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Original article

Ovine AQP_1: cDNA cloning, ontogeny, and control of renal gene expression*

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Abstract. The cDNA for the ovine aquaporin 1 (AQP_1) was obtained and found to be 97%, 88%, and 85%, respectively, homologous to the bovine, human, and rat AQP_1 cDNA. The level of total kidney mRNA expressed as a ratio to glyceraldehyde-3-phosphate dehydrogenase increased sevenfold from 60 days to 140 days of gestation (term=150 days) and reached adult values by 6 weeks after birth. Treatment of pregnant ewes (and their fetuses) at 64 and 74 days of gestation with dexamethasone (0.76 mg/h for 48 h) resulted in a small but statistically significant increase in AQP_1 mRNA only in the 74-day fetuses. By immunohistochemistry, it was shown that the increase in AQP_1 mRNA with dexamethasone resulted largely from an increase in maturity of the inner zone of the fetal renal cortex (i.e., more tubules) as well as stronger expression of AQP_1 in proximal tubules and thin descending limbs of loops of Henle. A similar effect occurred in fetuses infused for 3 days with angiotensin I (6.7 μg/h) in the last third of gestation.

Key words: Ovine – Aquaporin 1 – Dexamethasone – Angiotensin I – Fetus

Introduction

The adult kidney filters some 26 moles of sodium daily, and reabsorbs more than 99% of this, mostly (81%) in the proximal convoluted and straight tubules [1]. Water follows the sodium reabsorbed, and can do so rapidly, in the short time that the filtrate takes to pass through the proximal segments and thin descending limbs of the loops of Henle, because these renal segments contain large numbers of water channels, on both the apical and basolateral surfaces [2–4]. These water channels are composed of glycosylated and non-glycosylated forms of a peptide of approximately 270 amino acids (28 kilodaltons) known as aquaporin 1 (AQP_1). The highest concentration of AQP_1 protein (measured by enzyme-linked immunosorbent assay and expressed as femtomoles per millimeter) in the adult rat kidney is in the initial segment of the thin descending limb of the loops of Henle of the deepest cortical glomeruli in the inner stripe of the outer medulla [5]. The concentration of AQP_1 protein is less (approximately one-half that of outer medulla) in the thin descending limb segments in the inner medulla, then lower in the proximal tubule straight segments of the outer stripe of the outer medulla, and lowest overall in the proximal convoluted and straight tubules of the cortex. However, the overall total cortical content of AQP_1 is somewhat higher than that of the total medulla [5].

In the fetus the glomerular filtration rate (GFR) is only half that of the adult, so the filtered sodium load is halved, and a smaller percentage (51% vs. 81%) of the filtered sodium is reabsorbed in the proximal segments [1, 6]. Thus the requirement for water channel production is considerably less in the fetus than the adult. In addition, the fetal kidney is not fully developed, in terms of loop of Henle development, at birth, even in those species (human, sheep) in which nephrogenesis is complete before birth [7, 8]. Thus the amount of AQP_1 should be lower in the fetus than in the adult. In the rat, in which nephrogenesis is not complete before birth, and GFR is not maximal until 4 weeks after birth, there is only a slight amount of AQP_1 protein detected close to term–around day 18–19 [9, 10]. In the human fetus, however, some AQP_1 protein is present in proximal tubules of 12- to 13-week gestation fetal metanephroi (approximately 5% of adult cortical value) and at birth the total amount of AQP_1 protein is only 47% of the adult cortical value [11].

Most of the functional studies on fetal kidneys have been carried out in the chronically cannulated ovine fetus [7, 8, 12]. The genes for AQP_1 have been cloned in the human [13], rat [14], and cow [15]. In order to investigate further the regulation of AQP_1 production in the

* Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession no. AF009037

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                    10        20        30        40        50        60
bovaqp1.seq     ATGGCCAGCGAGTTCAAGAAGAAGCTCTTTTGGAGGGCGGTGGTGGCCGAGTTCCTGGCC
haqp1.seq       ATGGCCAGCGAGTTCAAGAAGAAGCTCTTCTGGAGGGCAGTGGTGGCCGAGTTCCTGGCC
rataqp1.seq     ATGGCCAGCGAAATCAAGAAGAAGCTCTTCTGGAGGGCTGTGGTGGCTGAGTTCCTGGCC
saqp1.seq       ATGGCCAGCGAGTTCAAGAAGAAGCTCTTTTGGAGGGCGGTGGTGGCCGAGTTCCTGGCC
                ***********  ********** ******** ******** ************

bovaqp1.seq     ATGATCCTCTTCATCTTCATCAGCATCGGTTCAGCCCTGGGCTTCCACTACCCAATAAAG
haqp1.seq       ACGACCCTCTTTGTCTTCATCAGCATCGGTTCTGCCCTGGGCTTCAAATACCCGGTGGGG
rataqp1.seq     ATGACCCTCTTCGTCTTCATCAGCATCGGTTCTGCCCTAGGCTTCAATTACCCACTGGAG
saqp1.seq       ATGATCCTCTTCATCTTCATCAGCATCGGTTCAGCCCTGGGCTTCCACTACCCAATAAAG
                * ** ******  ****************** ***** ****** * *****  *   *

bovaqp1.seq     AGCAACCAGACGACAGGTGCAGTCCAGGATAACGTGAAGGTGTCACTGGCCTTTGGGTTG
haqp1.seq       AACAACCAGACG------GCGGTCCAGGACAACGTGAAGGTGTCGCTGGCCTTCGGGCTG
rataqp1.seq     AGAAACCAGACG------CTGGTCCAGGACAATGTGAAGGTGTCACTGGCCTTTGGTCTG
saqp1.seq       AGCAACCAGACGACAGGTGCAGTCCAGGATAACGTGAAAGTGTCACTGGCCTTTGGGTTG
                *  *********         ******** ** ***** ***** ******** **  **

