Effect of water deprivation on aquaporin 4 (AQP4) mRNA expression in chickens (*Gallus domesticus*)

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ABSTRACT

Aquaporin (AQP) 4 is a member of the AQP gene family of water-selective transport proteins. We studied the effect of water deprivation on AQP4 gene expression in chickens. The nucleotide sequence of a chicken aquaporin 4 (AQP4) cDNA, that encodes a protein of 335 amino acids showed high homology to mammalian AQP4. Using Northern-blotting analysis, AQP4 mRNA in chickens was observed as a band of approximately 5.5 kb in several tissues in addition to the hypothalamus, proventriculus, kidney and breast muscle. Quantitative analysis by real-time RT-PCR analysis showed that the mRNA expression of AQP4 in the hypothalamus significantly increased after dehydration. On the other hand, the mRNA expression of AQP4 in the kidney significantly decreased after dehydration. This suggests that AQP4 may play a pivotal role in osmoregulation in the chicken brain.

Theme: Endocrine and autonomic regulation

Topic: Osmotic and thermal regulation

Keywords: chicken, aquaporin 4 (AQP 4), cDNA cloning, water deprivation, real-time PCR, gene expression

Aquaporins (AQP) are water-selective transport proteins that confer high-membrane water permeability to certain tissues in animals, plants and microorganisms. AQP4 cDNA has been isolated in rats [1, 2], humans [3], mice [4] and bovines [5]. Mammalian AQP4 is characterized by its high water permeability and mercurial-insensitive water channel [6]. The primary expression sites of AQP4 are glia limitans, the epdymallining system, cerebellum, hippocampal dentate gyrus, and supraoptic and periventricular nuclei in the hypothalamus [1, 7]. Recent studies on brain AQP4 have revealed the important role of AQP4 in brain water homeostasis. AQP4 is involved in blood-brain barrier (BBB) development, function and integrity [8]. AQP4-knockout mice studies showed that AQP4 plays a key role in osmotically driven water transport and the development of cytotoxic brain edema, and participates in the absorption of excess brain water and the resolution of vasogenic brain edema [9]. AQP4 is also proposed as a mechanism of central osmoreception corresponding to Verney's hypothalamic 'vesicular osmometers' [1, 10, 11]. In several peripheral tissues and cells, there are several reports that the gene expression of AQP1, 2, 3, and 5 are increased by hyperosmotic stress [12-18]; however, , there is an in vitro report that AQP4 and 9 mRNA levels are induced in the brain after hyperosmotic mannitol stress [19].

In birds, the quail AQP4 cDNA sequence was recently cited in the

computer database (accession number X80232), but little is known about the gene expression of AQP4. The purpose of this study was therefore to examine the gene expression of AQP4 in relation to water deprivation in chickens. First, as the quail AQP4 cDNA sequence contains only an open-reading frame and does not include the untranslated 3' region, we cloned a full-length nucleotide sequence of the chicken AQP4 cDNA homologue. Second, we examined the tissue distribution of AQP4 mRNA using Northern blotting and analyzed the changes in AQP4 mRNA levels after 2 days of water-deprivation treatment by real-time RT-PCR.

One-day-old male white leghorn chicks were obtained from a local commercial supplier (Chubu Kagaku Shizai Co., Ltd., Nagoya, Japan). The chicks were killed by decapitation and brain tissues were collected, frozen in liquid nitrogen and stored at -80℃ until RNA extraction. For the isolation of chicken AQP 4 cDNA, total RNA was extracted from the hypothalamus using TRIzol^{TM} reagent (Invitrogen, CA). The resulting pellet of total RNA was dissolved in water, and total RNA was measured by spectrophotometer at 260 nm. Reverse transcription was performed with 1 μg total RNA and poly-(T_{18}) using PowerScript (QIAGEN K.K., Tokyo, Japan).

