brains were quickly removed for RNA extraction. To dissect the SFO, brains were placed in a brain matrix and sliced midsagittally. The SFO tissue was bilaterally cut with microdissecting scissors according to Paxinos and Watson's atlas (4) and stored at −80 °C until use. Trunk blood was collected into heparinized tubes for the measurement of plasma osmolality.

Double in Situ Hybridization—To localize TTF-1 and AoGen mRNAs in the SFO, the brains of four rats were fixed by transcardiac perfusion of 4% parformaldehyde-borate buffer, pH 9.5, as recommended (23). After an overnight postfixation in the same fixative containing 10% sucrose, the brains were blocked and frozen at −85 °C until use. Thirty-μm sections were then prepared with a freezing sliding microtome and processed for hybridization as reported (24). The hybridization procedure was that recommended by Simmons *et al.* (23), as described earlier by us (24), using a [^{35}S]UTP-labeled TTF-1 cRNA probe (20) and a digoxygenin-UTP-labeled AoGen cRNA. The latter was transcribed from a 205-bp AoGen cDNA template generated by PCR amplification (see below for additional details). Following an overnight hybridization at 45 °C, the slides were washed and processed for digoxygenin detection as described (24). After dehydration, the slides were dipped in Ilford K5 emulsion (without defatting) instead of the NTB-2 emulsion used for isotopic hybridization and were exposed to the emulsion for 3 weeks at 4 °C. At this time, the slides were developed, quickly dehydrated, dried, and coverslipped for microscopic examination.

Combined Immunohistochemistry-in Situ Hybridization—To determine if TTF-1 is expressed in neurons or glial cells of the SFO, these cell types were identified by immunohistochemistry, and the presence of TTF-1 mRNA was assessed using the [^{35}S]UTP-labeled TTF-1 mRNA described above. Neurons were identified using a monoclonal antibody against NeuN, a neuron-specific nuclear protein (25). This antibody stains the nuclei of most neurons of the central and peripheral nervous system, with the exception of some subsets, such as cerebellar Purkinje cells, olfactory bulb mitral cells, and retinal photoreceptor cells (25). The antibody was purchased from Chemicon (Temecula, CA) and used at a 1:200 dilution. Astroglial cells were visualized with rabbit polyclonal antibodies against glial fibrillary acidic protein (GFAP) obtained from Dako (Carpinteria, CA) and used at a 1:500 dilution. The brains were fixed and sectioned as outlined above and then subjected to immunohistochemistry using a procedure described elsewhere (26, 27). A modification to the method was the addition of a ribonuclease inhibitor to the immunoreactions. The ribonuclease inhibitor SUPERaseIn (Ambion, Austin, TX) was added (at 100 units/ml) to the solutions containing both the primary and secondary antibodies. Prior detection of ribonuclease activity in the different solutions used for immunohistochemistry using a fluorometric RNase detection assay (RNaseAlert™ Lab Test kit, Ambion) demonstrated that only these two solutions had detectable ribonuclease levels. Upon completion of the immunohistochemical reaction, the sections were mounted on glass slides and were dried overnight in a vacuum oven. The next day they were hybridized with the TTF-1 cRNA. Control sections were incubated with a sense TTF-1 probe transcribed from the same plasmid but linearized on its 3′-end to transcribe the coding strand of TTF-1. Following posthybridization washes, the sections were exposed to NTB-2 emulsion for 3 weeks. At this time, the reaction was developed, and the sections were counterstained with 1% methyl green before microscopic examination.

TABLE I
EMSA oligodeoxynucleotide probes

Each sequence represents the sense strand of probes; core TTF-1 binding motifs are underlined.

Location in the 5′-flanking region of AoGen gene	Probe sequences
C	5′-CACTGCCCAGTCAAGTGTTCTTGA-3′
Cβ	5′-CACTGCCCAGTCACGCGTTCTTGA-3′
−643	5′-CCACACTCAAGGGCGGTG-3
−591	5′-CCATCCACAAGCCCAGAAC-3′
−446	5′-CAACCATTCAAGGTCGTCC-3′
−329	5′-CTTACGTCCCAAGGTCTGCATG-3′
−135	5′-CCCTGGCCTTGCTCCATC-3′
−125	5′-GCTCCATCTTGGCTAAGC-3′
−14	5′-AAGGCTGCTTGGTTCACCAG-3′

RNA Extraction and RNase Protection Assay (RPA)—RPA was used to quantitate changes in TTF-1 and AoGen mRNA abundance in the SFO and arginine vasopressin (AVP) mRNA in the supraoptic nucleus (SON). Total RNA was isolated as reported (28) and subjected to RPA using the procedure previously described by Ma *et al.* (29) with some modifications (30). The preparation of antisense RNA probes for TTF-1, AoGen, AVP, and cyclophilin followed a procedure described in detail elsewhere (20, 31, 32). RNA samples (5 μg/tube) or different amounts of *in vitro* synthesized TTF-1 and AoGen sense RNA (0.06–1 pg/tube) were hybridized with 500,000 cpm of ^{32}P-labeled TTF-1, AoGen, and AVP cRNA probes, respectively, for 18–20 h at 45 °C. The tissue RNA samples were simultaneously hybridized to a cyclophilin cRNA probe (5000 cpm/tube) to correct for procedural losses. At the completion of hybridization, the samples were treated with ribonucleases A and T1 to digest unhybridized RNA species. The protected cRNA fragments were separated on a polyacrylamide-urea gel (5% acrylamide, 7 M urea), and the hybridization signals were detected by exposure of dried gels to X-Omat x-ray film (Eastman Kodak Co.). The intensity of the signals obtained was quantified as reported (29, 30).

PCR Cloning of an AoGen cDNA Fragment and 5′-Flanking Region of the AoGen Gene—A cDNA fragment of AoGen was cloned by reverse transcription-PCR from rat liver RNA. The specific primer sets (sense primer, 5′-CCT GAA GGC CAC CAT CTT CT-3′; antisense primer, 5′-CAG GGT CTT CTC ATC CAC G-3′) were designed to generate a 205-bp AoGen cDNA fragment corresponding to nucleotides 74–278 in rat AoGen mRNA (NCBI GenBank™ data base, accession number L00091). A rat AVP cDNA fragment was also cloned by reverse transcription-PCR from the rat hypothalamic RNA. The specific primer sets (sense primer, 5′-GCA AGG CTT CTG GCC AGA CT-3′; antisense primer, 5′-CGC CAT GAT GCT CAA CAC TA-3′) were designed to generate a 283-bp AVP cDNA fragment corresponding to nucleotides 26–308 in rat AVP mRNA (NCBI GenBank™ data base, accession number M25646).