bovaqp1.seq     AGCATCGCCACGCTGGCCCAGAGCGTGGGCCACATCAGCGGTGCCCACCTCAACCCAGCC
haqp1.seq       AGCATCGCCACGCTGGCGCAGAGTGTGGGCCACATCAGCGGCGCCCACCTCAACCCGGCT
rataqp1.seq     AGCATCGCTACTCTGGCCCAAAGTGTGGGTCACATCAGTGGTGCTCACCTCAACCCAGCG
saqp1.seq       AGCATCGCCACGCTGGCCCAGAGTGTGGGCCACATCAGCGGTGCCCACCTCAACCCAGCC
                ******** ** ***** ** ** ***** ******** ** ** *********** **

bovaqp1.seq     GTCACACTGGGGCTCCTGCTCAGCTGCCAGATCAGTGTCCTCCGGGCCATCATGTACATC
haqp1.seq       GTCACACTGGGGCTGCTGCTCAGCTGCCAGATCAGCATCTTCCGTGCCCTCATGTACATC
rataqp1.seq     GTCACACTGGGGCTTCTGCTCAGCTGTCAGATCAGCATCCTCCGGGCTGTCATGTATATC
saqp1.seq       GTCACACTGGGGCTCCTGCTCAGCTGCCAGATCAGTATCCTCCGGGCCATCATGTACATC
                ************** ********** ********  ** **** **  ******* ***

bovaqp1.seq     ATTGCCCAGTGCGTGGGGGCCATCGTTGCCACTGCCATCCTCTCGGGCATCACCTCCTCT
haqp1.seq       ATCGCCCAGTGCGTGGGGGCCATCGTCGCCACCGCCATCCTCTCAGGCATCACCTCCTCC
rataqp1.seq     ATCGCCCAGTGTGTGGGAGCCATCGTTGCCTCCGCCATCCTCTCCGGCATCACCTCCTCC
saqp1.seq       ATTGCCCAGTGCGTGGGGGCCATCGTCGCCACTGTCATCCTCTCGGGCATCACCTCCTCT
                ** ******** ***** ******** *** * * ********* **************

bovaqp1.seq     CTGCCCGACAACTCGCTTGGCCTCAATGCGCTGGCCCCTGGCGTGAACTCGGGCCAGGGC
haqp1.seq       CTGACTGGGAACTCGCTTGGCCGCAATGACCTGGCTGATGGTGTGAACTCGGGCCAGGGC
rataqp1.seq     CTGCTCGAGAACTCACTTGGCCGAAATGACCTGGCTCGAGGTGTGAACTCCGGCCAGGGC
saqp1.seq       CTGCCTGACAACTCGCTTGGCCTCAATGCGCTGGCCCCTGGCGTGAACTCGGGCCAGGGC
                ***   *  ***** *******  ****  *****    ** ******** *********

bovaqp1.seq     CTGGGCATCGAGATCATCGGGACTCTGCAGCTGGTGCTGTGCGTGCTGGCCACCACCGAC
haqp1.seq       CTGGGCATCGAGATCATCGGGACCCTCCAGCTGGTGCTATGCGTGCTGGCTACTACCGAC
rataqp1.seq     CTGGGCATTGAGATCATTGGCACCCTGCAGCTGGTGCTGTGCGTTCTGGCTACCACTGAC
saqp1.seq       CTGGGCATCGAGATCATCGGGACTCTGCAGCTGGTGCTGTGTGTGCTGGCCACCACCGAC
                ******** ******** ** ** ** *********** ** ** ***** ** ** ***

bovaqp1.seq     CGGAGG---CGCCGTGACCTCGGGGGCTCTGGGCCCCTGGCCATTGGCTTCTCTGTGGCC
haqp1.seq       CGGAGG---CGCCGTGACCTTGGTGGCTCAGCCCCCCTTGCCATCGGCCTCTCTGTAGCC
rataqp1.seq     CGGAGG---CGCCGAGACTTAGGTGGCTCAGCCCCACTTGCCATTGGCTTGTCTGTGGCT
saqp1.seq       CGGAGGAGGCGCCGCGACCTCGGGGACTCCGGACCCCTTGCCATTGGCTTCTCTGTGGCA
                ******   ***** *** * ** * *** *  ** ** ***** *** * ***** **

bovaqp1.seq     CTGGGACATCTGCTGGCGATAGACTACACCGGCTGCGGTATTAACCCTGCCCGGTCCTTC
haqp1.seq       CTTGGACACCTCCTGGCTATTGACTACACTGGCTGTGGGATTAACCCTGCTCGGTCCTTT
rataqp1.seq     CTTGGACACCTGCTGGCCATTGACTACACTGGCTGTGGGATCAACCCTGCCCGGTCATTT
saqp1.seq       CTTGGACACCTGCTGGCGATAGACTACACTGGCTGCGGTATTAACCCTGCCCGGTCCTTC
                ** ***** ** ***** ** ******** ***** ** ** ******** ***** **

bovaqp1.seq     GGCTCCTCGGTGATTACGCACAATTTCCAGGACCACTGGATCTTCTGGGTGGGGCCGTTC
haqp1.seq       GGCTCCGCGGTGATCACACACAACTTCAGCAACCACTGGATTTTCTGGGTGGGGCCATTC
rataqp1.seq     GGCTCTGCTGTGCTCACCCGCAACTTCTCAAACCACTGGATTTTCTGGGTGGGACCATTC
saqp1.seq       GGCTCCTCGGTGATCACGCACAATTTCCAGGACCACTGGATCTTCTGGGTGGGGCCGTTC
                *****  * *** * ** * *** ***    ********** ********** ** ***