PCR was performed using a pair of primers designed from the human AQP4 cDNA sequence, namely, the sense and antisense primers were 5'- CAC ATC AAC CCC GCT GTG ACG GT-3' and 5'- CCA AAG

GAT CGG GCG GGG TTC AT-3', respectively. All subsequent PCR reaction steps were performed using ExTaq (Takara Bio. Inc., Kyoto) and a programmable thermocycler (GeneAmp2400, Appied Biosystems, Foster City, CA). The reaction was incubated for 30 cycles at 95˚C, 55˚C and 72˚C each for 0.5 min, with an elongated step of 5 min during the first 95˚C and the final 72˚C. To obtain the 5' and 3' regions of chicken AQP 4 cDNA, the RACE method was employed using a SMART RACE cDNA Amplification Kit (Clontech, CA). All PCR fragments were cloned into pGEM-T Easy vector (Promega, WI) for DNA sequencing.

One-day-old male white Leghorn chicks were obtained from a local commercial supplier (Chubu Kagaku Shizai, Co., Ltd., Nagoya, Japan). For Northern analysis, chicks were maintained with free access to food and water until 7 days old. They were killed by decapitation and tissues (brain, lung, heart, liver, proventriculus, duodenum, rectum, kidney and muscle) were collected. Total RNA was extracted from each tissue and 25 μg of total RNA was fractionated in 1.0% formaldehyde-agarose gel electrophoresis with 50 V constant. After electrophoresis, agarose gel was stained with ethidium bromide. Total RNA was transferred to a nylon membrane (Hybond N^+, Amersham, UK) by capillary action.

The membranes were pre-hybridized with hybridization buffer (50% formamide, 5 x SSC, 1 x Denhardt's solution, 25 mg/ml salmon sperm DNA and 10% dextran sulfate) for 2 hr at 42℃. After

prehybridization, the membranes were incubated with hybridization buffer containing a [α^{32}P]-dCTP-labeled DNA probe for 18 hr at 42℃. The AVT cDNA insert, 270 bp of the distal 3' glycopeptide part of the AVT gene was labeled with [α^{32}P]-dCTP (ICN International) using a *Bca*BEST™ Labeling Kit (Takara Bio, Kyoto, Japan). After hybridization, these membranes were washed three times, with a final washing in 0.1 x SSC containing 0.1% SDS at 60℃ for 15 min. The hybridized membranes were exposed to x-ray film with an intensifying screen (Amersham Inc., UK) for 2 to 7 days at -70℃.

One-day-old male white leghorn chicks were maintained with free access to food and water until 7 days old, after which they were divided into two groups, (1) free access to food and water (control group) and (2) free access to food without water (dehydration group), and kept for 2 days. Tissue samples (hypothalamus, proventriculus, kidney and breast muscle) were taken and total RNA was extracted from the tissue. The extracted total RNA was treated with DNase and reverse transcribed into cDNA. Real-time PCR was carried out using a Smart Cycler II System (Takara Bio Inc., Japan) using Quantitect SYBR Green PCR (QIAGEN K.K., Japan). PCR reactions were subjected to the following thermal protocol: (97℃ × 5 s; 60℃ × 10 s; 72℃ × 30 s) × 50 cycles. The relative expression was analyzed according to the $\Delta\Delta C_t$ method using the S17 gene for normalization [20]. The chicken AQP4 primers were 5'-CCA TCG TGG

GAG CTG GCA TCC TCT A-3'and 5'-AAT GTA ATT ATC AGC TCC ACC AGG A-3'. The chicken arginine vasotocin (AVT) primers were 5'-GCA GTG AGC AGG CAG AAG AGG-3' and 5'-GCT CAG AGG CCA GGC TGC TTG-3' [21]. The chicken ribosome protein S17 primers were 5'-AAG CTG CAG GAG GAG GAG AGG-3' and 5'-GGT TGG ACA GGC TGC CGA AGT-3' [22].

All values are the mean ± SEM. Statistical differences were analyzed using the t-test at $p<0.05$.

Full-length cloned chicken AQP4 cDNA and deduced amino acid sequences are shown in Fig. 1. The cDNA was 1940 bp long and encoded a chicken AQP4 protein consisting of 335 amino acid residues. The predicted amino acid sequence of chicken AQP4 cDNA contains six transmembrane domains and two NPA motifs (Asn-Pro-Ala) preserved in the AQP4 cDNA of mammals [1, 2, 3, 4, 5]. The deduced amino acid sequence of chicken AQP4 shows 98.8% homology to that of quail AQP4 (accession number X80232) and 84.5% homology to human AQP4 [3] (Fig. 2). The avian-deduced amino acid sequence of AQP4 is 12 amino acids longer than that of mammals [1, 2, 3, 4, 5] (Fig. 2).

