2. Materials & Experimental procedures

2.1 Material

2.1.1 Chemicals, cells, animals, antibodies, and kits

2-mercaptoethanol	Carl Roth, Karlsruhe, Germany
8-arginine-vasopressin	Peptide chemistry, FMP-Berlin, Germany
Acetic acid	Merck, Darmstadt, Germany
Acrylamide, Bisacrylamide	Serva, Heidelberg, Germany
Agarose (Ultra PURE)	Life Technologies, Karlsruhe, Germany
α^{32}-P desoxy-cytosinetriphosphate (6000 Ci/mmole)	Amersham Pharmacia Biotech, Freiburg, Germany
Ammoniumpersulfate (APS)	Sigma-Aldrich, Steinheim, Germany
BamHI	Life Technologies, Karlsruhe, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Steinheim, Germany
Bromephenol blue	Sigma-Aldrich, Steinheim, Germany
Cacodylic acid, sodium salt	Carl Roth, Karlsruhe, Germany
Chloroform	Merck, Darmstadt, Germany
Collagenase CSLII	Sigma-Aldrich, Steinheim, Germany
Coomassi Brilliant blue G250	Carl Roth, Karlsruhe, Germany
Dibuturyl-cyclo-AMP	Sigma-Aldrich, Steinheim, Germany
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich, Steinheim, Germany
Disodium hydrogenphosphate	Merck, Darmstadt, Germany
Dithiothreitol (DTT)	Sigma-Aldrich, Steinheim, Germany
dried milk powder (low-fat)	Néstle AG, Frankfurt, Germany
Dulbecco´s modified Eagle´s medium	Sigma-Aldrich, Steinheim, Germany
E.coli (JM 109)	Stratagene, Heidelberg, Germany
Ethanol	J.T. Baker, Deventin, The Netherlands
Ethidium bromide	Sigma-Aldrich, Steinheim, Germany
Ethylendiamine-tetraacetate (EDTA)	Carl Roth, Karlsruhe, Germany
Fetal calf serum (FCS)	Gibco BRL, Karlsruhe, Germany
Ficoll Type 400	Amersham Pharmacia Biotech, Freiburg, Germany
Formamide	Merck, Darmstadt, Germany
FuGENE 6 Transfection Reagent	Roche Diagnostics, Mannheim, Germany
Gelatine (fish skin)	Sigma-Aldrich, Steinheim, Germany

Glucose	Merck, Darmstadt, Germany
Glutamine	Life Technologies, Karlsruhe, Germany
Glycerine	Sigma-Aldrich, Steinheim, Germany
Glycine	Carl Roth, Karlsruhe, Germany
Herring-sperm DNA	Promega, Heidelberg, Germany
Hyaluronidase	Roche Diagnostics, Mannheim, Germany
Hydrochloric acid	Merck, Darmstadt, Germany
ImmuMount	Shandon, Pittsburgh, U.S.A
Isopropanol	J.T. Baker, Deventin, The Netherlands
Lactacystine	Calbiochem, Beeston, U.K.
Lumi-Light solution	Roche Diagnostics, Mannheim, Germany
Methanol	J.T. Baker, Deventin, The Netherlands
MG-132	Calbiochem, Beeston, U.K.
Nitrocellulose membrane (Nytrane NY 13 N)	Schleicher & Schuell, Dassel, Germany
Non-essential amino acids	Sigma-Aldrich, Steinheim, Germany
Oligonucleotides	BioTeZ, Berlin, Germany
Ovalbumine	Sigma-Aldrich, Steinheim, Germany
Paraformaldehyde	Merck, Darmstadt, Germany
PD 98059	Promega, Madison, U.S.A
pEGFP-N1 vector	Clontech, Heidelberg, Germany
Penicillin	Sigma-Aldrich, Steinheim, Germany
Phosphoric acid	Merck, Darmstadt, Germany
Polyvinylpyrrolidone	Merck, Darmstadt, Germany
Ponceau S	Roche Diagnostics, Mannheim, Germany
Potassium chloride	Sigma-Aldrich, Steinheim, Germany
Potassium-dihydrogen phospate	J.T. Baker, Deventin, The Netherlands
Protein ladder, 10 - 250 kDa, prestained	BioRad, Munic, Germany
Protein ladder, 10 kDa	Life Technologies, Karlsruhe, Germany
PVDF-filters, Immobilon-P	Millipore Corporation, Bedford, U.S.A.
Rats (Wistar-Schönwalde)	DIMED, Schönwalde, Germany
RNA-ladder, 0.24-9.5 kb	GibcoBRL, Karlsruhe, Germany
Rottlerin	Calbiochem, Beeston, U.K.
SB 203580	Promega, Madison, U.S.A
Sephadex G50	Amersham Pharmacia Biotech, Freiburg, Germany
Sodium chloride	Sigma-Aldrich, Steinheim, Germany