bovaqp1.seq     ATCGGAGCAGCCCTGGCAGTGCTCATCTATGACTTCATCCTGGCGCCACGCAGCAGTGAC
haqp1.seq       ATCGGGGGAGCCCTGGCTGTACTCATCTACGACTTCATCCTGGCCCCACGCAGCAGTGAC
rataqp1.seq     ATTGGGAGTGCCCTGGCAGTGCTGATCTATGACTTCATCCTGGCCCCACGCAGCAGCGAC
saqp1.seq       ATCGGAGCAGCCCTGGCAGTGCTCATCTATGACTTCATCCTGGCCCCACGCAGCAGCGAC
                ** **    ******** ** ** ***** ************** ************ ***

bovaqp1.seq     CTCACAGACCGCGTGAAGGTGTGGACCAGCGGTCAGGTGGAGGAGTATGACCTGGATGCC
haqp1.seq       CTCACAGACCGCGTGAAGGTGTGGACCAGCGGCCAGGTGGAGGAGTATGACCTGGATGCC
rataqp1.seq     TTTACAGACCGCATGAAGGTGTGGACCAGTGGCCAAGTGGAGGAGTATGACCTGGATGCT
saqp1.seq       CTCACAGACCGCGTGAAGGTGTGGACCAGCGGTCAGGTGGAGGAGTATGACCTGGATGCC
                 * ********* ********** ** ** ***********************

bovaqp1.seq     GACGACATCAACTCCAGGGTGGAGATGAAGCCCAAATAA
haqp1.seq       GACGACATCAACTCCAGGGTGGAGATGAAGCCCAAATAG
rataqp1.seq     GATGATATCAACTCCAGGGTGGAGATGAAGCCCAAATAG
saqp1.seq       GACGACATCAACTCCAGGGTGGAGATGAAGCCCAAATAG
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A

Fig. 1. A Comparison of the ovine AQP_1 cDNA (saqp1) nucleotide (nt) sequence with that of the bovine (bovaqp1), human (haqp1), and the rat (rataqp1). The additional segments of ACAGGT and AGG are observed at nt 133–138 and nt 487–489 of the ovine cDNA, respectively. **B** Comparison of the ovine amino acid sequence with the bovine, human and, rat sequences. The similarities between the four sequences are indicated by *asterisks*

fetal kidney, it was necessary to obtain the ovine cDNA for AQP_1, and to study the normal ontogeny.

When neonatal rats (day 4) were treated with betamethasone, there was a doubling of renal AQP_1 protein [16]. It is hypothesized that this increase was due to increased tubule length rather than increased expression per cell. It has been shown that treatment of the pregnant ewe and fetus at 70–75 days of gestation (where term is approximately 150 days) with dexamethasone for 2 days increases tubular growth and medullary development [17]. To test the hypothesis that increased tubular development would be accompanied by increased AQP_1 mRNA expression, kidneys from the previous study [17] were also examined for AQP_1 mRNA and protein expression. In a preliminary report [18], it was shown that angiotensin II (Ang II) could increase AQP_1 mRNA expression, temporarily, in an immortalized transformed rat proximal tubule cell line, via an action on angiotensin type I (AT_1) receptors. From previous studies [19], renal RNA was available from ovine fetuses which had been infused for 3 days, in the last third of gestation, with Ang I the precursor of Ang II. AQP_1 mRNA levels were quantitated in 5 of these fetuses, together with 5 control animals from the same study.

Materials and methods

Animals. All experimental and surgical procedures were approved by the animal ethics committee of the Howard Florey Institute in accordance with the regulations of the National Health and Medical Research Council of Australia. Fetuses of 21 pregnant Merino ewes, of known mating date, were used as well as 12 lambs, 1–10 weeks of age. Ten of the ewes carried twin fetuses which were treated as separate animals. For the ontogeny study, ewes, with fetuses, and lambs were not subjected to any other experiment. Tissue was collected from 4 fetuses at each of 60, 100, and 140 days. For the comparison of the effects of dexamethasone, 8 ewes, 3 carrying twins, were infused with dexamethasone (Decadron Phosphate Shock Pak, Merck, Sharp and Dohme, Boronia, Australia) at 0.76 mg/h for 48 h prior to collection of tissues. Four ewes were 64±1 days of gestation at the time of tissue collection and 4 were 74±1 days. Control animals (6 ewes, 9 fetuses) were infused with isotonic saline at 0.38 ml/h for 48 h prior to tissue collection. Physiological studies on these sheep have been reported previously [17]. In the Ang I study, Ang I (6.7 μg/h in 0.19 ml saline) or isotonic saline (0.19 ml/h) was infused for 3 days into chronically cannulated ovine fetuses at 110–120 days of gestation (term=145–150 days) as described previously [19]. Renal tissue from 5 controls and 5 Ang I-infused fetuses was used for AQP_1 mRNA quantitation.