The proximal promoter of the rat AoGen gene (−689 to +49 bp) to be employed for promoter analysis was also cloned by PCR from rat liver

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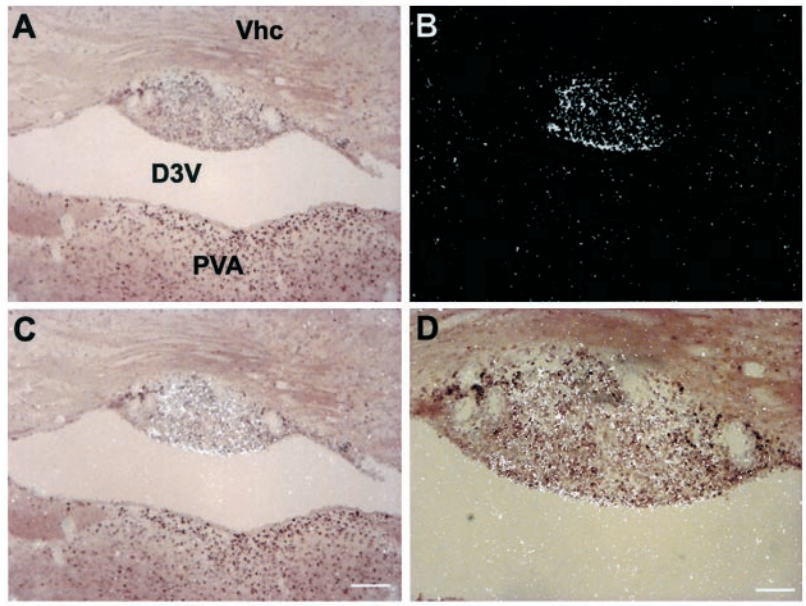

FIG. 1. **Localization of TTF-1 and AoGen mRNA in the rat SFO.** Coronal sections were simultaneously hybridized with a digoxygenin-labeled AoGen cRNA probe (*purple*) and a ^{35}S-labeled TTF-1 cRNA probe (*white grains*). *A*, bright field image showing abundance of AoGen mRNA-containing cells in the SFO and in the anterior thalamic PVA. *B*, dark field image showing that TTF-1 mRNA is only expressed in the SFO. *C*, merged image showing the colocalization of TTF-1 and AoGen mRNAs in cells of the SFO and the absence of TTF-1 mRNA in the PVA. *D*, a higher magnification image highlighting the colocalization of TTF-1 and AoGen mRNAs in cells of the SFO. *Vhc*, ventral hippocampal commissure; *D3V*, dorsal third ventricle; *bars*, 200 μm (*A–C*) and 100 μm (*D*).

DNA using the sequence information deposited in the NCBI GenBank™ data base (accession number M12113). The sense primer was 5′-GGA TCC ACC CGT CTC ATT CTC-3′, and the antisense primer was 5′-CAA GAG GGC TCT GCT TAC CTT-3′. The resulting DNA fragment was inserted into the luciferase reporter plasmid (pGL-3 basic; Promega, Madison, WI), and its sequence was confirmed by DNA sequencing. Mutant AoGen promoter constructs carrying deletions of the TTF-1 binding motifs were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions; the intended mutations were confirmed by sequencing.

Cell Culture and Assays for Luciferase Activity—AoGen promoter analysis was performed in C6 glioma cells, because they endogenously express AoGen (see Supplementary Fig. 1). C6 cells were grown in Dulbecco's modified Eagle's medium/F-12 containing 10% fetal bovine serum. Twenty-four h after seeding the cells in six-well plates, they were transiently transfected with the AoGen promoter-luciferase reporter construct (AoGen-P) using LipofectAMINE (Invitrogen) along with different concentrations (100–500 ng/well) of the expression vector, pcDNA 3.1-zeo (Invitrogen), containing the TTF-1 coding region (TTF-1-pcDNA) (20, 22). Transfection efficiency was normalized by cotransfecting the β-galactosidase reporter plasmid (pCMV-β-gal; Clontech, Palo Alto, CA) at 20 ng/ml. The transfected cells were harvested 48 h after transfection for luciferase and β-galactosidase assays as reported (20).

Electrophoretic Mobility Shift Assay (EMSA)—The procedure for expression and purification of the TTF-1 homeodomain (TTF-1HD) protein has been described (33). Double-stranded oligodeoxynucleotides, labeled with ^{32}P at the 5′ terminus, were used as probes for the EMSA. Sequences of the oligodeoxynucleotides used are shown in Table I. Oligodeoxynucleotides C and Cβ were used as a positive and negative control, respectively (34). EMSA was performed as previously described (22, 33, 34). Electrophoretically separated signals corresponding to the protein-bound and free DNA were quantitated with Multi-analyst software. Binding of TTF-1HD to oligodeoxynucleotides representing different regions of the AoGen promoter was expressed as a percentage of TTF-1HD binding to oligodeoxynucleotide C, which contains the core TTF-1 binding domain and flanking region of the thyroglobulin gene promoter (17).

To determine endogenous binding activity of nuclear extracts from the SFO to the TTF-1 binding domains in the 5′-flanking region of AoGen gene, nuclear protein fractions from the rat SFO were prepared according to the method of Andrews and Faller (35), utilizing the mixture of protease inhibitors recommended by Kuhn *et al.* (36). The binding assay was performed as previously described (20), using 15 μg of protein and 20,000 cpm of probe. To further confirm the presence of immunoreactive TTF-1 in the nuclear extracts, the proteins were incubated with 1 μl of undiluted TTF-1 antiserum (NeoMarkers, Fremont, CA) or preimmune serum for 30 min at room temperature before initiating the binding reactions.

Intracerebral Administration of Antisense (AS) TTF-1 ODN and Assessment of Water Intake and Urine Output—To determine whether TTF-1 controls AoGen synthesis *in vivo* and hence has a physiological role in the regulation of water intake and urine excretion, a phosphorothioate AS TTF-1 ODN (GenoTech Corp., Daejeon, Korea) was delivered in the vicinity of the SFO. The AS TTF-1 ODN used to disrupt TTF-1 synthesis (5′-GAC TCA TCG ACA TGA TTC GGC GTC-3′) was directed against the sequence surrounding the first ATG codon of TTF-1 mRNA as previously reported (22). As a control, a scrambled (SCR) sequence of identical base composition was used (5′-AGT CCT ACT CGG TAC GTA TGC AGC-3′). For the intracerebral injection, the ODNs were diluted to a final concentration of 100 ng/μl artificial cerebrospinal fluid (22). Under pentobarbital (7.5 mg/kg body weight) and ketamine hydrochloride (25 mg/kg body weight) anesthesia, a polyethylene cannula (outer diameter, 1.05 mm; inner diameter, 0.35 mm) was stereotaxically implanted into the brain with its opening protruding onto the top of the SFO (coordinates: *AP* = 1.3 mm caudal to the bregma; *V* = 4.3 mm from the dura mater; *L* = 0.0 mm from the midsagittal line) as recommended (37). After a week of recovery, the ODNs (2 nmol in 4 μl of artificial cerebrospinal fluid) were injected with a Hamilton syringe, and water intake, urine excretion, and body weight were measured. One day following a single ODN administration, total RNA or nuclear proteins were extracted from the SFO to determine AoGen mRNA and TTF-1 protein content. To determine the effect of AS TTF-1 ODN on renin-induced water intake, renin (1 milliunit/2 μl) was injected 4 h after AS ODN injection through the same cannula, and water intake was determined for 1 h, beginning 1 h after the renin injection.

Western Blot Analysis of TTF-1 Protein—Nuclear protein extracts were prepared according to the method of Andrews and Faller (35), and Western blots using TTF-1 antiserum were performed as reported (22). The proteins were detected with an ECL kit (Amersham Biosciences) according to the protocol provided by the manufacturer; membranes were exposed to x-ray film for 3 min. Standardization of applied protein concentration was done by Western blotting of β-tubulin with specific antibody (Sigma).

Determination of Plasma AVP and ANG II—Plasma AVP and ANG II levels were determined by radioimmunoassay using radioimmunoassay kits (AVP kit from Diasorin Inc., Stillwater, MN; ANG II kit from Mitsubishi Kagaku Bio-Clinical Laboratories Inc., Tokyo, Japan) and the procedure provided by each manufacturer. Blood samples were collected in ice-cold tubes containing EDTA 13 h after ODN injection, and the plasma was separated by centrifugation before the assays. The resulting values are expressed as pg/ml.