Sodium citrate	Merck, Darmstadt, Germany
Sodium hydroxide	Carl Roth, Karlsruhe, Germany
Sodium phospate	Merck, Darmstadt, Germany
Sodium-dihydrogen phosphate	Merck, Darmstadt, Germany
Sodiumdodecyle sulfate (SDS)	Carl Roth, Karlsruhe, Germany
Sodium-hydrogen carbonate	Sigma-Aldrich, Steinheim, Germany
Sorbitol	Sigma-Aldrich, Steinheim, Germany
Streptomycine	Sigma-Aldrich, Steinheim, Germany
T4-ligase	New England Biolabs, Frankfurt, Germany
Tetramethylendiamine (TEMED)	Sigma-Aldrich, Steinheim, Germany
Tris(hydroxymethyl)aminomethane	Sigma-Aldrich, Steinheim, Germany
Triton X-100	Sigma-Aldrich, Steinheim, Germany
TRIzol reagent	Gibco-BRL, Karlsruhe, Germany
Tween-20	Sigma-Aldrich, Steinheim, Germany
Ultroser	Life Technologies, Karlsruhe, Germany
Urea	Gibco-BRL, Karlsruhe, Germany
WT-10 cells	a kind gift of P.M.T.Deen
XhoI	Life Technologies, Karlsruhe, Germany
<u>Antibodies:</u>	
Cy3-conjugated goat anti-rabbit antibodies (Fa/b fragments, affinity purified)	Dianova, Hamburg, Germany
peroxidase-conjugated goat anti-rabbit antibodies (Fa/b fragments)	Dianova, Hamburg, Germany
rabbit anti-AQP2 antiserum	FMP-Berlin, Germany
rabbit anti-pCREB antibodies	New England Biolabs, Frankfurt, Germany
rabbit anti-TonEBP antiserum	a kind gift of Drs. A Rao and C. Lopéz-Rodríguez, Harvard Medical School, Boston, U.S.A.
peroxidase-conjugated mouse anti-Actin antibodies	Oncogene Research Products, Cambridge, U.S.A.
<u>Kits:</u>	
GeneClean II	Dianova, Hamburg, Germany
Terminal-transferase labelling Kit	Roche Diagnostics, Mannheim, Germany
Megaprime labelling Kit	Amersham Pharmacia Biotech, Freiburg, Germany
QuiaPrep Spin Miniprep Kit	Qiagen, Hilden, Germany

2.1.2 Apparatus and software

AA-gel cast-stand	Hoefer Mighty Small SE 245
AA-gel electrophoresis chamber	Mighty Small, Amersham Pharmacia Biotech
Agarose gel electrophoresis chambers	Hoefer HE99X-15-1.5; Hoefer HE33
Centrifuges	Beckmann TLK 100
	Beckmann Optima L70
	Sorvall RC 285
	Haereus Biofuge pico
Clean bench	BDK
Geiger-counter	FHT 111M, Contamat
Incubator	Heareus
Lumi-Imager F1	Roche Diagnostics
Microscopes	Zeiss Axiovert
	LSM Zeiss KS 400
Peristaltic pump (Gilson minipuls 3)	Gilson
Perspex hybridization containers	manufactured at the FMP-Berlin
PhosphorImager	Storm 830, Amersham Pharmacia Biotech
Photometer	GeneQuantII, Pharmacia Biotech
Pipettes	Eppendorf
Potter	Potter S, B.Braun Biotech International
Power supplies	Bio-Rad, Amersham Pharmacia Biotech
Rotator	Stuart Scientific, Blood tube rotator SB1
Scales	Scalterl SBA52
	Mettler Toledeo AG245
Scintillator	Wallac 1409
Sequencer (Abi Prism)	Applied Biosystems
Semi-dry Western Blot chamber	Trans Blot SD, Bio-Rad
Shaker	Edmund Bühler, Swip SM 25 DIGI
Sonicator	Sonopuls UW 2040, Bandelin Electronics
Speed vac	Dobsen Technologies
Stove	Memmert
Thermocycler	Perkin Elmer, GeneAmp 9700
Thermomixer	Eppendorf Thermomixer 5436
Transilluminator	Hoefer, Macro Vue UV-20

Video camera	Zeiss Sensicam
Vortexer	Janke&Kunkel IKA labortechnik
Water bath	Stuart Scientific, Shaker Bath SBS 30

Software:

AxioVision	Zeiss
Exel 2000	Microsoft
GeneTool 1.0	BioTools
GraphPad Prism 3.02	GraphPad software
Image Quant 5.1	Amersham Pharmacia Biotech
Photoshop 5.0	Adobe
Powerpoint 2000	Microsoft
Wiacalc	Wallace and Os
Word 2002	Microsoft

2.2 Experimental Procedures

2.2.1 Preparation of primary cultures of IMCD cells from rat inner medullae

Phosphate buffered saline (PBS; without Ca^{2+} and Mg^{2+})

0.8 g NaCl
0.2 g KCl
0.2 g KH_2PO_4
1.15 g Na_2HPO_4
adjust pH to 7.4 ad 1 l

IMCD cells were obtained from renal inner medullae of Wistar rats (2-3 month old, 200-250 g; both sexes). The animals were anaesthetized with CO_2 and killed by decapitation. Both kidneys were removed and chilled in ice-cold phosphate buffered saline (PBS; 0.8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, ad 1 l; pH 7.4). Each kidney was sliced in two halves and the inner medulla, which stands out against the surrounding tissue by its lighter color, was excised. The inner medullae were collected in a culture dish containing 0.5 ml of PBS and subsequently minced with a razor blade. The minced tissue was transferred to a 50 ml Falcon tube and incubated with calcium free PBS containing 0.2 % (w/v) hyaluronidase and 0.2 % (w/v) collagenase CLSII for 1.5 h at 37°C in a shaker (350 U/min). A sterile glass pipette was used to gently shear apart isolated cell clumps that occasionally remained after digestion. The cell suspension was centrifuged for 7 min at 1200 x g, the pellet was washed in PBS and spun down two times and cells were subsequently transferred to 5 ml medium with 600 mosmol/l (based on Dulbecco´s modified eagles medium elevated to 600 mosmol/l by addition of 100 mM NaCl and 100 mM urea, termed 600N) and seeded onto collagen type IV coated culture dishes at an approximate density of 7.5 x 10^4 cells/cm^2. Cells were cultured at 37°C under a 5 % CO_2 - atmosphere. High osmolality medium (600N) was used to select for IMCD cells. One day after seeding desired culture media were applied. Media were changed every 24 h. Confluent cells were harvested for diverse analysis 5 to 7 days later.