Ewes, fetuses, and lambs were killed by an overdose of sodium pentobarbitone (Lethabarb, Arnolds, Boronia, Australia – 100 mg/kg body weight). Fetal, maternal, and lamb kidneys were removed and snap-frozen in liquid nitrogen, then stored at –80° C prior to use. One kidney from each fetus in the dexamethasone/saline protocol was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 4 h. These organs were washed in phosphate buffer for 8 h, dehydrated in alcohol, and embedded in paraffin.

cDNA cloning. Oligonucleotide primers were synthesized on an Applied Biosystems Oligonucleotide Synthesizer (Perkin Elmer, N.J., USA). Four primers were selected with high homology between the rat and human cDNA of AQP_1-CHIP (Genbank accession no: M77829 and X67948, respectively). Sense primers A:CAGCATGGCCAGCGAGTTCAAGA [nucleotide (nt) +35 to +57 of the human], B:CAGCGGCGCCCACCTCAACCC (nt +248 to +268) and antisense primers, C:TGTCGTCGGC ATCCAGGTCATAC (nt +794 to +817), and D:TCTATTT GGGCTTCATCTCCACCCTGGAGT (nt +820 to +849). cDNA was reverse transcribed from 5 μg of sheep outer cortex adult kidney RNA using random hexamer primers and AMV reverse transcriptase (Promega, Madison, Wis., USA). The cDNA was amplified by polymerase chain reaction (PCR) at 94° C for 1 min 55° C for 2 min, and 72° C for 2 min, 30 cycles. The PCR products were electrophoresed on an agarose gel, eluted, and then subcloned into pBluescript II KS+ (Stratagene, Calif., USA) in the polylinker region and transformed into bacteria. Positive clones were then selected and plasmids isolated and sequenced.

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                    10        20        30        40        50        60
bovaqp1.pep     MASEFKKKLFWRAVVAEFLAMILFIFISIGSALGFHYPIKSNQTTGAVQDNVKVSLAFGL
haqp1.pep       MASEFKKKLFWRAVVAEFLATTLFVFISIGSALGFKYPVGNNQT--AVQDNVKVSLAFGL
rataqp1.pep     MASEIKKKLFWRAVVAEFLAMTLFVFISIGSALGFNYPLERNQT--LVQDNVKVSLAFGL
saqp1.pep       MASEFKKKLFWRAVVAEFLAMILFIFISIGSALGFHYPIKSNQTTGAVQDNVKVSLAFGL
                **** ***************  ** ********** **   ***   *************

bovaqp1.pep     SIATLAQSVGHISGAHLNPAVTLGLLLSCQISVLRAIMYIIAQCVGAIVATAILSGITSS
haqp1.pep       SIATLAQSVGHISGAHLNPAVTLGLLLSCQISIFRALMYIIAQCVGAIVATAILSGITSS
rataqp1.pep     SIATLAQSVGHISGAHLNPAVTLGLLLSCQISILRAVMYIIAQCVGAIVASAILSGITSS
saqp1.pep       SIATLAQSVGHISGAHLNPAVTLGLLLSCQISILRAIMYIIAQCVGAIVATVILSGITSS
                ********************************  ** *************  ********

bovaqp1.pep     LPDNSLGLNALAPGVNSGQGLGIEIIGTLQLVLCVLATTDRRRR-DLGGSGPLAIGFSVA
haqp1.pep       LTGNSLGRNDLADGVNSGQGLGIEIIGTLQLVLCVLATTDRRRR-DLGGSAPLAIGLSVA
rataqp1.pep     LLENSLGRNDLARGVNSGQGLGIEIIGTLQLVLCVLATTDRRRR-DLGGSAPLAIGLSVA
saqp1.pep       LPDNSLGLNALAPGVNSGQGLGIEIIGTLQLVLCVLATTDRRRRRDLGDSGPLAIGFSVA
                *  **** * ** ******************************** *** * ***** ***

bovaqp1.pep     LGHLLAIDYTGCGINPARSFGSSVITHNFQDHWIFWVGPFIGAALAVLIYDFILAPRSSD
haqp1.pep       LGHLLAIDYTGCGINPARSFGSAVITHNFSNHWIFWVGPFIGGALAVLIYDFILAPRSSD
rataqp1.pep     LGHLLAIDYTGCGINPARSFGSAVLTRNFSNHWIFWVGPFIGSALAVLIYDFILAPRSSD
saqp1.pep       LGHLLAIDYTGCGINPARSFGSSVITHNFQDHWIFWVGPFIGAALAVLIYDFILAPRSSD
                ********************** * * **  *********** *****************

bovaqp1.pep     LTDRVKVWTSGQVEEYDLDADDINSRVEMKPK
haqp1.pep       LTDRVKVWTSGQVEEYDLDADDINSRVEMKPK
rataqp1.pep     FTDRMKVWTSGQVEEYDLDADDINSRVEMKPK
saqp1.pep       LTDRVKVWTSGQVEEYDLDADDINSRVEMKPG
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B **Fig. 1B**

DNA sequencing. Double-stranded DNA sequencing was performed using an ABI 373A Sequencer (Perkin Elmer). The ovine AQP_1 sequence was confirmed by comparison with known human and rat AQP_1 cDNA sequences in the Genbank DNA database with ANGIS (Australian National Genomic Information Services).