A single band of approximately 5.5 kb was detected in the chicken hypothalamus, proventriculus, kidney and breast muscle (Fig. 3). There were no bands in the lung, heart, liver, duodenum and rectum.

Two days of dehydration treatment significantly increased plasma

osmolality in chickens (control group, 321.5 ± 2.4 mOsmol; water deprivation group, 357.4 ± 3.4 mOsmol, $p<0.05$). Real-time RT-PCR analysis showed that mRNA levels of hypothalamus AVT increased after water-deprivation treatment ($p<0.05$), concurrently with a significant increase in AQP4 mRNA levels in the hypothalamus ($p<0.05$) (Fig. 4). On the other hand, AQP4 mRNA levels in the kidney significantly decreased ($p<0.05$). There were no significant differences in AQP4 mRNA levels in the proventriculus and breast muscle.

Full-length chicken AQP4 cDNA encoded the 335 predicted amino acid residues. The encoded protein has several unique characteristics common to other AQP4, consisting of 6 transmembrane domains and 2 NPA motifs as reported previously in mammals. Jung *et al*. [1] suggested that rat AQP4 cDNA has two potential initiation sites, but we failed to isolate a clone corresponding to rat M23 from the chicken hypothalamus.

This study reported that hyperosmotic stimulation increased hypothalamus AQP4 mRNA levels in chickens. The brain was the primary expression site of AQP4 in these chickens, similar to rat AQP4 mRNA distribution [6]. In rats, a quantitative analysis study showed that AQP4 mRNA expression was highest in the brain, followed by the eye, trachea, lung, stomach, kidney, and skeletal muscle [6]. In this study, we could not find AQP4 mRNA in the lung, but the order of AQP4 mRNA levels was similar to the results in rats. As reported previously [23. 24],

water-deprivation treatment increased plasma osmolality and hypothalamic mRNA levels of AVT, an antidiuretic hormone in birds. AQP4 was proposed as a mechanism of central osmoreception corresponding to Verney's hypothalamic 'vesicular osmometers' [1, 10, 11]. AQP4 is located in a number of brain regions associated with the osmoregulation of vasopressin secretion and thirst, including SON and SFO; however, little is known about the regulation of AQP4 gene expression in the brain in relation to osmolalityvariation. Recently, Arima et al. [19] demonstrated that, in cultured rat astrocytes, hyperosmotic manitol induced AQP4 mRNA expression but hyperosmotic NaCl did not. This study showed for the first time that hyperosmolality induced AQP4 gene expression in the brain. The mechanism of hyperosmolality-induced AQP4 mRNA expression is not clear. Recently, Nierman et al. [25] reported that vasopressin has a pivotal role in the modulation of water flux in the neocortex and suggested that the effect of vasopressin may be mediated by AQP4 modulation. Therefore, the results of increased AQP4 mRNA levels by dehydration in this study may be induced by increased AVT.

It is now recognized that AQP2 is responsible for antidiuretic hormone-induced increases in water permeability in the kidney collecting duct [26]. AQP4 also plays a role in basolateral membrane water movement in the kidney, because water permeability in the inner medullary collecting duct in AQP4 knockout mice fell compared to normal mice [27].

Therefore, AQP4 may also play a role in the kidney of chickens; however, decreased AQP4 mRNA levels in the kidney after water deprivation suggest that AQP4 does not have a pivotal anti-diuretic function in the kidney of chickens.

This study clearly showed that dehydration induced AQP4 gene expression in the chicken hypothalamus; however, we did not examine the distribution of AQP4 in the chicken brain and the cell type of AQP4-expressing cells. Further study is necessary to understand whether AQP4 is involved in the mechanism of central osmoreception in chickens.

Acknowledgements

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FIGURE LEGENDS

Fig. 1. The nucleotide sequence and deduced amino acid sequence of chicken AQP4 cDNA. Both strands were completely sequenced. NPA motifs (open box) are shown. Six transmembrane regions are indicated by underlining. The termination codon is marked with an asterisk.