Statistics—Changes in water intake and urine excretion were analyzed by a two-way analysis of variance followed by the Student Neuman-Keuls multiple comparison test. Changes observed between two groups were analyzed by Student's *t* test.

RESULTS

TTF-1 and AoGen mRNA Are Coexpressed in the SFO—Double *in situ* hybridization demonstrated that TTF-1 mRNA (white grains) is abundantly expressed in AoGen mRNA-

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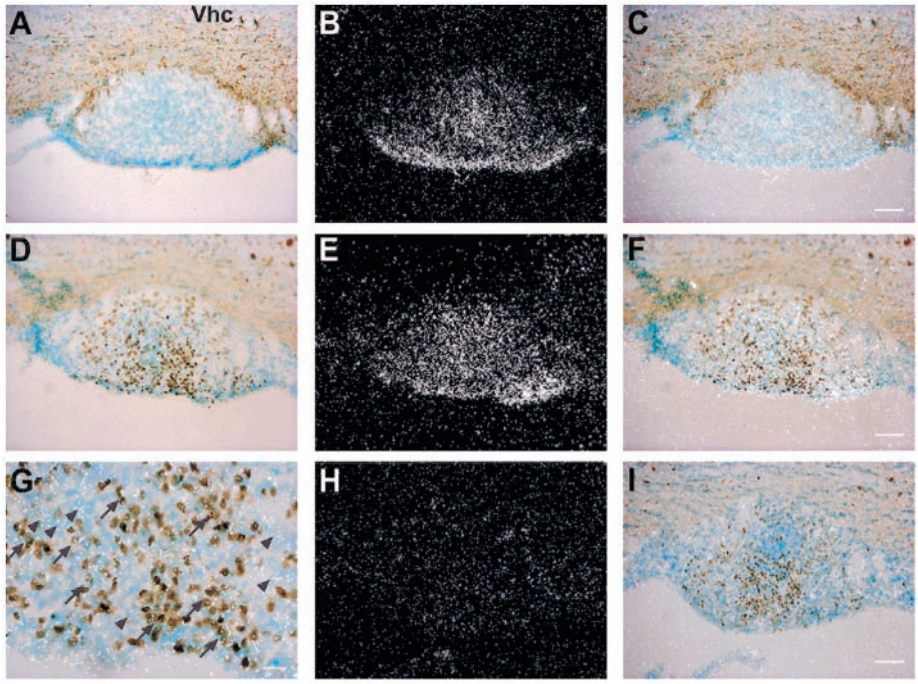

FIG. 2. **Detection of TTF-1 mRNA in neurons and nonneuronal cells of the rat SFO.** TTF-1 mRNA (*silver grains*) was detected with a ^{35}S-labeled cRNA probe. Astroglial cells (*brown*) were identified by immunohistochemistry using specific antibodies against the astrocytic marker GFAP (*A* and *C*). Neurons (also *brown*) were identified using antibodies against the neuron-specific nuclear protein NeuN (*D*, *F*, *G*, and *I*). *A*, bright field image showing the absence of GFAP-positive cells (*brown*) in the innermost portion of the SFO. Most GFAP-positive cells are seen in the region that separates the SFO from the ventral hippocampal commissure (*Vhc*). *B*, dark field image showing the presence of TTF-1 mRNA-containing cells in the SFO. *C*, merged image showing TTF-1 mRNA in GFAP-negative cells of the SFO. *D*, neurons are mostly localized to the central region of the SFO. *E*, dark field image showing the presence of TTF-1 mRNA in cells located in the central region of the SFO. *F*, merged image showing that whereas TTF-1 mRNA is expressed in some neurons, most of the hybridization signal is present in nonneuronal cells of the SFO (*blue* nuclear staining with no brown NeuN immunoreactivity). *G*, higher magnification of a merged image showing the presence of TTF-1 mRNA in some neurons (NeuN-positive cells) of the SFO (*arrows*) and its expression in many nonneuronal cells (*arrowheads*) of the organ. *H*, dark field image showing the absence of hybridization of a TTF-1 sense probe to cells in the SFO. *I*, merged image showing the absence of hybridization of the sense TTF-1 RNA probe to either neurons or nonneuronal cells of the SFO. *Bars*, 100 μm (*A–F*, *H*, and *I*) and 20 μm (*G*).

containing cells of the SFO (*purple*) (Fig. 1). In contrast, no TTF-1 hybridization signal was detected in AoGen mRNA-containing cells of the anterior thalamic paraventricular nucleus (PVA). TTF-1 mRNA was abundant in cells throughout the central region of the SFO, but AoGen mRNA-containing cells located in the lateral regions showed scarcely any TTF-1 hybridization (Fig. 1, *C* and *D*).

TTF-1 mRNA Is Present in Neurons and Nonneuronal, GFAP-negative Cells of the SFO—To determine whether the TTF-1 gene is expressed in neurons or glial cells of the SFO, we performed combined immunohistochemistry-*in situ* hybridization experiments. As shown in Fig. 2, most (if not all) GFAP-positive cells detected with the polyclonal antibodies used were confined to the internal portion of the SFO in a region that separates the SFO from the ventral hippocampal commissure and had no detectable TTF-1 signal (Fig. 2, *A–C*). In contrast, neurons identified by the presence of NeuN immunoreactivity were mostly located in the central portion of the SFO (Fig. 2*D*), and some of them were TTF-1 mRNA-positive (Fig. 2, *F* and *G*). Surprisingly, most TTF-1 mRNA-positive cells contained neither NeuN nor GFAP immunoreactivity (Fig. 2, *C–G*), suggesting that they are tanycytes and/or astrocytic tanycytes known to make up the majority of cells present in circumventricular organs such as the SFO (38), the median eminence of the hypothalamus (39), and the subcommissural organ (40). Although these cells are considered to be glial, astrocytic tanycytes (39) are for the most part GFAP-negative (40, 41). This cellular distribution is shown at a higher magnification in Fig. 2*G*. Sections incubated with the sense TTF-1 RNA probe did not show specific hybridization signals (Fig. 2, *H* and *I*).

Both TTF-1 and AoGen mRNA Content in the SFO Increased after Water Deprivation—In agreement with the hybridization histochemistry results, both TTF-1 and AoGen mRNAs, simultaneously detected by RPA (Fig. 3*A*), were present in the SFO (Fig. 3*B*). Water deprivation significantly increased both mRNA levels compared with the euhydrated control group (Fig. 3*C*), as assessed after 48 h of water deprivation. In contrast to the relatively small (but statistically significant) change found in TTF-1 mRNA levels, the content of TTF-1 protein was greatly increased by water deprivation (Fig. 3*D*). In harmony with these changes, water-deprived rats showed a significant increase in plasma osmolality and a decrease in body weight compared with control animals (Table II).

Identification of Putative TTF-1 Binding Motifs in the Rat AoGen Promoter—To find potential TTF-1 binding motifs in the 5′-flanking region of the rat AoGen gene, the promoter sequence generated by PCR amplification (see above) was analyzed using a search program provided on the World Wide Web at molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html. Several putative consensus motifs for transcription factors were detected (Fig. 4), including binding sites for CCAAT-box/enhancer-binding protein (C/EBP), cAMP-response element binding protein, GATA-1, and glucocorticoid receptor. Based on the sequence identity with reported conserved motifs of TTF-1 binding domains (42), seven putative TTF-1 binding domains were detected (Fig. 4, *double underline*).