RNA isolation and northern blot. RNA was isolated from ovine fetal kidney [20]. RNA was electrophoresed on 1% agarose gels in 20 mM MOPS (3-[*N*-morpholino]propanesulfonic acid), pH 7.0/8 mM sodium acetate/1 mM ethylenediaminetetra-acetic acid disodium salt (Na_2EDTA), pH 8.0/2.2 M formaldehyde (all reagents from Sigma, St. Louis, Mo., USA). The denatured RNA was then transferred to Hybond-C Super (Amersham Life Sciences, Buckinghamshire, England, UK) membranes by capillary elution. Filters were baked at 80° C for 2 h to fix the RNA and then prehybridized for at least 1 h. The ovine cDNA was labelled with [alpha ^{32}P] dCTP (3000 Ci/mmol, 1 Ci=37 gbq, NEN Research Products, Boston, Mass., USA). The filters were then hybridized overnight at 65° C in 5×SSC, 5× Denhardt's reagent (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), 1 mM Na_2EDTA, 1% sodium dodecyl sulfate (SDS), and 100 µg/ml heat-denatured salmon sperm DNA (all reagents from Sigma). Membranes were washed twice in 2×SSC, 0.1% at 65° C and then twice in 0.1×SSC, 0.1% SDS at 65° C for 15 min each. The filters were exposed on to a phosphorimaging plate, which was subsequently developed on a Fuji BAS2000 Bio-Imaging Analyzer (Fuji Photo Film, Tokyo, Japan). The filter was then later exposed to autoradiography with Hyperfilm (Eastern Kodak, New Haven, Conn., USA) for 3 days. The membranes were then stripped by washing in boiling 0.1×SSC, 0.1% SDS and then reprobed with an internal standard, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The radioactivity present in each band was quantitated, and the ratio of the sample to the standard was calculated.

Statistical analysis. Means and standard errors are given throughout. The unpaired *t*-test was used to assess statistical significance in the dexamethasone or Ang I and control fetuses. An analysis of variance with post-hoc testing was used to assess the ontogenic changes in fetuses and lambs [21].

Morphology. To demonstrate the developmental changes in renal morphology, sections (4-µm) of kidneys at 60, 75, 100 and 140 days were cut and stained with hematoxylin and eosin.

Immunohistochemistry. The distribution of AQP_1 protein was assessed in control and dexamethasone-treated fetuses at 64 and 74 days, and control and Ang I-treated fetuses at 110–120 days. Sections (4-µm) of paraffin-embedded kidney were cut and mounted on 0.1% gelatinized slides. The avidin-biotin immunoperoxidase method, as previously described [22], was used for immunostaining. Briefly, sections were deparafinized and rehydrated, then immersed in 0.3% hydrogen peroxide for 30 min followed by washing in 0.1 M phosphate buffer. Using a humid chamber, non-specific antigens were blocked by use of 10% normal horse serum (NHS). Serial sections were incubated with AQP_1 antibody (used at 1:200 as in [23], kindly donated by Dr. Mark Knepper, National Institute of Health) raised in rabbit and diluted in 2% NHS in 0.1 M phosphate buffer. Separate sections were also incubated without primary antibody to act as a negative control. After washing, sections were incubated with anti-rabbit secondary antibody (Vector, Burlingame, Calif., USA) diluted 1:200 in phosphate buffer for 45 min. Sections were again washed before a 45-min incubation with 1:100 avidin-biotin complex (Vector, Burlingame, Calif., USA). Sections were then incubated in 5 mg/ml 3,3,-diaminobenzidine tetrahydrochloride dihydrate containing 0.01% hydrogen peroxide for 3 min, counterstained with Meyer's haematoxylin for 30 s, differentiated in Scott's tap water, dehydrated in alcohol, and mounted. Sections from control, dexamethasone-, and Ang I-treated animals were run in each assay.

Image acquisition. To obtain light micrographs, a Sony 3CCD (charge-coupled device) color video camera DXC-930P (Sony Australia, Melbourne) coupled to a Nikon Microphot microscope (FSE, Melbourne, Australia) was used. The images were analyzed using the microcomputer imaging device M2 image analyzer (Imaging Research, St. Catherines, Canada) and printed on a Fujix Bas HG-printer (Berthold Australia, Bundoora, Victoria, Australia)

Results

Analysis of the AQP_1 cDNA

The reverse transcription-PCR experiment on sheep outer cortex adult kidney RNA gave an 819-base pair cDNA fragment corresponding to nt +35 to +849. Figure 1A and B shows the nucleotide and deduced amino acid sequences of the ovine AQP_1 cDNA respectively compared with the bovine, human, and rat sequences. The ovine cDNA has an initiating ATG and contains an open reading frame with a stop codon at +819. Computer search of the Genbank DNA database identified a strong homology with the known bovine, human, and rat sequenc-

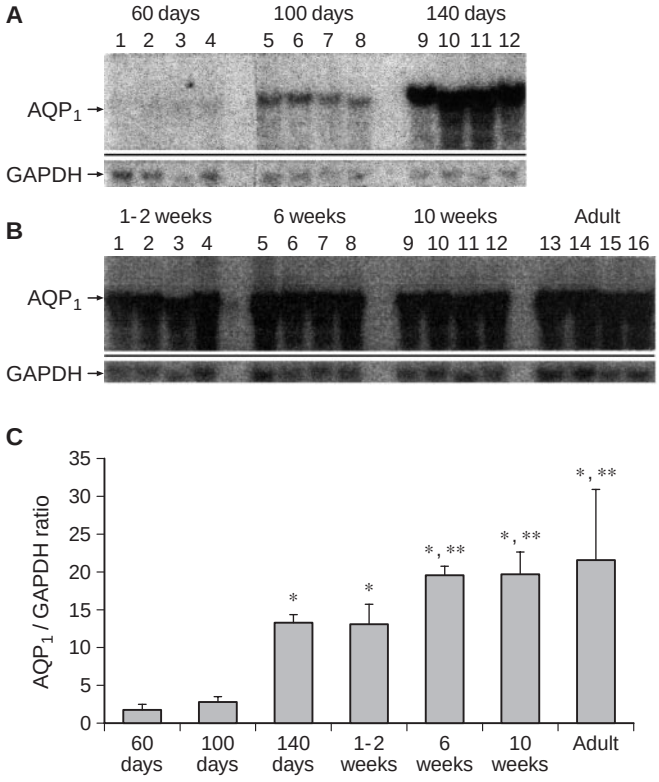

Fig. 2A–C. Northern blot analyses of AQP_1 total RNA isolated from sheep at a range of ages; 15 µg of RNA was used in each lane. The blot was hybridized with a ^{32}P-labelled fragment [517 base pairs (bp)] of the ovine AQP_1 cDNA clone or with the ovine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe. The 3.0-kilobase (kg) signal is identified by *arrows.* **A** Fetal kidney 60, 100, and 140 days of gestation; **B** lamb and adult kidney. **C** The ratio of AQP_1 to GAPDH standard (mean±SEM). * $P<0.001$ pre 140 days of gestation compared with 140 days of gestation onwards; * $P<0.05$ pre 1–2 weeks compared with 6 weeks onwards