Fig. 2. Amino acid alignment of chicken AQP4 (accession number U68063), quail AQP 4 (X80232), rat AQP4 (U14007), human AQP 4 (U63611-U63623) and mouse AQP 4 (M23633). Reversed letters indicate identical amino acids. NPA motifs (asterisk) are shown. Six transmembrane regions (TM 1-6) are indicated by underlining.

Fig. 3. Northern blotting of chicken AQP 4 mRNA expression in the tissues of 7-day-old male chicks. Upper panel represents an autoradiogram of mRNA transcripts of chicken AQP 4. Lower panel represents ethidium bromide-stained S18 and S28 ribosomal RNA as a loading control.

Fig. 4. Real-time RT-PCR analysis of AVT (A) and AQP4 (B) mRNA expression after 2 days' dehydration treatment. AVT and AQP4 mRNA expression was analyzed according to the $\Delta\Delta C_t$ method [9], using the

S17 gene for normalization. Results are represented as the mean ± SEM (n = 4-5) relative values to the mean of the control group. An asterisk shows significant difference from the control group (t-test, $p<0.05$).

Figure 1

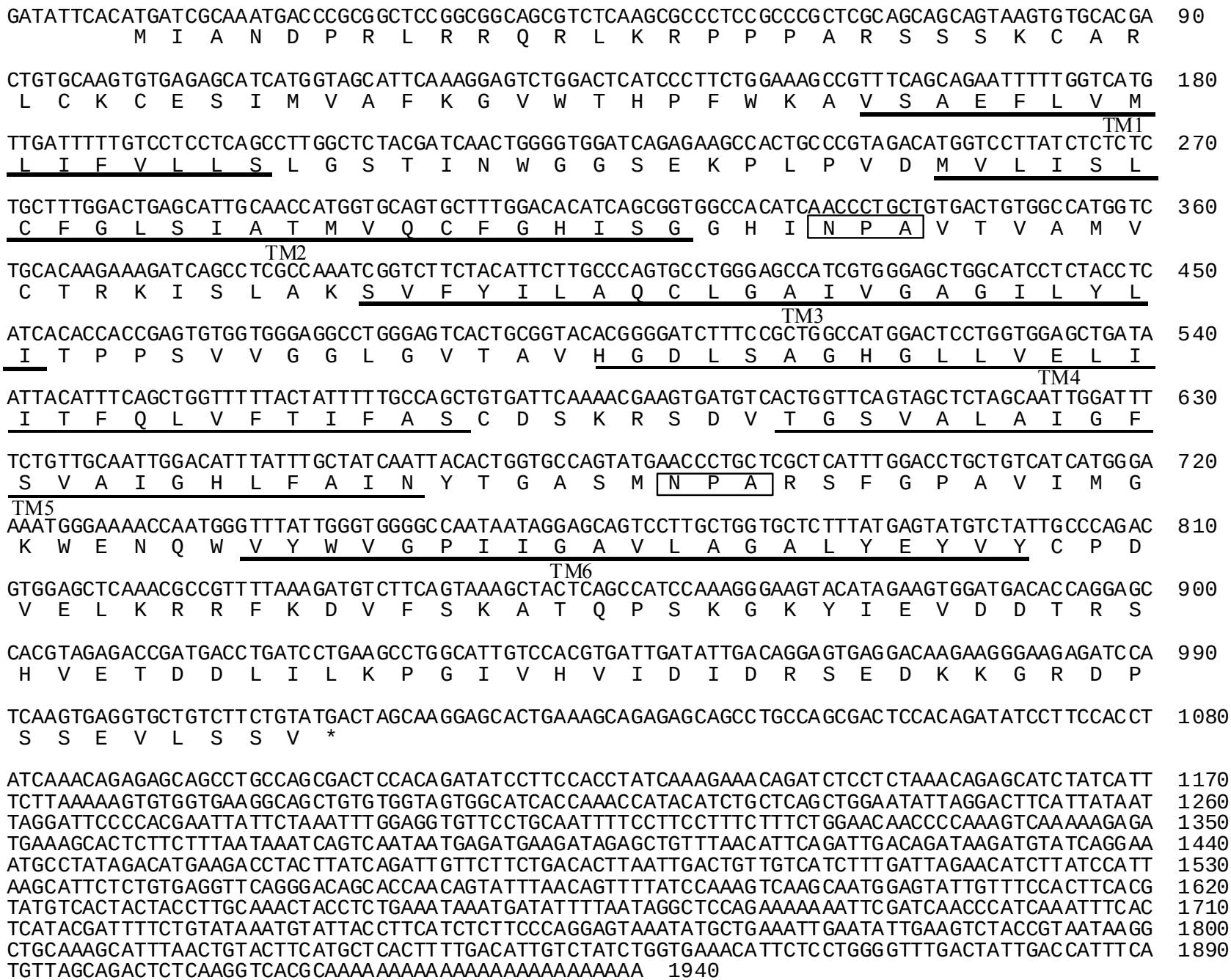
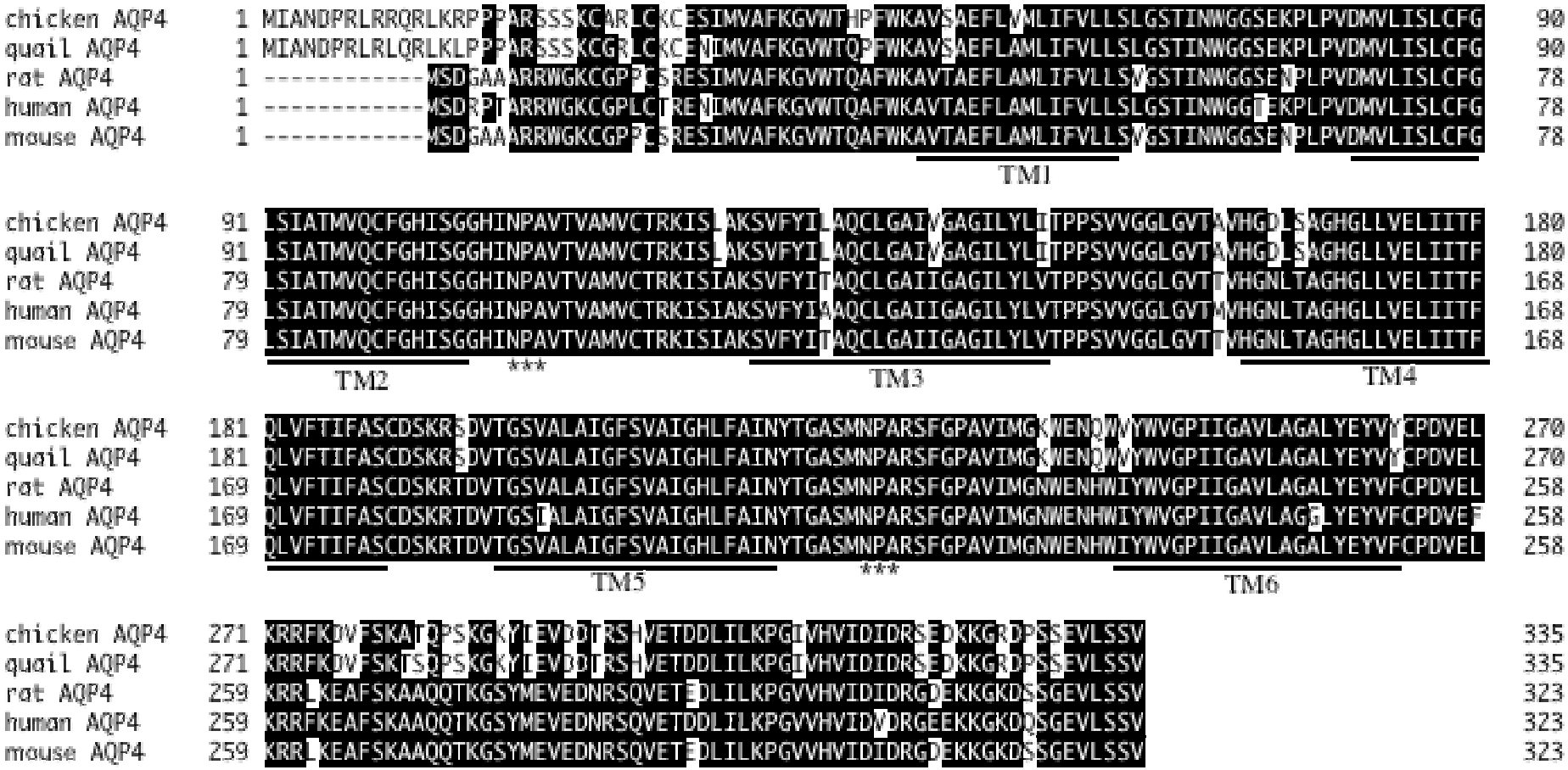