2.2.2 Coating of glass and plastic ware with collagen type IV for cell culture

From a collagen type IV stock solution (0.5 mg/ml), one milliliter was solubilized in sterile-filtrated acetic-acid (1:20, v/v). To yield a collagen density of approximately 2 μg/cm^2 on the growth area, 0.25 ml/cm^2 of the collagen solution was filled into the dishes to be coated and incubated for 1 h at RT. For the coating of glass coverslips, four cover slips were placed in a

culture dishes with a diameter of 35 mm and 2 ml of collagen solution was added. Subsequently, culture containers were rinsed with sterile PBS.

2.2.3 Media used for cell culture

All media used were based on Dulbecco´s modified eagles medium (DMEM 5523; containing 110 mmol/l NaCl; 300 mosmol/l). Moreover, the medium contained 4.5 % glucose (instead of 1 %) and was routinely supplemented with penicillin (0.5 U/ml), streptomycin (0.5 µg/ml), glutamin (2 mM), 1 % non-essential amino acids and 1 % Ultroser instead of 10 % FCS. This basis medium, termed 300N, contained, if not indicated otherwise, 500 µM DBcAMP to maintain AQP2 expression, which was otherwise not detectable 2-3 days after seeding (Maric et al. 1998, personal observation). Media of elevated osmolalities (600 and 900 mosmol/l after equimolar addition of NaCl and urea) were termed 600N and 900N respectively, or, if sorbitol was used to elevate osmolality, 600S and 900S. Other media used were termed as follows. The first number indicates the osmolality of the medium [in mosmol/l]. Whether NaCl, sorbitol (S) or urea (U) was used is indicated behind the first number describing the medium´s osmolality. The amounts of NaCl or sorbitol and/or urea [in mM] added to 300N medium to elevate medium osmolality are indicated by the two numbers separated by a slash (see also table on next page).

WT-10 cells (Madin-Darby canine kidney cells stably transfected with human AQP2 cDNA, driven by cytomegalovirus (CMV) promoter (kindly provided by P.M.T. Deen, Deen et al., 1997), were cultured without DBcAMP in 300N with 5 % FCS instead of Ultroser and confluent cell monolayers were passaged every 4-5 days. Six days before membrane preparation, WT-10 cells were split 1 : 10 and seeded on culture dishes in either 300N or 600N medium.

Media used for cultivation of IMCD cells:

300N medium (300 mosmol/l)	DMEM 5523 (Dulbecco´s modified eagles medium) 3.5 g glucose 2 g sodium hydrogencarbonate adjusted to pH 7.4 with 1 N HCl 0.5 IU/ml penicillin 0.5 µg/ml streptomycin 2 mM glutamin 1 % (w/v) non-essential amino acids 1 % (v/v) Ultroser (for IMCD cells) 500 µM DBcAMP (if not indicated otherwise!)

300N medium elevated with NaCl
(amounts in [g] for 100 ml medium

			final osmolality/tonicity (in mosmol/l)
300N +	10 mM	NaCl (0.058 g)	~320
300N +	20 mM	NaCl (0.117 g)	~340
300N +	35 mM	NaCl (0.204 g)	~370
300N +	50 mM	NaCl (0.292 g)	~400
300N +	100 mM	NaCl (0.584 g)	~500
300N +	150 mM	NaCl (0.876 g)	~600
300N +	200 mM	NaCl (1.168 g)	~700
300N +	250 mM	NaCl (1.460 g)	~800
300N +	300 mM	NaCl (1.752 g)	~900

300N medium elevated to 600 mosmol/l with NaCl / urea / sorbitol
(amounts in [g] for 100 ml medium)

600N (control)	100 mM 100 mM	NaCl urea	0.584 g 0.6 g	~600
600 S	300 mM	sorbitol	5.465 g	~600
600 U	300 mM	urea	1.8 g	~600
600S/U 200/100	200 mM 100 mM	sorbitol urea	3.643 g 0.6 g	~600
600NaCl/S 50/200	50 mM 200 mM	NaCl sorbitol	2.92 g 3.643 g	~600
600NaCl/U 50/200	50 mM 200 mM	NaCl urea	2.92 g 1.2 g	~600
600NaCl 150/0	150 mM	NaCl	0.876 g	~600

300N medium elevated to 900 mosmol/l with NaCl / urea / sorbitol
(amounts in [g] for 100 ml medium)

900N	200 mM 200 mM	NaCl urea	1.168 g 1.2 g	~900
900 NaCl/U 250/100	250 mM 100 mM	NaCl urea	1.46 g 0.6 g	~900

2.2.4 Protein analysis

2.2.4.1 Determination of protein concentrations by the method of Bradford *(1976)*

Using ovalbumin as a protein standard, a series of samples were run in parallel with the samples of unknown protein concentration for calibration. Duplicates were used for the samples used for calibration and the samples to be determined. Proteins were denatured with NaOH (see Table 1) and incubated for either 10 min at 60°C or 1 h at 37°C.