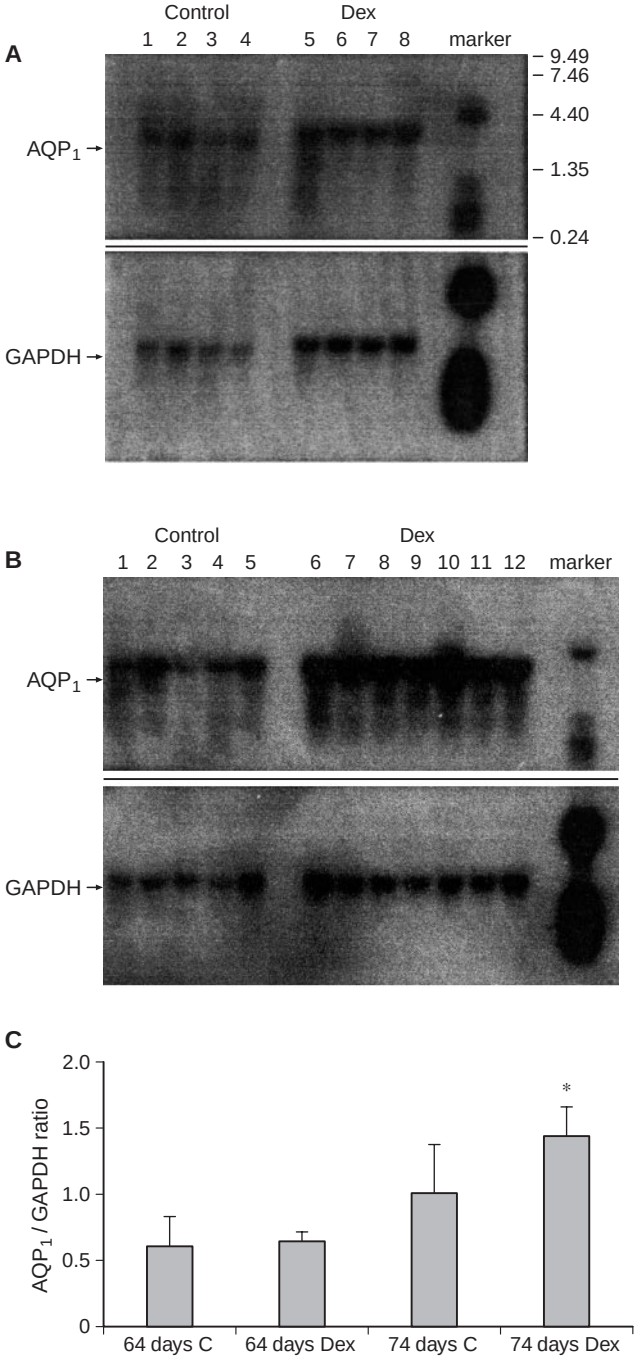

Fig. 3A–C. Northern blot analyses of AQP_1 total RNA isolated from sheep fetal kidney in control (*C*) and dexamethasone (*Dex*) treated animals at 64 and 74 days of gestation; 15 µg of RNA was used in each lane. The size of the RNA markers in kb is shown at the right. The blot was hybridized with a ^{32}P-labeled fragment (517 bp) of the ovine AQP_1 cDNA clone or of the ovine GAPDH probe. The 3.0-kb signal is identified by *arrows*. **A** 64 days of gestation. RNA isolated from fetal kidney of saline-infused (*lanes 1–4*) and dexamethasone-treated (*lanes 5–8*) fetuses; **B** 74 days of gestation. RNA isolated from fetal kidney of saline-infused (*lanes 1–5*) and dexamethasone-treated (*lanes 6–12*) fetuses; **C** the ratio of AQP_1 to GAPDH standard in 64 and 74 days of gestation fetuses (mean±SEM). * $P<0.05$

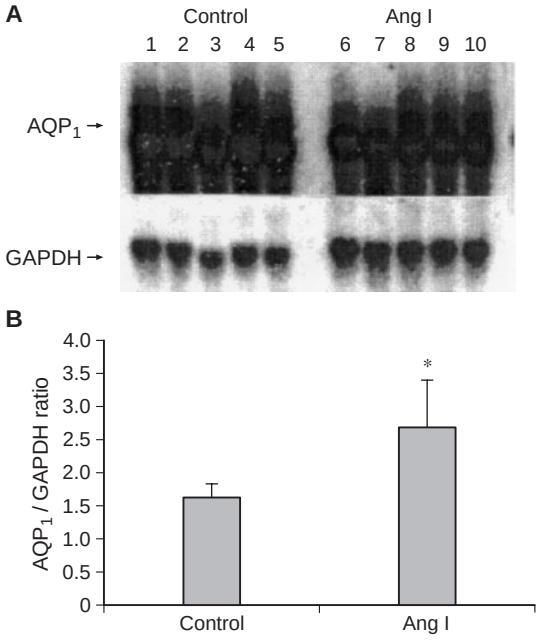

Fig. 4A, B. Northern blot analyses of AQP_1 total RNA isolated from sheep fetal kidney in control and angiotensin I (*Ang* I)-treated animals at 110–120 days of gestation. The blot was hybridized with the same 517-bp ovine cDNA and with the ovine GAPDH probe. **A** 110–120 days of gestation, RNA isolated from fetal kidney of saline-infused (*lanes 1–5*) and Ang I-treated (*lanes 6–10*) fetuses; **B** the ratio of AQP_1 to GAPDH standard (mean±SEM). * $P<0.01$