TTF-1 Transactivates AoGen Promoter Activity—Functional analysis of the AoGen promoter demonstrated that this promoter is transcriptionally active over a wide range of concentrations in C6 cells (Fig. 5*A*). Cotransfection with different concentrations of a TTF-1 expression vector resulted in a dose-dependent increase in AoGen promoter activity (Fig. 5*B*). En-

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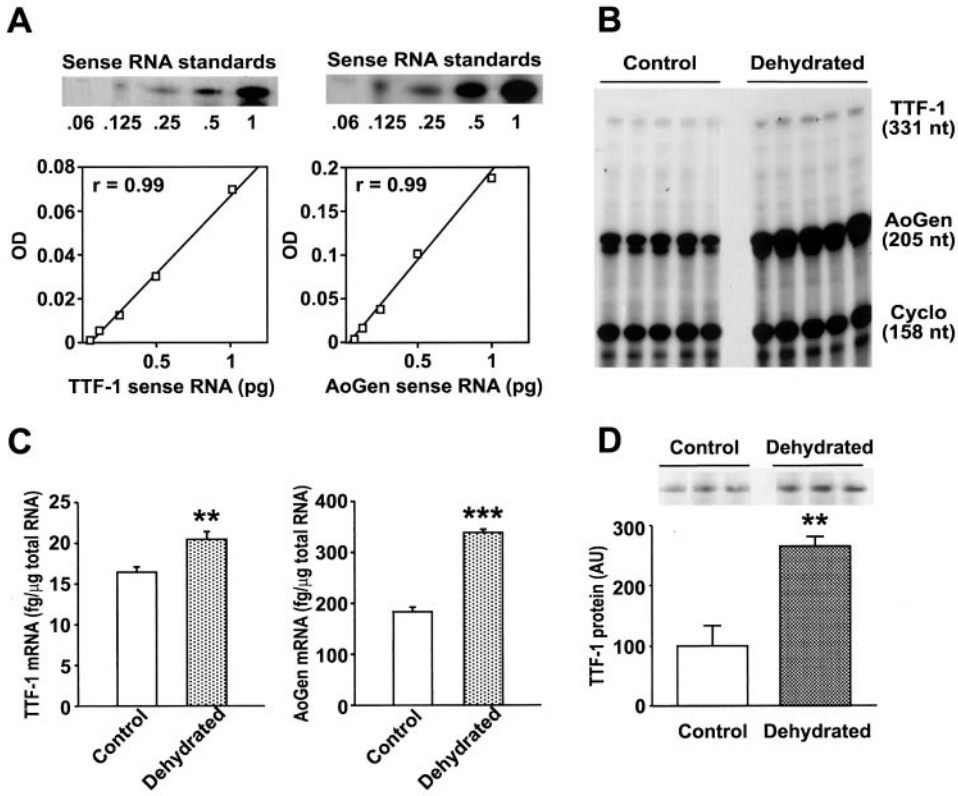

FIG. 3. **Changes in TTF-1 and AoGen mRNA levels in the SFO induced by water deprivation.** *A*, autoradiographs of TTF-1 and AoGen mRNA standards generated by hybridization of [^{32}P]UTP-labeled cRNA probes to increasing amounts of *in vitro*-transcribed TTF-1 and AoGen sense RNA standards, followed by ribonuclease digestion and gel electrophoresis to separate the protected mRNA species. Linear regression analyses of the hybridization signals are depicted *below* the *autoradiograms*. *B*, representative autoradiograms demonstrating the increase in TTF-1 and AoGen mRNA content in the SFO induced by water deprivation (*Dehydrated*). In all cases, cyclophilin mRNA (*Cyclo*) was used as an internal mRNA control to normalize the signals generated by the TTF-1 and AoGen mRNAs. *C*, quantification of the increase in TTF-1 and AoGen mRNA levels induced by a 2-day water deprivation in the SFO of adult rats. *D*, effect of water deprivation on the TTF-1 protein level detected by Western blot analysis. Each *bar* represents the mean of two experiments, each performed with 3–5 animals. *Vertical lines* represent S.E. **, $p < 0.01$; ***, $p < 0.001$ *versus* control. *r*, correlation coefficient; *AU*, arbitrary unit; *OD*, optical density.

dogenous expression of AoGen mRNA in C6 cells was also increased by expressing TTF-1 (Supplementary Fig. 1).

TTF-1 Binds to TTF-1 Putative Binding Domains in the 5′-Flanking Region of AoGen Gene—EMSAs were performed to determine the ability of TTF-1HD to recognize the putative TTF-1 binding domains present in the AoGen promoter. Double-stranded oligodeoxynucleotide probes (Table I), containing the presumptive TTF-1 binding motifs and their flanking sequences shown in Fig. 4, were employed. Of the seven putative binding motifs, four were recognized by TTF-1HD (Fig. 6*A*). The site at −643 showed the strongest signal, whereas the sites at −446, −125, and −14 had relatively weak signals. The sites at −591, −329, and −135 showed no binding activity and thus cannot be considered as TTF-1 binding domains.

Nuclear proteins from the rat SFO strongly bound the oligonucleotide probes (−446, −125, and −14) (Fig. 6*B*) that contained the binding motifs used by TTF-1 to transactivate the AoGen promoter (see below) (Fig. 7). The interaction of the labeled probes with SFO nuclear proteins was reduced by the addition of a 50-fold excess of unlabeled oligonucleotide C. In contrast, an oligonucleotide carrying a mutated core TTF-1-binding sequence (Cβ) was ineffective. Preincubation of SFO nuclear proteins with a TTF-1 antibody delayed the migration of the protein-DNA complex, indicating that TTF-1 is indeed part of this complex.

Loss of AoGen Transactivation by TTF-1 after Deletion of the TTF-1 Binding Core Motif at −125—To determine whether the sites at −643, −446, −125, and −14, recognized by TTF-1HD in EMSA assays, are required for TTF-1 to transactivate the AoGen promoter, we deleted each of them by site-directed mutagenesis and examined the ability of TTF-1 to transactivate the mutated promoters. As shown in Fig. 7, TTF-1-dependent transactivation of the AoGen promoter appeared to mostly require the core motif at −125, since deletion of this site resulted in an almost complete loss of TTF-1-dependent transactivation. Whereas deletion of either the −446 or −14 sites partially diminished TTF-1-induced AoGen promoter activity, no decrease was found after deletion of the −643 site. A triple deletion, including the −125 site, decreased TTF-1 transactivational activity to a level similar to that caused by the deletion of the −125 site alone. Deletion of all four TTF-1 binding domains completely abolished TTF-1-dependent activation of the AoGen promoter.

TABLE II
Changes in body weight and plasma osmolality by water deprivation

	Body weight	Plasma osmolality
	g	*mOsm/kgH_2O*
Control, 0 h	267.8 ± 3.3	
Water deprivation, 0 h	265.2 ± 4.0	
Control, 48 h	278.2 ± 4.8	304.2 ± 2.6
Water deprivation, 48 h	221.8 ± 2.8	320.8 ± 2.1[a]

[a] $p < 0.01$ *versus* control animals.