Sample reagent (PR CBB):
Coomassi Brilliant blue (0.1 g) was solubilized in 50 ml 95 % (v/v) EtOH, aqua dest. was added to 800 ml and the solution was incubated o/n in the dark at RT. Subsequently, 100 ml 85 % H_3PO_4 was added and the final volume was adjusted with aqua dest. to 1 l. Before use, the solution was filtered through a paper filter.

Tab.1:

	Sample No.	Prot.amount [µg]	Standard ovalbumine [µl] **[0.1 mg/ml]**	H_2O [µl]	2 N NaOH [µl]	PR CBB [µl]
samples for calibration:						
	1	0	0	50	50	1000
	2	0	0	50	50	1000
	3	1	10	40	50	1000
	4	1	10	40	50	1000
	5	2	20	30	50	1000
	6	2	20	30	50	1000
			[1 mg/ml]			
	7	5	5	45	50	1000
	8	5	5	45	50	1000
	9	10	10	40	50	1000
	10	10	10	40	50	1000
	11	20	20	30	50	1000
	12	20	20	30	50	1000
	13	40	40	10	50	1000
	14	40	40	10	50	1000
			Probe	H_2O	2 N NaOH	
samples of unknown prot. conc.:			[µl]	[µl]	[µl]	
	A		5	45	50	1000
	A		5	45	50	1000

After addition of CBB PR, samples were incubated for 10 min at RT. Extinction at 595 nm was measured with a photometer. The samples No. 1 and 2 in Table 1 were used for calibration of the photometer. The calibration curve was calculated using wiacalc (Wallac and Os, 1989). The

program then calculates the protein concentration of samples using the calibration curve. Protein concentrations were used only, when in the range of the calibration curve.

2.2.4.2 Protein preparations

Total membrane preparation: All procedures were performed at 4°C. Cells cultured in culture dishes with a diameter of 60 mm were rinsed twice with ice cold phosphate-buffered saline (PBS), scraped off the culture dishes, and were homogenized in 1.5 ml ice-cold PBS with a glass/teflon homogenizer (10 strokes, 750 rpm; Potter S, B. Braun Biotech International). For membrane preparations, the homogenates were centrifuged at 800 x g for 5 min to remove nuclei and debris; the supernatant was centrifuged at 200,000 x g for 1 h and the resulting pellet (membrane fraction) was solubilized in ice cold PBS. Protein concentration of samples were determined in duplicates by the method of Bradford (see Table 1; Bradford, 1976). Samples (15 μg) were subjected to SDS-PAGE (12 % acrylamide in separating gels).

Total homogenates: Cells grown on 24-well plates (2 cm^2/well) were rinsed twice with ice cold PBS. Cells in each well were lyzed with 40 μl of modified Laemmli buffer (4 % SDS w/v instead of 2 %) and a sheet of parafilm was placed between the plate and the lid that was additionally sealed with tape to avoid drying out. Plates were incubated at RT for 18 h on a rotator (tilted ~30°). The solubilized material of each well was transferred with a pipette into 1.5 ml eppendorff cups and subsequently sonicated (with a Sonopuls UW 2070, Bandelin electronic, Berlin, Germany; 5 sec. at 40 % power) to shear chromosomal DNA. Homogenates were allowed to settle overnight at 4°C and were then subjected to SDS-PAGE (total homogenate from 1 cm^2 of confluent cell monolayer was loaded per lane onto SDS-PAGE gels; 12 % or 8 % acrylamide in separating gels).

2.2.4.3 Size separation of proteins by SDS-PAGE

4 x Laemmli-sample buffer:

10 %	(v/v)	glycerine
2 %	(w/v)	SDS
5 %	(v/v)	2-mercapto-ethanol
0.01 %	(w/v)	brome-phenol blue
60 mM		Tris/HCl (pH 6.8)

Transfer buffer:

0.1 %	(w/v)	SDS
50 mM		Tris
150 mM		glycine

pH adjusted to 7.4 with HCl

Volumes of reagents used for 2 AA gels

separation gel	12 %	8 %	stacking gel	
30 % (w/v) AA, 0.8 % (w/v) BAA	4.5 ml	3.0 ml	835 µl	30 % (w/v) AA, 0.8 % (w/v) BAA
Tris/HCl 0.75 M, pH 8.8	5.625 ml	5.625 ml	625 µl	Tris/HCl 0.025 M, pH 6.8
20 % SDS	56.5 µl	56.5 µl	25 µl	20 % SDS
Temed	5.65 µl	5.65 µl	5 µl	Temed
H_2O	1 ml	2.5 ml	3.5 ml	H_2O
APS 10 % (w/v)	79 µl	79 µl	25 µl	APS 10 % (w/v)

The solutions for 12 % or 8 % SDS PAGE mini-gels (1 mm thickness) were put together as shown above. Using Pharmacia gel cast chambers, separation gels were cast first, covered with isopropanol and incubated for 30 min to allow for polymerization. Subsequently, isopropanol was discarded and stacking gels were cast. Samples (15 µg protein) as well as the appropriate molecular weight standard were mixed with ¼ (v/v) of sample buffer and boiled for 5 min. Samples were loaded and electrophoresis was performed for 1 h at 20 mA per gel.