es. The ovine nucleotide sequence showed 97%, 88%, and 85% homology to the bovine, human, and rat sequences, respectively (Fig. 1A), while with the ovine amino acid sequence, homology was 98%, 90% and 88% (Fig. 1B). The conserved amino acid residue cysteine 189 was also observed, which is common to water channels. Additional segments of ACAGGT representing threonine and glycine at nt 133–138 were found only in the bovine sequence, while AGG representing arginine at nt 487–489 were not found in the bovine, human, and rat sequences.

Northern analysis

Northern analysis of total kidney mRNA from fetal sheep showed a single transcript of 2.9–3.0 kilobases (Fig. 2A) and was observed throughout development (Fig. 2A, B). The amount of AQP_1 mRNA, as determined by Northern blot and expressed as a ratio to GAPDH mRNA, increased approximately sevenfold from 100 to 140 days of gestation, at which stage it was about 61% of the value in kidneys from adult pregnant sheep (Fig. 2C). By 6 weeks after birth the values were not significantly different from adult values.

The amount of AQP_1 mRNA in the fetal kidneys of ewes treated with dexamethasone at 64 days of gestation did not change (Fig. 3A, C). However, in the kidneys of fetuses at 74 days of gestation (Fig. 3B, C), there was an increase of 43% in dexamethasone-treated animals com-