Figure 2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quail AQP4 1 MIANDPRLRLQRLKLPPPARGSSKCGRLCKCENIMVAFKGVWTQPFWKAVSAEFLAMLIFVLLSLGSTINWGGSEKPLPVDMVLISLCFG 90
rat AQP4 1 ----------MSDGAAARRWGKCGPPCSRESIMVAFKGVWTQAFWKAVTAEFLAMLIFVLLSVGSTINWGGSENPLPVDMVLISLCFG 78
human AQP4 1 ----------MSDRPTARRWGKCGPLCTRENIMVAFKGVWTQAFWKAVTAEFLAMLIFVLLSLGSTINWGGTEKPLPVDMVLISLCFG 78
mouse AQP4 1 ----------MSDGAAARRWGKCGPPCSRESIMVAFKGVWTQAFWKAVTAEFLAMLIFVLLSVGSTINWGGSENPLPVDMVLISLCFG 78
TM1
chicken AQP4 91 LSIATMVQCFGHISGGHINPAVTVAMVCTRKISLAKSVFYILAQCLGAIVGAGILYLITPPSVVGGLGVTAVHGDLSAGHGLLVELIITF 180
quail AQP4 91 LSIATMVQCFGHISGGHINPAVTVAMVCTRKISLAKSVFYILAQCLGAIVGAGILYLITPPSVVGGLGVTAVHGDLSAGHGLLVELIITF 180
rat AQP4 79 LSIATMVQCFGHISGGHINPAVTVAMVCTRKISIAKSVFYITAQCLGAIIGAGILYLVTPPSVVGGLGVTTVHGNLTAGHGLLVELIITF 168
human AQP4 79 LSIATMVQCFGHISGGHINPAVTVAMVCTRKISIAKSVFYIAAQCLGAIIGAGILYLVTPPSVVGGLGVTMVHGNLTAGHGLLVELIITF 168
mouse AQP4 79 LSIATMVQCFGHISGGHINPAVTVAMVCTRKISIAKSVFYITAQCLGAIIGAGILYLVTPPSVVGGLGVTTVHGNLTAGHGLLVELIITF 168
TM2