Effect of AS TTF-1 ODN on AoGen mRNA Content in the SFO—To determine whether an *in vivo* decrease in TTF-1 availability results in a diminished steady-state level of AoGen mRNA, a single injection of AS TTF-1 ODN or its corresponding scrambled DNA sequence was administered intracere-

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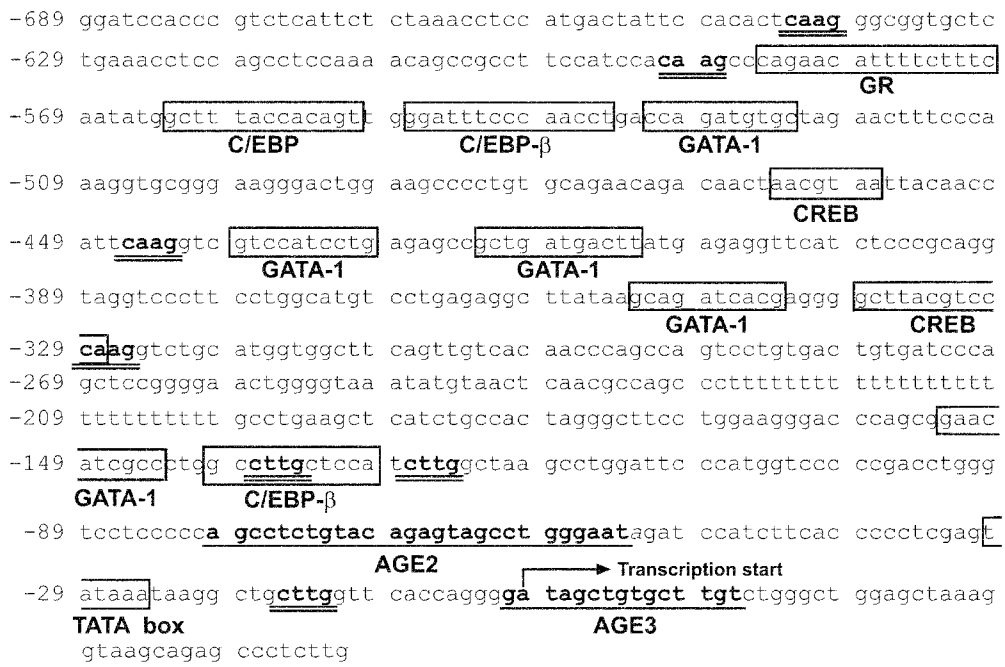

FIG. 4. **Nucleotide sequence of the 5′-flanking region of the rat AoGen gene.** Nucleotides are numbered by assigning position +1 to the transcriptional start site. Potential regulatory factors based on sequence analysis (GenBank™ accession number M12113) are indicated with *boxes*, and the putative TTF-1 binding motifs (5′-CAAG-3′ and 5′-CTTG-3′) are *double underlined*. The TATA box and transcription start site are indicated. AoGen gene-activating elements (*AGE*) (14) 2 and 3 are indicated. CREB, cAMP-response element-binding protein; GR, glucocorticoid receptor.

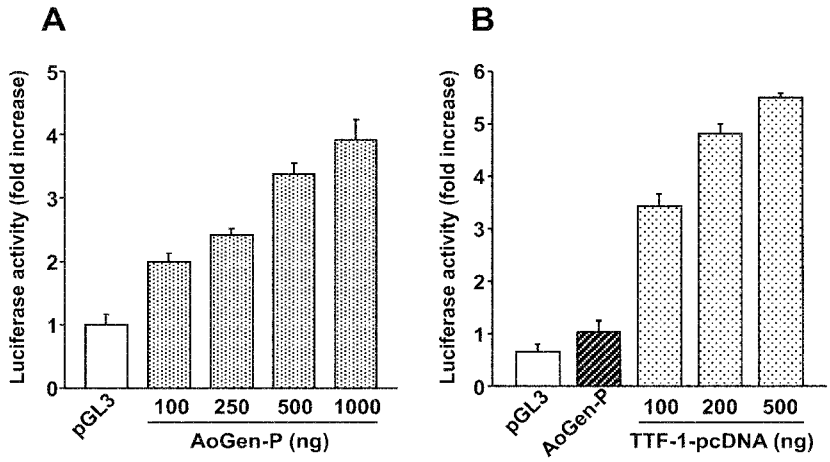

FIG. 5. **Transactivation of the 5′-flanking region of AoGen gene by TTF-1.** Luciferase reporter constructs (pGL3) containing the 5′-flanking region of the AoGen gene (AoGen-P) were co-transfected into C6 cells with an expression vector carrying the rat TTF-1-coding region (TTF-1-pcDNA) at the final concentrations indicated. The cells were harvested for luciferase and β-galactosidase assays 48 h after transfection. *A*, dose-related increase in AoGen promoter activity following transfection of different amounts of AoGen-P. *B*, transactivation of the AoGen promoter by different concentrations of TTF-1. Results are the means ± S.E. of at least six wells per group.

brally, targeting the SFO region of 2-month-old male rats. One day after the injection, SFOs were collected for measurement of TTF-1 protein and AoGen mRNA. As shown in Fig. 8*A*, the TTF-1 antibody detected a ~40-kDa band similar in size to TTF-1, as previously reported for the rat hypothalamus (22). TTF-1 AS ODN-treated animals showed a significant decrease in TTF-1 protein content as compared with both sham-operated controls and SCR ODN-injected animals (Fig. 8, *A* and *B*). Importantly, AoGen mRNA levels were significantly decreased in the SFO of AS TTF-1 ODN-injected rats, as determined by RPA (Fig. 8*C*). However, no change in AoGen mRNA level was observed in the PVA (Fig. 8*D*). Thus, in keeping with its transactivational activity *in vitro*, TTF-1 is required for maintaining AoGen expression specifically in the SFO *in vivo*.

Effect of AS TTF-1 ODN on Water Intake and Urinary Excretion—To determine whether the decrease in AoGen gene expression, a result of TTF-1 synthesis inhibition in the SFO, has physiological consequences, we measured water intake and urinary excretion as indices of body fluid homeostasis. Daily water intake was measured every day for 4 days, beginning 2 days before the ODN injection. As shown in Fig. 9, water consumption remained steady until the time of injection. Twenty-four h after the AS TTF-1 ODN injection, there was a dramatic reduction in daily water intake (Fig. 9*A*). To further characterize this effect, a detailed profile of water intake after ODN injection, assessed as cumulative water intake, was obtained at 3-h intervals for 24 h. In keeping with the reduction in daily water intake, the AS TTF-1 ODN injection decreased cumulative water intake compared with controls (Fig. 9*B*). Significant differences between the AS ODN-injected and two control groups began 10 h after ODN injection and persisted for the remainder of the 24-h period studied.

Renin, an enzyme that converts AoGen to ANG I, is a potent dipsogen that increases water intake within 1 h of its systemic administration. The renin-induced increase in water intake was partially suppressed by the administration of AS TTF-1 ODN 4 h before renin injection (Fig. 9*C*).