2.2.4.4 Semi-dry Western blotting

Semi-dry blotting buffer, pH 9-9.4

20 %	(v/v)	MeOH
48 mM	(w/v)	Tris
39 mM	(v/v)	glycine
0.0375 % SDS	(w/v)	SDS
ad 1 l		H_2O

TBS:

10 mM	Tris
150 mM	NaCl

pH adjusted to 7.4 with HCl

TBST:

10 mM		Tris
150 mM		NaCl
0.05 %	(v/v)	Tween-20

pH adjusted to 7.4 with HCl

Using a semi-dry Western blot apparatus, size-separated proteins were transferred to Immobilon-P PVDF-membranes. Protein transfer and equal loading was verified by Ponceau-red staining (not shown). Briefly, membranes cut in the dimensions of the minigel, were placed in EtOH for 2 min, rinsed with H_2O and equilibrated for 5 min in blotting buffer. Within the semi dry blotting apparatus the membrane was placed on top of a Whatman paper soaked in

blotting buffer and covered with the minigel. Another wet Whatman paper was put on top of the gel and eventually trapped air bubbles were displaced by rolling a glass rod over the blotting stack. Electrophoretic transfer of proteins was performed at 10 V for 30 min to 2 h (for large proteins).

2.2.4.5 Immunochemistry

Stripping buffer for PVDF membranes

10 ml	0.025 M	Tris/HCl, pH 6.8
2 g		SDS
0.308 g		DTT
ad 100 ml		H_2O

Membranes were blocked for one hour in blocking buffer (tris-buffered saline (TBST) containing 5% w/v low-fat dried milk), incubated with the desired primary antibodies and subsequently washed in TBST (5 x 8 min). *Primary antibodies* - Rabbit polyclonal antiserum against AQP2 (Liebenhoff and Rosenthal, 1995) which, besides AQP2, also detects the histone H2A1 (Jo et al., 1997), was used for 1 h at RT, 1 : 1,500 in blocking buffer. Rabbit polyclonal antiserum against phosphorylated CREB (New England Biolabs, Frankfurt am Main, Germany), as well as anti-TonEBP rabbit polyclonal antiserum (a kind gift of A. Rao and C. López-Rodríguez, Harvard Medical School; López-Rodríguez et al., 1999) was used overnight at 4°C, 1 : 500 in TBST containing 5 % BSA. As secondary antibodies, peroxidase-coupled goat anti-rabbit $F_{(a/b)}$ fragments (Dianova, Hamburg, Germany) were used (1 : 2,000 in blocking buffer, 1 h at RT). Blots were washed in TBST (5 x 8 min) and then incubated for 5 min in Lumi-Light solution (Roche Diagnostics, Mannheim, Germany). Chemiluminescence was visualized and band densities were quantified using a Lumi-Imager F1 (Roche Diagnostics, Mannheim, Germany). *Stripping and reprobing of Western blots.* Blots were washed in TBST to remove residual Lumi-Light solution. Subsequently 100 ml 2.5 mM Tris/HCl, pH 6.8 with 2 % SDS (w/v) were heated to 70°C in a plastic dish placed in a water bath. Then, after 0.308 g DTT were dissolved in 100 ml of the stripping solution, blots were added and incubated at 70°C under shaking for 30 min. Blots were rinsed twice in TBST, blocked and reprobed with the desired antibodies. *Statistical analysis.* Statistical evaluation was carried out by ANOVA, using GraphPad PRISM software (GraphPad Software Inc., San Diego, U.S.A.). GraphPad PRISM software was also used to perform non-linear regression analysis on the data obtained in time course experiments. Results are expressed as mean % signal intensity ± SE of control.

2.2.5 Nucleic acid analysis

2.2.5.1 Isolation of total RNA from IMCD cells *(using TRIzol Reagent, GIBCO-BRL, Karlsruhe, Germany, according to the manufacturer´s instructions)*

Total RNA was prepared from IMCD cells grown in 60 mm culture dishes. Briefly, residual medium was displaced by two rinses with ice-cold PBS before addition of 2 ml of TRIzol Reagent to each dish. Only new, RNAse free-plastic ware was used for the preparation. Within the reagent, cells were scraped off the plates with a teflon scraper and incubated for 10 min at RT. The cell lysate yielded from one 60 mm dish was equally distributed to two 2 ml Eppendorf cups. Chloroform (500 µl) was added to each lysate fraction before vortexing (20 sec). The fractions were centrifuged in a table centifuge (13000 rpm, for 6 min at RT). The upper aqueous phase was carefully transferred to a new 2 ml cup, mixed with an equal same volume of isopropanol and was incubated at RT for 20 min. Subsequently, precipitated RNA was spun down (12000 x g, for 20 min at 4°C). The pellet was washed twice with 75 % v/v ethanol in DEPC-treated aqua bidest. In between, centrifugation was repeated (12000 x g, for 20 min at 4°C). The etanol was allowed to evaporate at 37°C and RNA was dissolved in an adequate volume of DEPC-treated aqua bidest. RNA concentration was determined using a photometer (1 OD_{260nm}=40 µg/ml). RNA samples were stored at -80°C.

2.2.5.2 Horizontal agarose gel electrophoresis of DNA *(according to Sambrook et al., 1989)*

50 x TAE buffer

242 g	Tris
57.1 ml	Acetic acid
100 ml	0.5 M EDTA, pH 8.0
ad 1 l	H_2O

For size-separation of DNA, 1 % agarose gels were used. Therefore, the needed amount of agarose was boiled in 1 x TAE buffer using a microwave. The solution was allowed to cool off to ~60°C and 0.1 µl/ml of ethidium-bromid was added. DNA samples were mixed with $1/6^{th}$ of their volume with sample buffer (0.25 % brome-phenol blue, 40 w/v glucose in aqua bidest). Electrophoresis was carried out using 1 x TAE running buffer and a voltage of 80 - 120 V. Size-separated DNA fragments, stained in the gel by ethidium-bromide, was visualized by UV-light using a transilluminator or a Lumi-Imager (Roche Diagnostics, Mannheim, Germany).