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TM3
TM4
chicken AQP4 181 QLVFTIFASCDSKRSDVTGSVALAIGFSVAIGHLFAINYTGASMNPARSFGPAVIMGKWENQWVYWVGPIIGAVLAGALYEYVFCPDVEL 270
quail AQP4 181 QLVFTIFASCDSKRSDVTGSVALAIGFSVAIGHLFAINYTGASMNPARSFGPAVIMGKWENQWVYWVGPIIGAVLAGALYEYVFCPDVEL 270
rat AQP4 169 QLVFTIFASCDSKRTDVTGSVALAIGFSVAIGHLFAINYTGASMNPARSFGPAVIMGNWENHWIYWVGPIIGAVLAGALYEYVFCPDVEL 258
human AQP4 169 QLVFTIFASCDSKRTDVTGSIALAIGFSVAIGHLFAINYTGASMNPARSFGPAVIMGNWENHWIYWVGPIIGAVLAGGLYEYVFCPDVEF 258
mouse AQP4 169 QLVFTIFASCDSKRTDVTGSVALAIGFSVAIGHLFAINYTGASMNPARSFGPAVIMGNWENHWIYWVGPIIGAVLAGALYEYVFCPDVEL 258
TM5
***
TM6
chicken AQP4 271 KRRFKDVFSKAIQPSKGKYIEVDDTRSHVETDDLILKPGIVHVIDIDRGEDKKGKDPSSEVLSSV 335
quail AQP4 271 KRRFKDVFSKTSQPSKGKYIEVDDTRSHVETDDLILKPGIVHVIDIDRGEDKKGKDPSSEVLSSV 335
rat AQP4 259 KRRLKEAFSKAAQQTKGSYMEVEDNRSQVETDDLILKPGVVHVIDIDRGDEKKGKDSSGEVLSSV 323
human AQP4 259 KRRFKEAFSKAAQQTKGSYMEVEDNRSQVETDDLILKPGVVHVIDVDRGEEKKGKDQSGEVLSSV 323
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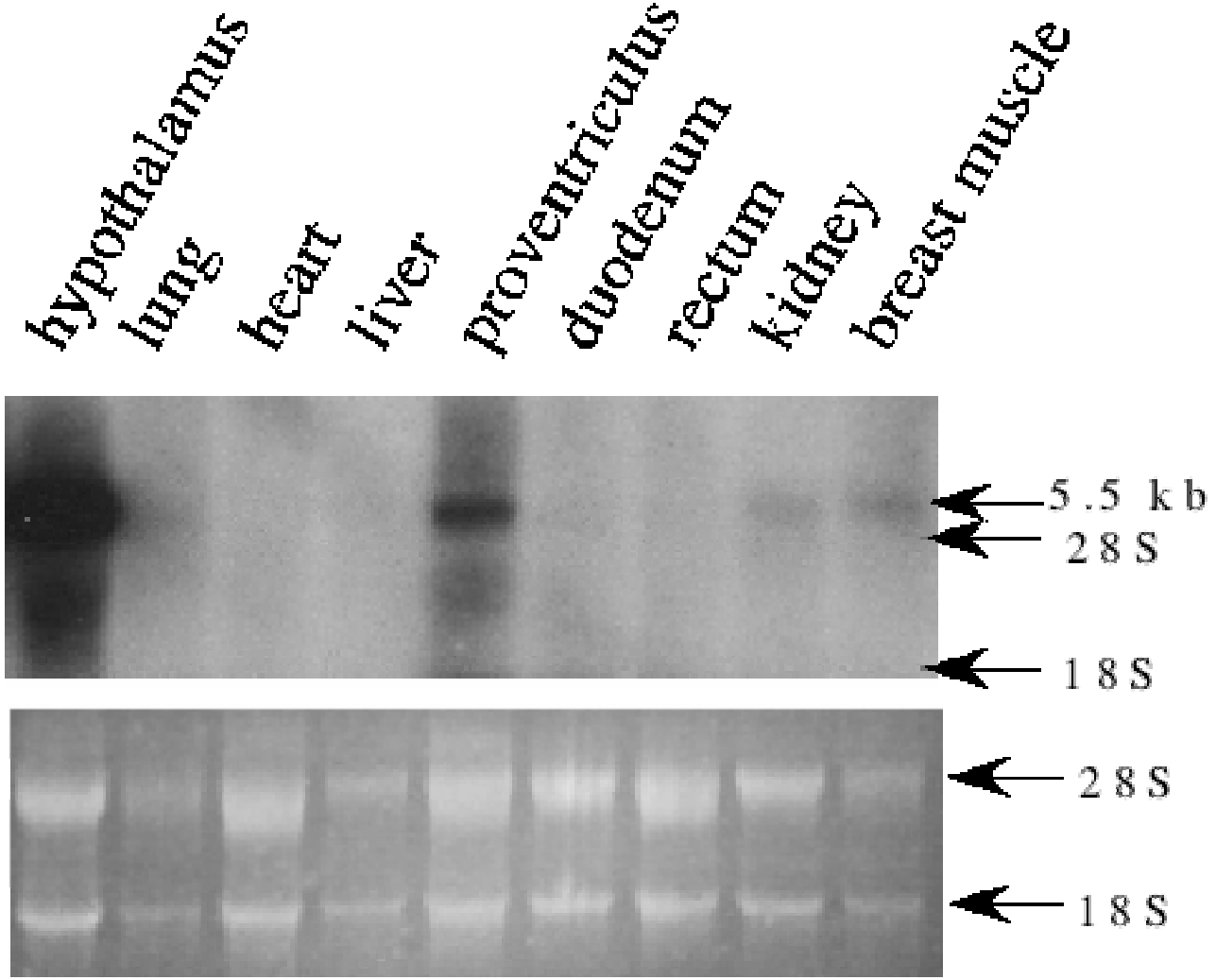
Figure 3