Urinary volume, determined in a metabolic cage at daily intervals, was significantly increased 1 day after the injection of AS TTF-1 ODN (Fig. 10*A*). In contrast, no change in body

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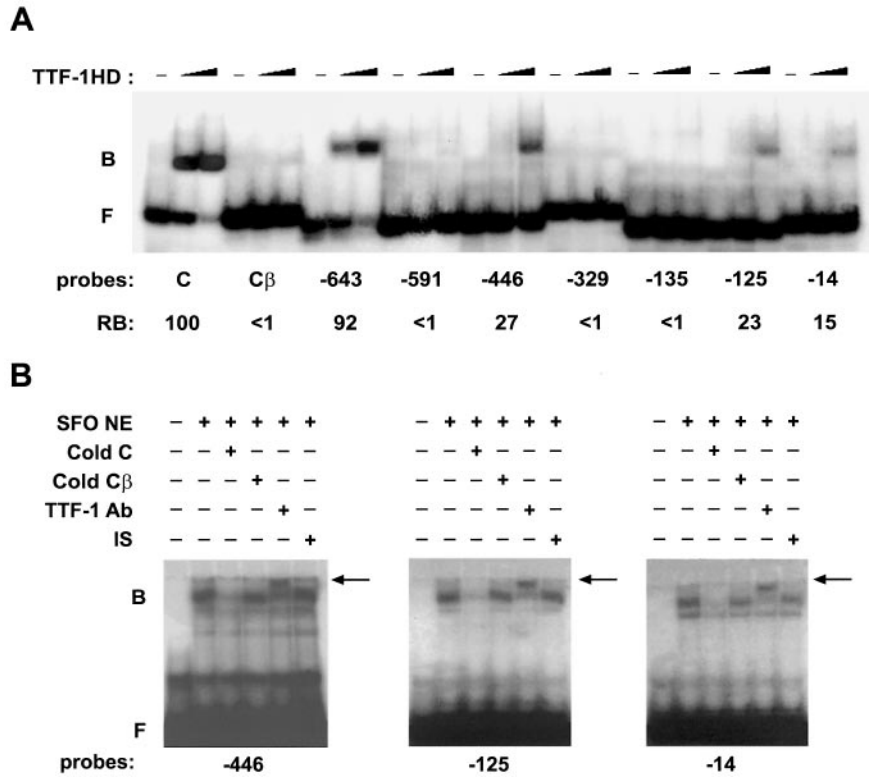

FIG. 6. **EMSAs.** EMSAs were performed with double-stranded oligomer probes containing the putative TTF-1 binding core motifs shown in Table I. *A*, TTF-1HD was used at two concentrations: 75 nM (*second lanes*); 150 nM (*third lanes*). The DNA probes were used at a final concentration of 5 mM. Relative binding activities, calculated as a percentage of TTF-1HD binding to probe C, are indicated (*RB*). *B*, SFO nuclear extracts (*SFO NE*) were incubated with oligonucleotide probes indicated *below* the *autoradiograms*, in the presence (+) or absence (−) of a 50-fold excess of cold oligonucleotide C and Cβ and TTF-1 antibody (*TTF-1 Ab*) or preimmune serum (*IS*). Incubation of nuclear proteins with a TTF-1 antibody prior to the protein-DNA binding reaction delays (supershifts) the migration of the protein-DNA complex (*arrow*). *B*, protein-bound DNA; *F*, free DNA.

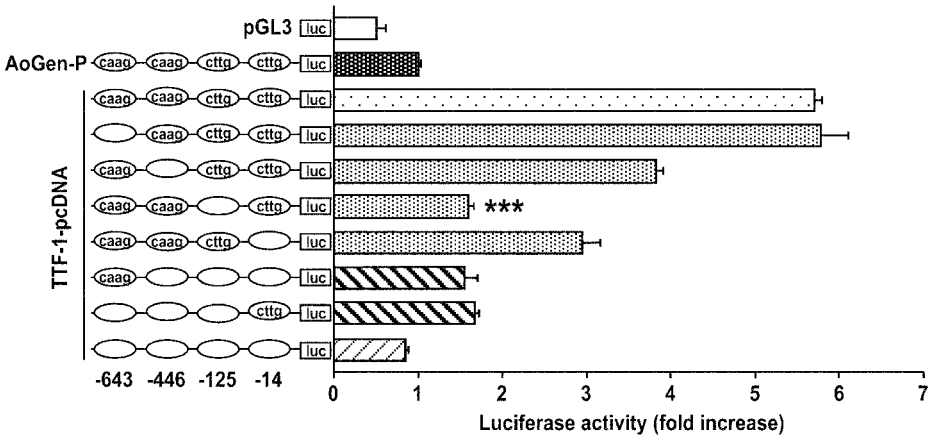

FIG. 7. **Effect of site-specific deletion of core TTF-1 binding motifs on the transactivation of the AoGen promoter (AoGen-P) by TTF-1.** Four single mutants and three combination mutants, with core TTF-1 binding motifs deleted, were cotransfected with 500 ng of the TTF-1 expression vector (*TTF-1-pcDNA*). The positions of the deleted binding sites are indicated. The data are means ± S.E. of 12 wells per construct. ***, $p < 0.001$ *versus* wild type promoter construct.

weight occurred during this period (Fig. 10*B*).

Effect of AS TTF-1 ODN on Plasma AVP and ANG II Levels—Blood samples were collected 13 h after the ODN injection (*i.e.* at the time when cumulative water intake begins to decrease in response to the AS ODN against TTF-1 mRNA) (Fig. 9*B*, *open arrow*). Whereas plasma AVP levels declined after the AS ODN injection (Fig. 11*A*), plasma ANG II titers increased (Fig. 11*B*). The AS ODN treatment also induced a significant decrease in AVP mRNA content in the SON (Fig. 11, *C* and *D*).

DISCUSSION

This study demonstrates that TTF-1, a homeodomain-containing transcription factor required for the embryonic development of the diencephalon and some ventral structures of the telencephalon, plays a critical role in the homeostatic regulation of fluid balance in adult animals. TTF-1 exerts this regulatory function within a remarkably discrete region of the brain, the SFO, where it transactivates AoGen gene expression. Although previous studies demonstrated the persistence of TTF-1 mRNA in discrete anatomical domains of the postnatal hypothalamus (20, 21), they did not identify a major extrahypothalamic site of expression. We now report a more detailed *in situ* hybridization analysis that reveals an additional (and unexpected) locus of TTF-1 expression in the SFO. Importantly, TTF-1 mRNA is present in AoGen-expressing cells of the SFO, a colocalization that provides the anatomical basis for the possibility that TTF-1 and AoGen may be functionally related in this highly specialized brain region.

The existence of a hierarchical relationship between TTF-1 and AoGen was initially suggested by our identification of consensus TTF-1 binding motifs in the 5′-flanking region of the AoGen gene. These binding motifs are intermingled with canonical binding sites for transcription factors already known to control AoGen gene expression such as glucocorticoid receptor, cAMP-response element-binding protein, and C/EBP. Perhaps the most important regulators of AoGen synthesis are C/EBP-related transcription factors. Several members of the C/EBP family have been shown to regulate AoGen gene expression in liver (43–45). Some of them are important for the liver-specific expression of AoGen (46, 47), and especially DBP, a liver-enriched transcription factor, binds to the proximal AoGen

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promoter and increases its activity (46). A recent report that shows that a subpopulation of African-Americans at high risk for hypertension have a point mutation of the C/EBP binding domain in the AoGen promoter provides genetic evidence for its critical role in the regulation of AoGen synthesis (48).