2.2.5.3 Recovery of DNA fragments from agarose gels *(using the Geneclean II kit, Dianova, Hamburg, Germany, according to the manufacturers instructions).*

TE buffer

10 mM	Tris-HCl, pH 7.4
1 mM	EDTA, pH 8.0

The band containing the fragment of the expected size was excised on a transilluminator using a sharp scalpel. The agarose lump containing the DNA was weighed in an Eppendorf cup, mixed with 3 times the amount of sodium jodid and melted (50°C, 15 min). Subsequently, 5 µl of well homogenized glassmilk was added, the sample was incubated 10-20 min on ice and was occasionally inverted. Glass particles with adherent DNA fragments were spun down for 20 sec at 13000 rpm using a table centrifuge, the liquid was discarded, the pellet was vortexed with 50 times the volume of chilled wash solution and spun down again. This step was repeated three times. The pellet was dissolved in TE-buffer (10 µl, pH 8.0), incubated for 3 min and subsequently spun down. The DNA containing aqueous phase was transferred to a new cup, the sediment was again dissolved in 10 µl of TE-buffer, spun down and the aqueous phase was pooled with the beforehand obtained. The sample was spun down again, the aqueous phase was transferred to a new cup and the DNA concentration was then determined using a photometer. Samples were stored at -20°C.

2.2.5.4 Gel electrophoresis of RNA and Northern blot analysis

denaturing buffer			Tris/HCl, pH 6.8
	5.4 µl	40 % (w/v)	Glyoxal (deionized, pH 5.0
	16 µl		DMSO
	3 µl	0.1 M	Na_3PO_4 buffer, pH 7.0

Northern blot analysis with 15 µg total RNA was essentially performed as described (Hanski et al., 1996). All used glass containers were backed for 5 h at 240°C to denature RNAses. All aqueous solutions used with RNA, were produced with tridest. H_2O, which was treated o/n with 0.1 % (v/v) DEPC and subsequently sterilized. Before agarose gels were cast (1 % agarose in 10 mM Na_3PO_4 –buffer), the gel-cast apparatus was cleaned with a 10 % w/v SDS-solution and rinsed with DEPC-treated aqua bidest. *Electrophoresis of RNA.* The running buffer (10 mM Na_3PO_4 buffer) contained 0.5 µg/ml ethidium bromide for staining of nucleic acids. Volumes of RNA samples was reduced to ~5.4 µl by vacuum centrifugation. The samples as well as 5.4 µl of the molecular weight standard were each incubated for 1 h in denaturing buffer and then placed on ice. To each denatured sample, 4 µl of sample buffer (50 % v/v glycerin; 10 mM

Na_3PO_4 buffer, pH 7.0 and 0.1 % w/v brome-phenole blue) was added. Samples were loaded onto the gel and size separation of RNA was carried out at 3-4 V/cm over 3-4 h. During electrophoresis, the running buffer was circulated in direction of RNA migration by means of a peristaltic pump (Gilson Minipuls 3) to avoid acidification of the buffer and thus RNA degradation.

20 x SSC		20 x SSPE		50 x Denhard´s	
175 g	NaCl	174 g	NaCl	1%	Ficoll
88.2 g	sodium citrate	27.6 g	NaH_2PO_4	1%	Polivinylpyrrolidone
ad 1 l H_2O		7.4 g	EDTA	1%	BSA
		6.5 ml	10 N NaOH		
		ad 1 l H_2O			

Transfer of RNA to nylon membranes - Northern blotting. RNA was transferred from the gel onto a nylon-membrane by overnight Northern blotting using 20 x SSC as transfer buffer. Briefly, the nylon membrane (exceeding the size of the gel by 0.5 cm on each side) was rinsed in DEPC-H_2O and equilibrated for 10 min in 2 x SSC. The two reservoirs of the electrophoresis chamber were filled with transfer buffer. A piece of Whatman paper (as wide as the chamber) was soaked in 2 x SSC and put on the crosspiece of the chamber with both narrow sides reaching into the transfer buffer. The gel was placed on the Whatman paper and by rolling a sterile glass rod over the gel, trapped air was gently squeezed out. The nylon membrane was placed on the gel and air inclusions were rolled out. Three Whatman papers soaked in 2 x SSC, followed by 2 dry ones and a 10 cm thick paper towel stack were piled onto the membrane. A glass plate with a 500 g weight was put horizontally on top of the tower. After overnight transfer, the membrane was washed in 6 x SSC, dried at RT and RNA was subsequently crosslinked to the membrane by incubation at 80°C for 2 h.