hypothalamus
lung
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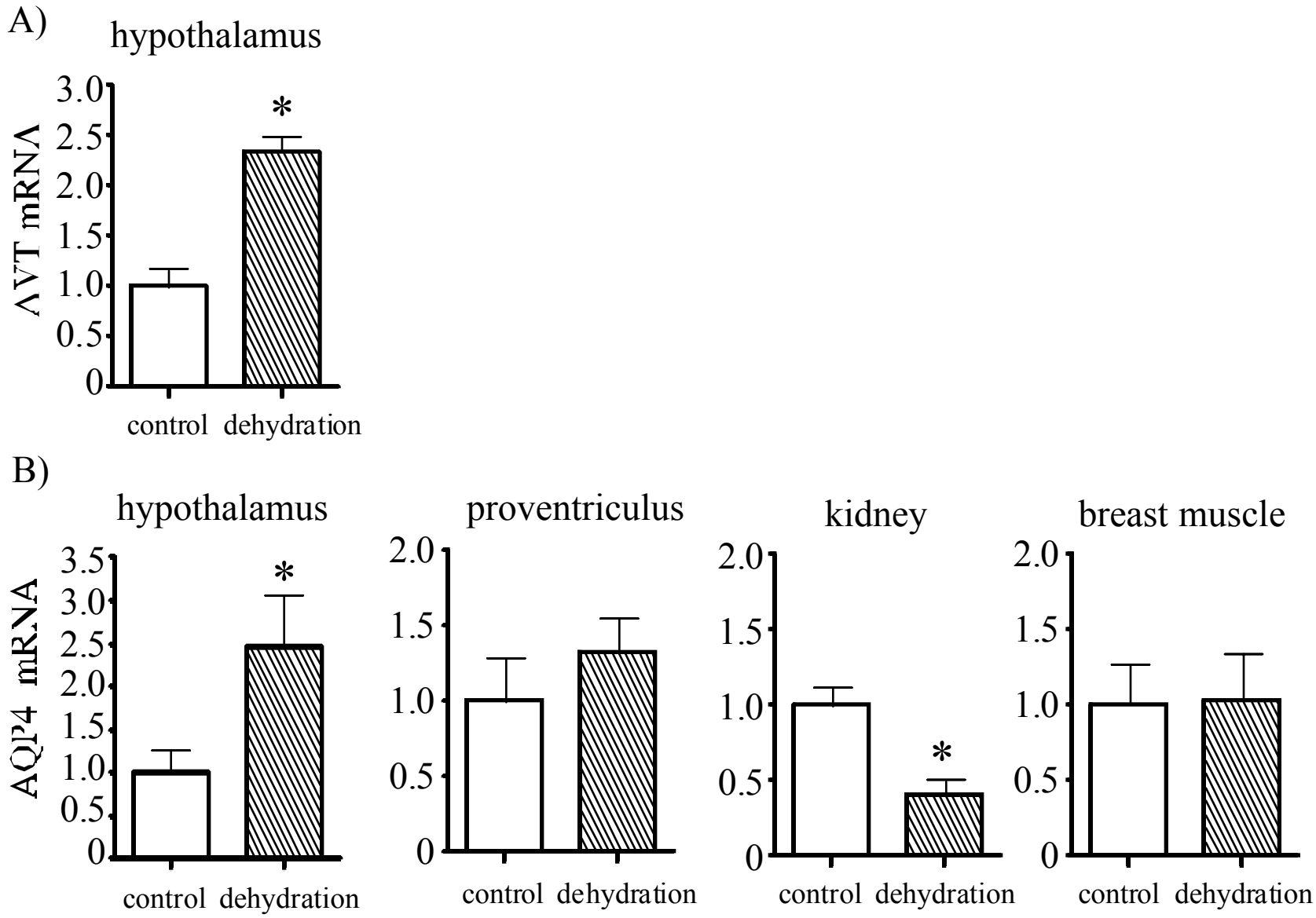
duodenum
rectum
kidney
breast muscle
5.5 kb
28S
18S
28S
18S

Figure 4


A)
hypothalamus
AVT mRNA
3.0
2.5
2.0
1.5
1.0
0.5
0
*
control
dehydration
B)
hypothalamus
AQP4 mRNA
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
control
dehydration
proventriculus
2.0
1.5
1.0
0.5
0
control
dehydration
kidney
2.0
1.5
1.0
0.5
0
*
control
dehydration
breast muscle
2.0
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1.0
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0
control
dehydration