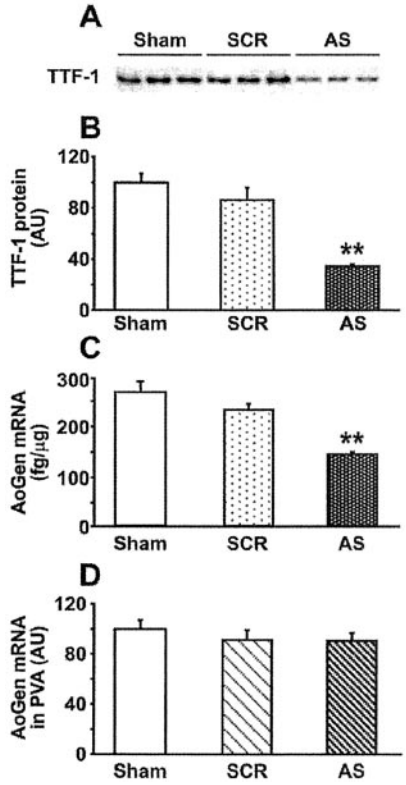

FIG. 8. **Effect of TTF-1-synthesis blockade by an AS TTF-1 ODN on AoGen mRNA levels in the rat SFO.** AS TTF-1 ODN was injected immediately dorsal to the SFO. One day after the ODN injection, RNA and protein were extracted from the SFO, and TTF-1 protein and AoGen mRNA levels were determined by Western blot analysis and RPAs, respectively. *A*, representative Western blot showing a decrease in TTF-1 protein caused by AS TTF-1 ODN (*AS*) administration. *B*, AS ODN significantly decreased TTF-1 compared with the sham-operated (*Sham*) and SCR ODN-injected groups (*SCR*). *C*, AoGen mRNA levels were significantly decreased by AS ODN. *D*, lack of changes in AoGen mRNA content in the anterior thalamic PVA after administration of the AS ODN. Data are represented as means ± S.E. ($n = 6$). *AU*, arbitrary unit. **, $p < 0.01$ *versus* both control groups.

Although the 5′-flanking region of the AoGen gene has seven putative TTF-1 binding domains, identified as such because of their similarity to the canonical TTF-1 binding motif present in TTF-1 target genes (33, 42, 49), only four of them recognize the TTF-1 homeodomain protein. Surprisingly, the binding affinity of these sites for TTF-1HD does not correlate well with their biological activity. Whereas the site at −643 showed the strongest binding activity, deletion of the site at −125, which had only 23% TTF-1HD binding activity as compared with the canonical TTF-1 binding site in the thyroglobulin promoter (oligomer C), was sufficient to prevent TTF-1 transactivation of the AoGen promoter. In contrast, mutation of the −643 site had no effect. This could be due to differences in binding and transcription activity in an *in vivo* three-dimensional DNA structure and the *in vitro* binding between DNA and protein in an EMSA and/or to the requirement, for full binding activity, of protein domains outside the homeodomain. Regardless of the explanation for this discrepancy, the results are not without precedent, since domains with relatively weaker binding activities have been reported to be important in the *in vivo* action of homeodomain-containing proteins (50). Our results also show that in addition to the site at −125, binding domains at −446 and −14 are important but not critical for the transactivation of AoGen by TTF-1. Deletions of the core motifs at −446 and −14 resulted in less AoGen promoter transactivation by TTF-1, but significant activity still remained. Of interest in this context is the presence of putative GATA-1 binding sites near those of TTF-1 at −446 and −125. Recent data showed that GATA-6 (51, 52) and GATA-4 (51) directly interact with TTF-1 to cooperatively transactivate TTF-1 target genes. It is possible that a similar interaction may contribute to the transcriptional regulation of AoGen expression by TTF-1 in the SFO. Further study will be necessary to clarify this potential interaction.

Cells containing TTF-1 mRNA were not observed in the PVA, whereas many AoGen-positive cells were detected in this region. The absence of TTF-1 mRNA in these cells suggests that TTF-1-dependent regulation of AoGen expression is limited to the SFO. The present histochemical data show that most TTF-1 mRNA-containing cells are located in the central portion of the SFO, a region that has few, if any, GFAP-positive cells but is composed by a majority of cells immunonegative for both GFAP and the neuronal marker NeuN. Because this region of the SFO is mostly composed of modified ependymoglial cells (38), which

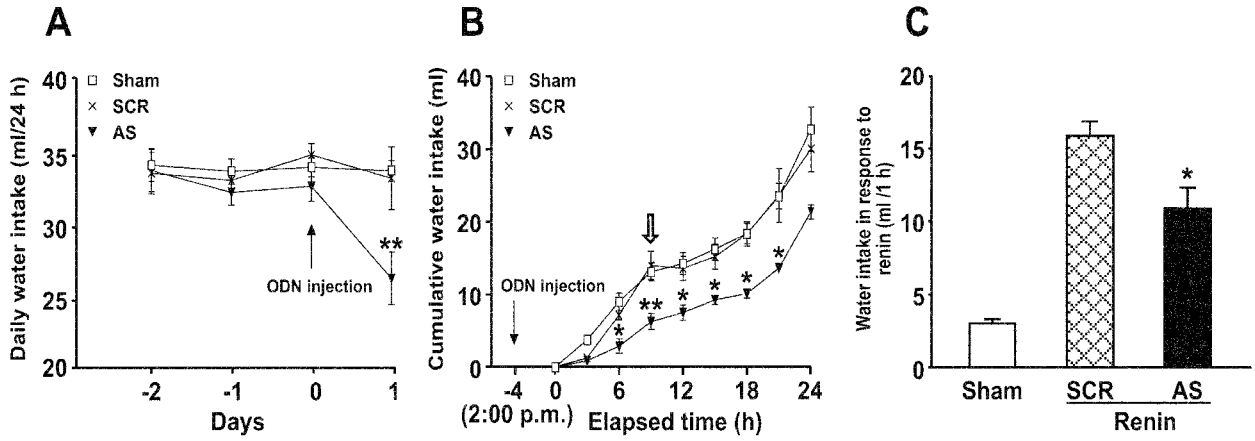

FIG. 9. **Effect of TTF-1 synthesis blockade on water intake.** Water intake was measured in rats receiving an intracerebral injection of AS TTF-1 ODN (*AS*) or a scrambled sequence (*SCR*). *A*, total daily water intake by individual animals was measured for a day, after a 4-h recovery period from the ODN injection at 2:00 p.m. **, $p < 0.01$ *versus* sham-operated (*Sham*) and SCR ODN-injected animals. *B*, cumulative water intake was measured for a day at 3-h intervals after 4 h from the ODN injection at 2:00 p.m. *, $p < 0.05$; **, $p < 0.01$ *versus* both control groups at each time point. The *open arrow* in *B* indicates the time of blood sampling for the determination of plasma AVP and ANG II. *C*, renin-induced water intake was inhibited by AS TTF-1 ODN (*AS*) administered 4 h earlier. Sham-operated animals were not injected with renin and did not receive ODN injections. *, $p < 0.05$ *versus* SCR. All data are presented as means ± S.E. ($n = 21$ for *A* and *B*; $n = 7$ for *C*).

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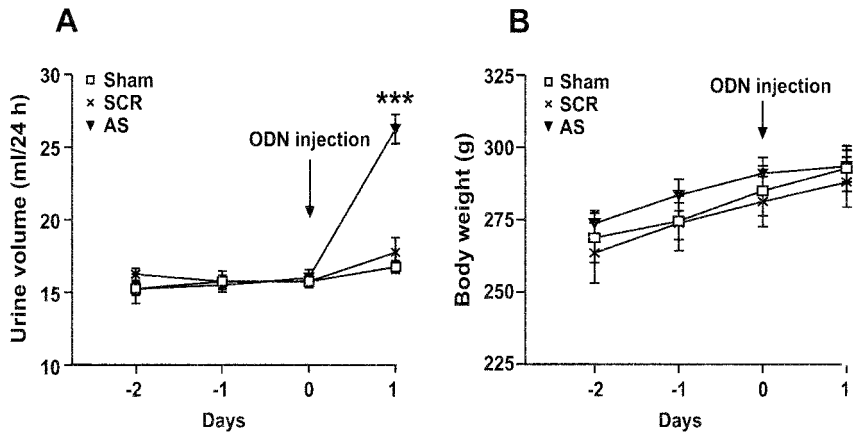

FIG. 10. **Effect of TTF-1 synthesis inhibition on the urine excretion.** *A*, total daily urine excretion from individual animals was measured for a day, after a 4-h recovery period from ODN injection at 2:00 p.m. *B*, body weight was not affected by the treatment. ***, $p < 0.001$ *versus* both controls. Data were obtained from 21 animals for each group and are represented as means ± S.E.