Pre-hybridization/Hybridization solution

50 ml	20 x	SSPE
100 ml		Formamide (deionized)
40 ml	50 x	Denhardt´s
2 ml	10 %	SDS

Pre-hybridization and hybridization. For pre-hybridization, membranes were placed in Perspex containers filled with 0.1 ml/cm^2 membrane area of hybridization buffer containing 100 µl/ml denatured herring-sperm DNA. Membranes were incubated for 3 h at 42°C in a water bath. Hybridization was carried out overnight at 42 °C using a heat-denatured ^{32}P-labelled rat AQP2

cDNA (40 million CPM/blot) mixed with 100 µl of denatured herring-sperm DNA. Hybridization was followed by three consecutive washes with 2 x SSC and 0.1 % (w/v) SDS, 1 x SSC and 0.1 % (w/v) SDS, and 0.1 x SSC and 0.1 % (w/v) SDS, each for 20 min at 50°C. *Detection of radioactivity.* Signals were visualized using a STORM 830 PhosphorImager and quantified using Image Quant 5.1 software (Amersham Pharmacia Biotech). For standardization of AQP2 mRNA signals, blots were stripped by boiling in 2 x SSC and subsequently re-hybridized with a ^{32}P-labelled 44 bp cDNA fragment specific for 18s rat rRNA (40 million CPM/blot, 5´ ACG AAT GCC CCC GGC CGT CCC TCT TAA TCA TGG CCT CAG TTC CG 3´); terminaltransferase end-labeled according to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). For semi-quantitative analysis, band densities for AQP2 mRNA were standardized to the corresponding 18s rRNA signals. Standardized signals were expressed as mean % signal intensity ± SE of controls (IMCD cells cultured in 600N) for each set of experiments. Statistical evaluation was carried out by ANOVA, using GraphPad PRISM software (GraphPad Software Inc.).

2.2.5.5 Radioactive random prime labeling of cDNA fragments *(using the Megaprime Labeling Kit, Amersham Pharmacia Biotech, Freiburg, Germany, according to the manufacturers´ instructions)*
25-50 ng of DNA, together with 5 µl of nonaprimer solution and 2 µl of aqua tridest. were denatured at 95°C for 5 min and instantly chilled on ice. 10 µl of 10 x reaction buffer, 25.5 µl aqua tridest., 2.5 µl [α-^{32}P]dCTP (6000 Ci/mmole), and 2 µl kleenow enzyme were added and the sample was incubated at 37°C for 15 min. For determination of radioactivity, 1 µl of the sample was added to 9 µl of TE in a scintillator tube. Labeled DNA was subsequently separated from unincorporated nucleotides using Sephadex G 50 columns.

2.2.5.6 Purification of [α-^{32}P]dCTP- labeled cDNA fragments using Sephadex G 50 columns Sephadex G 50 was soaked overnight in TE-buffer, pH 7.6. Excess liquid was removed, the sediment was degassed using subpressure and filled into glass pipettes closed at the tapered end with a small glass ball. Before loading of the labeled DNA, 3-5 ml of TE-buffer, pH 8.0 were run through the column. As the DNA solution had entered the column, it was chased with TE-buffer, pH 8.0. The blue fraction, containing the labeled cDNA fragments, and the green fraction bearing free dNTP´s were separately collected and radioactivity of 1 µl/fraction was counted using a scintillator. Of the labelled cDNA, the volume equalling to 40 million CPM was used for hybridization.

2.2.5.7 Sequence searches and alignments

DNA sequences for the human and rat AQP2 promoters, as well as large fragments of human chromosome 12, were obtained by using the NCBI nucleotide search (http://www.ncbi.nlm.nih.gov). Alignments were performed using GeneTool™ software (version 1.0, BioTools incorporated) or the NCBI-blast search function (http://www.ncbi.nlm.nih.gov/blast). Motifs for transcription factor binding sites were located within genomic DNA sequences using the search function in Microsoft Word™. Because the program does not allow to search for motifs in which a certain position can be occupied by variable bases, it was used only to search for the invariable parts of the motif and for each hit, final alignment was done by eye.

2.2.5.8 Generation of TonEBP-constructs: GFP-TonEBP and GFP-dominant-negative TonEBP

A human c-mycTonEBP construct, a kind gift of Drs. Anjana Rao and Christina López-Rodríguez (Harvard Medical School), was the source for PCR-amplification of the full-length human TonEBP (NCBI Acc. No. AF346509; Dalski et al., 2001) and a truncated, dominant-negative form of TonEBP (DNTonEBP). DNTonEBP consists of the N-terminal 472 AA, containing the DNA binding domain but not the transactivation domain (Woo et al., 2002, López-Rodríguez et al., 2001). To allow for cloning into the pEGFP-N1 vector (Clontech), specific primers with 5´ overhangs (underlined) containing a 5´XhoI cutting site or 3´ BamHI site, respectively, were used for amplification of DNTonEBP and TonEBP (see below). Construct identity was verified by sequencing (Abi Prism 310; Applied Biosystems, Weiterstadt, Germany) using vector sequencing primers and gene specific primers.

Primers used for amplification of TonEBP and DNTonEBP:

TonEBP fwd:	5´GAC TCA GAT CTC GAG CTC ATG GGC GGT GCT TGC AGC TCC3´
TonEBP rev:	5´GGT GGC GAC CGG TGG ATC CG AAA GGA GCC AGT CAA GTT GTT 3´
DN-TonEBP rev:	5´GGT GGC GAC CGG TGG ATC CG AGC TGC TGC TGG GTC TGG AGT 3´

PCR products were isopropanol-precipitated, dried by vacuum-centrifugation, solubilized in tridest. H_2O and DNA concentration was determined by UV-measurement at 260 nm. Vector DNA (pEGFP-N1, Clontech) as well as DNTonEBP and TonEBP PCR products were digested with XhoI and BamHI overnight at 37°C to obtain sticky ends for ligation. After digestion, PCR products and vector were size-separated in a 1 % agarose gels containing 0.1 % ethidium bromide. DNA was visualized on a UV-screen and bands containing the fragments of interest were cut out. DNA was subsequently recovered from the gel using either QiaPrep spin

columns (Quiagen, Hilden, Germany), or the Geneclean II kit (Dianova, Hamburg, Germany), according to the manufacturer´s instructions. DNA concentration was subsequently determined by UV-absorption measurement. Ligation of pEGFP-N1 vector DNA with DN-TonEBP or TonEBP, was performed at 16°C using T4-DNA ligase according to the manufacturers instructions (Roche Diagnostics, Mannheim, Germany).

BamHI/XhoI digestion:

10 µl	(100 ng/µl)	vector DNA or cleaned PCR product
5 µl		H_20
2 µl		10 x BamHI-buffer
2 µl		10 x BSA
0.5 µl		BamHI
0.5 µl		XhoI
20 µl		

Ligation:

5 µl	(100 ng/µl)	vector DNA
10 µl	(100 ng/µl)	insert DNA
2 µl		T4-10 x buffer
2 µl		T4 ligase
1 µl		H_20
20 µl		

2.2.6 Transfection of competent bacteria

Competent E.coli (strain JM 109), stored at –80°C, were thawed on ice. To 100 µl of E.coli suspension, 5 µl of the appropriate ligation reactions containing either the DNTonEBP-GFP or TonEBP-GFP DNA constructs were added. Bacteria were incubated on ice for 30 minutes, subsequently heat-shocked (90 seconds at 42°C), and then chilled on ice for 2 minutes. Pre-warmed LB-medium was added (1 ml) and the mixture was incubated for 1 h at 37°C. The bacteria were spun down in a table centrifuge (3000 rpm for 1 minute), the supernatant was discarded and the bacteria-pellet was re-suspended in approximately 50 µl of remaining medium. The bacteria were plated out on kanamycin-containing LB-agar-plates to select for transformed bacteria that obtained kanamycin-resistance with the pEGFP-N1 constructs. Plates were incubated overnight at 37°C. Single colonies were picked with a 200 µl pipette-tip, transferred to a glass test-tube containing 5 ml of LB-medium with kanamycin and incubated on a shaker at 37°C overnight. Mini-preparations of DNA were performed using a MiniPrep DNA-Isolation kit (QIAprep Spin Miniprep Kit, Quiagen) according to the manufacturers instructions. Recovered plasmid DNA sequences were verified by sequencing (Abi Prism 310; Applied Biosystems, Weiterstadt, Germany).

2.2.7 Immunofluorescence studies

Fixation buffer:

final concentration		stocks	volumes
100 mM	cacodylate	400 mM	12.5 ml
100 mM	sucrose	1 M	5 ml
10 %	PFA		12 ml
0.2 M	NaOH	2 M	0.5 ml
	adjust pH to 7.5 with HCl		ad 50 ml H_2O

Immunofluorescence experiments were essentially performed as previously described for the detection of AQP2 in IMCD cells (Maric et al., 1998). AQP2 or TonEBP was detected by fluorescence microscopy (Leica DMLB microscope with Sensicam 12 Bitled CCD camera, Bensheim, Germany) using anti-AQP2 rabbit polyclonal antiserum (Liebenhoff and Rosenthal, 1995) or anti-TonEBP rabbit polyclonal antiserum (kindly provided by A. Rao and C. López-Rodríguez; López-Rodríguez et al., 1999) and Cy3-conjugated anti-rabbit secondary antibodies. *Fixation and permeabilization of IMCD cells.* Briefly, IMCD cells were rinsed twice with sterile PBS to remove the culture medium. For fixation, cells were incubated for 30 min in cacodylate-sucrose-paraformaldehyde buffer. Subsequently, cells were rinsed twice and then subjected to permeabilization using 0.1 % Triton X-100 in PBS for 5 min. Again, cells were rinsed twice and the coverslips were transferred with a fine pair of tweezers to a coverslip stand, and bathed in PBS for 2 x 10 min to remove residual detergent or paraformaldehyde. *Immunocytochemistry.* Subsequently, cells were blocked with 0.2 % teleostier-gelatine in PBS for 20 min at 37°C. For incubation of cells with primary antibodies, cover slips were transferred to a numerated transparency placed on top of two layers of wet Whatman paper in a tupper-ware box to avoid drying of preparations. Before adding 35 µl of primary antibody solution (anti-AQP2 antiserum, 1 : 600; anti-TonEBP antiserum 1 : 500, anti-actin antibodies 1 : 500) to each cover slip, residual PBS was drained off the coverslips by placing the edge of the glass to an absorbent tissue. Cy3-conjugated goat anti-rabbit antibodies (1 : 600) were used as secondary antibodies. The box was closed and preparations were incubated with the primary antibodies for 1 h at 37°C. Residual primary antibody solution was drained off the coverslips, which were then placed on cover slip stands and bathed in PBS (3 x 15 min). *Mounting.* Cells were washed in PBS (3 x 15 min), residual PBS was drained off, each cover slip was dipped in aqua dest. in order to wash away salts and was subsequently mounted (cell face down) on an ethanol-rinsed microscope slide using a drop of immu-Mount™. *Visualization.* Cy3 fluorescence was visualized by fluorescence microscopy

using a Zeiss KS 400 laser scanning microscope or a Leica DMLB microscope with coupled to a Sensicam 12 Bitled CCD camera (PCO CCD imaging). *Quantification of signal intensities.* For quantification of signal intensities within the nuclei and the cytoplasm of IMCD cells, the programm Zeiss KS 400, version 3.0 (Carl Zeiss Vision) was used. Data were evaluated using Microsoft Exel™.