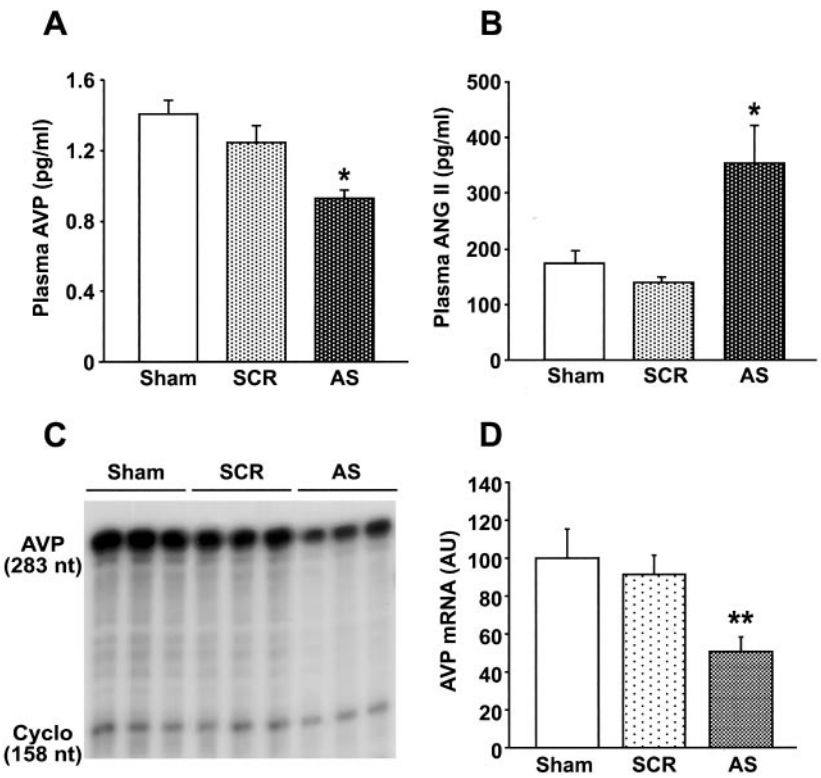

FIG. 11. **Effect of TTF-1-synthesis inhibition on the plasma AVP and ANG II levels and AVP mRNA level in the SON.** *A*, determined at 13 h after ODN injection, plasma AVP level was significantly decreased by the AS TTF-1 ODN compared with controls. *B*, AS TTF-1 ODN significantly increased plasma ANG II level. *C*, representative autoradiograms of RPAs, demonstrating the decrease in AVP mRNA content in the SON induced by the administration of AS TTF-1 ODN. *Cyclo*, cyclophilin; *nt*, nucleotides. *D*, quantification of the decrease in AVP mRNA level at 13 h after the AS ODN injection. *, $p < 0.05$; **, $p < 0.01$ *versus* both controls. Data represent means ± S.E. ($n = 5–6$).

in other CVOs are not recognizable by GFAP staining (40, 41), it is likely that they represent a major site of TTF-1 expression. The median eminence, another ventricular organ, also contains numerous ependymoglial cells of the astrocytic tanycyte type (39), which for the most part are GFAP-negative (41). Thus, the present results indicate that TTF-1 and its target gene AoGen are mainly expressed in ependymoglial cells and neurons of the SFO.

Water deprivation leads to an up-regulation of ANG II receptor binding and AT_{1A} receptor gene expression (5, 53) as well as an increase in steady state AoGen mRNA levels in the rat SFO (5). The simultaneous increase in TTF-1 mRNA and protein and AoGen mRNA observed in the SFO after 2 days of water deprivation suggests that dehydration up-regulates AoGen synthesis in the SFO via activation of TTF-1 production. This interpretation is supported by the results of blocking TTF-1 synthesis via *in vivo* administration of antisense oligodeoxynucleotides. This treatment resulted in two key alterations in body fluid homeostasis: inhibition of water intake and increased urine excretion (the latter reflecting a decrease in renal water reabsorption). These two changes represent the major homeostatic responses to hypervolemia and hypoosmolality and are regulated by ANG II secreted from both the SFO and peripheral sources (1).

The SFO seems to be mainly responsible for increasing water ingestion in response to circulating ANG II and other dipsogenic inputs, since destruction of the SFO results in the loss of drinking in response to intravenous ANG II (54, 55). Destruction of the SFO or infusion of an ANG II antagonist into the SFO also prevented water consumption in response to adrenergic activation (56). Because some angiotensin-sensitive structures involved in water drinking behavior lie inside the blood-brain barrier and cannot be directly reached by circulating ANG II (1), SFO-intrinsic angiotensins appear to play an important role in the regulation of these structures. In fact, inhibition of brain ANG II synthesis via an intracerebral injection of AS AoGen ODN (57) results in a decreased water intake, as predicted by the concept that brain ANG II is important for the regulation of water consumption. In addition to showing that blockade of TTF-1 synthesis targeted to the SFO decreases water intake, our results also show that AS TTF-1 ODN significantly decreased the renin (a potent dipsogen)-induced in-

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crease of water intake. Thus, AoGen synthesized in the SFO under the facilitatory control of TTF-1 may serve as a renin substrate and play an important role in the central regulation of water drinking behavior.

AVP-secreting magnocellular neurons terminate in the posterior pituitary, where AVP, the major hormone required for water conservation through its antidiuretic effect, is stored and released into the bloodstream in response to increases in osmolality or hypovolemia (58). Peripheral administration of ANG II can stimulate AVP release by acting on CVOs such as the SFO or directly on the pituitary; intracerebral injection of ANG II can also cause AVP release (59). Ablation of the SFO abolished the stimulatory effect of peripherally administered ANG II on AVP release (60, 61), indicating that the central mechanism used by ANG II to stimulate AVP release requires the SFO. By showing that TTF-1 synthesis blockade by AS ODN injection reduces AoGen expression in the SFO, decreases the AVP mRNA content of the SON, and reduces circulating plasma AVP levels, while increasing urine excretion, the present results suggest that AoGen production is required for the SFO-dependent regulation of AVP synthesis and secretion. Accordingly, AoGen produced in the SFO appears to be an important regulator of AVP-dependent renal water retention.

Surprisingly, in contrast to the decreased AVP level, plasma ANG II level was significantly increased by the AS TTF-1 ODN injection. This increase may be due to a homeostatic response coping with a transient hypovolemia induced by decreases both in drinking and renal reabsorption. Hypovolemia is sensed by vascular stretch receptor and then triggers an increase in circulating ANG II level (1, 62).

In conclusion, our results show that the AoGen gene expressed in cells of the SFO is under the transcriptional regulation of TTF-1, a homeodomain gene previously thought to be required only for the basal forebrain morphogenesis. Our data also demonstrate that this regulatory mechanism plays an important role in the control of body fluid homeostasis. We anticipate that identification of this novel control system will provide new insights into the understanding of the central processes that, operating within the brain, regulate body fluid homeostasis in normalcy and disease.

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