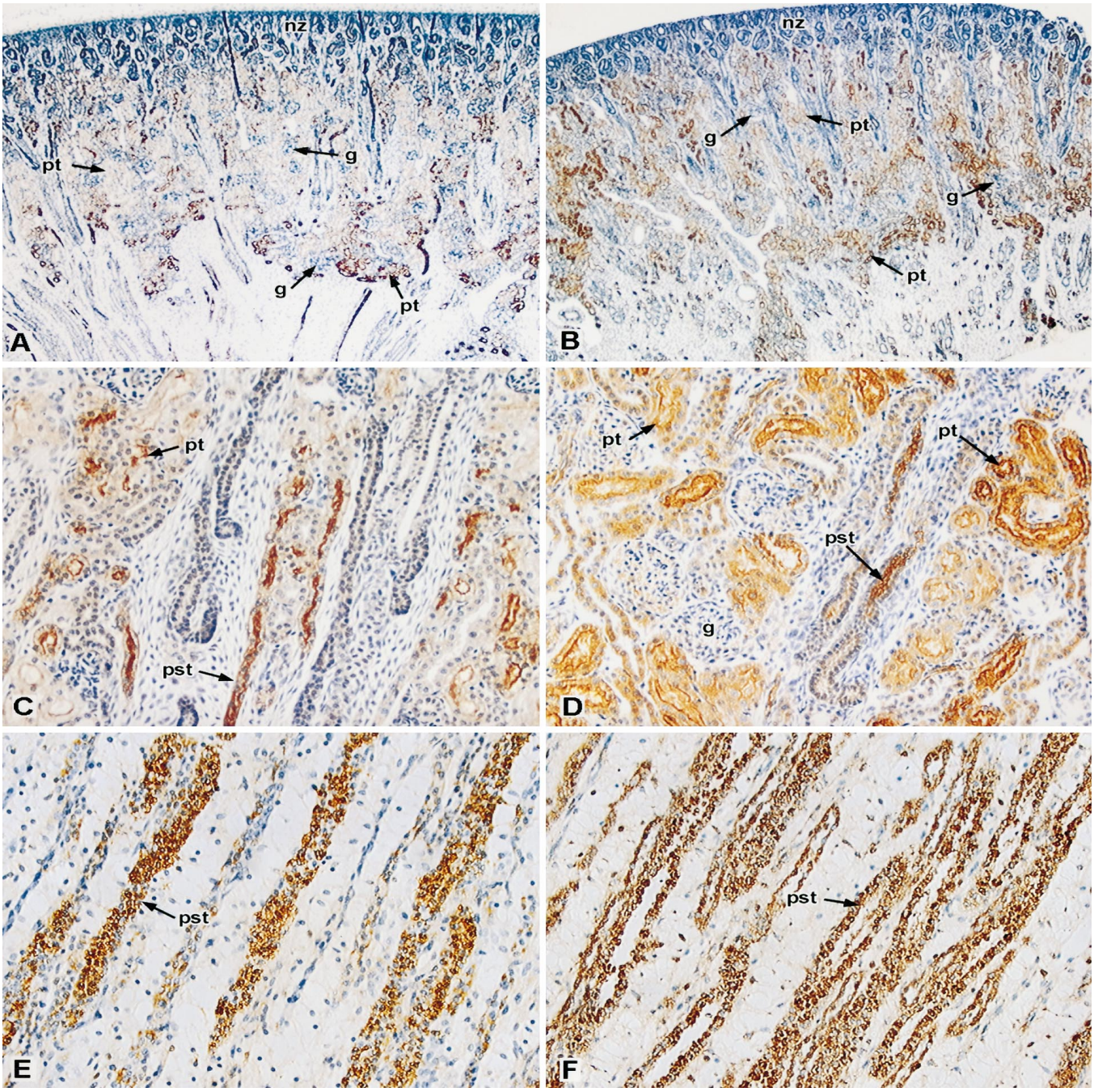

Fig. 5A–F. Effects of dexamethasone treatment on fetal kidney at 74 days and Ang I treatment at 100–120 days of gestation. When compared with controls (**A**), dexamethasone treatment (**B**) induces maturation of the fetal kidney as shown by the decrease in the nephrogenic zone (*nz*) and an increase in the maturity and numbers of proximal convoluted tubules (*pt*) containing an increased AQP_1 immunoreactivity (original magnification ×40). A similar difference between control (**C**) and dexamethasone-treated (**D**) fetal kidneys is observed at higher magnification (×400), although the intensity of immunoreactive AQP_1 in the proximal straight tubules (*pst*) appears to be identical. There is no staining in the renal glomeruli (*g*). Ang I treatment (**F**) produced very little, if any, difference in staining intensity observed in control sections (**E**), but more of the (original magnification ×200) proximal straight tubules showed immunoreactivity

pared with control animals, which was significant ($P<0.05$). As shown in Fig. 4, AQP_1 mRNA was increased significantly ($P<0.01$) by the 3-day infusion of Ang I. The ratio of AQP_1 mRNA to GAPDH mRNA increased from 1.6±0.1 to 2.7±0.2.

Immunohistochemistry

As shown in Fig. 5, the AQP_1 mRNA was translated into protein: at 74 days of gestation (Fig. 5A, C) there was weak immunostaining of some proximal tubules and more intense staining of some of the proximal straight tubules in control tissue. In the section from a dexameth-

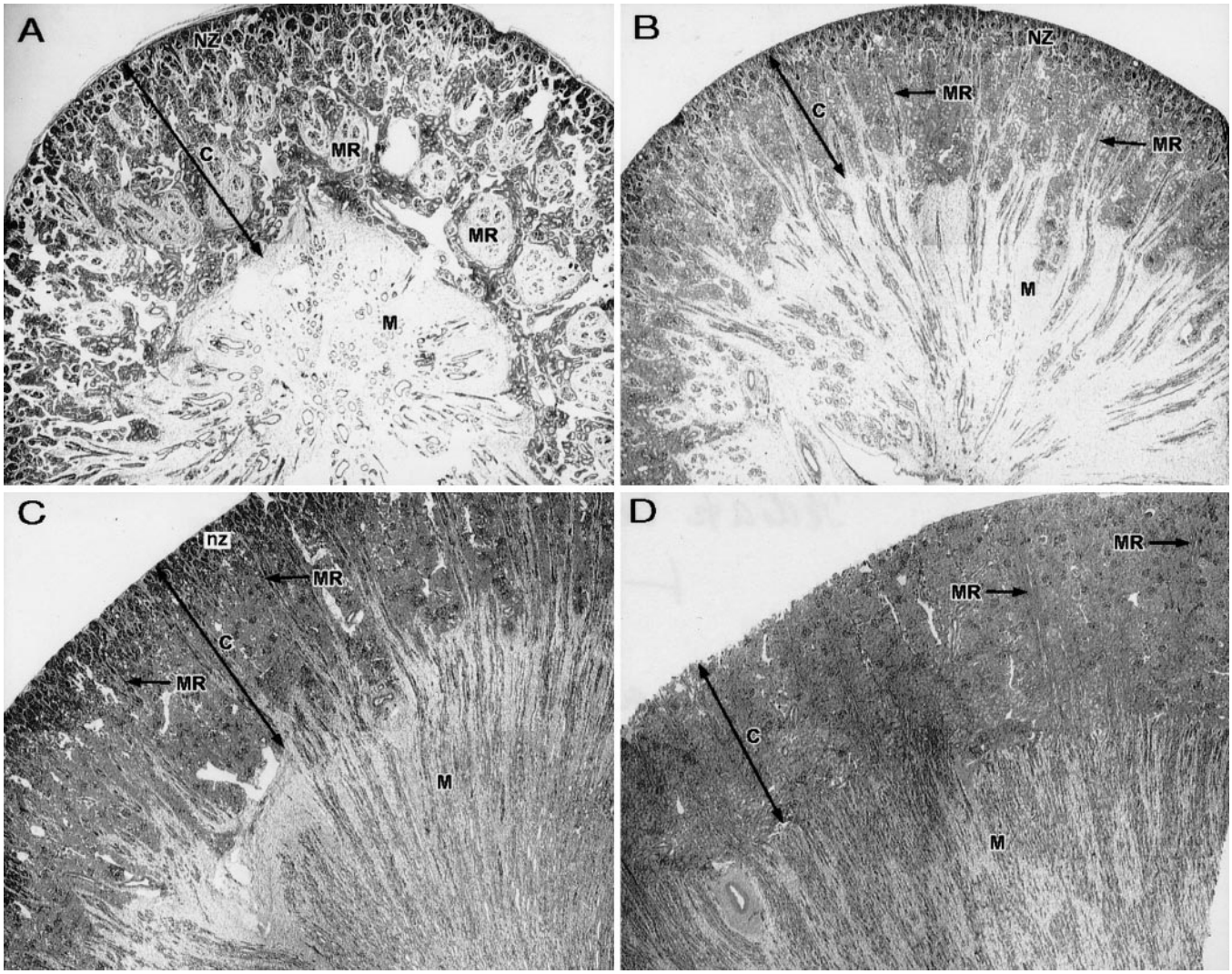

Fig. 6. Comparison of fetal sheep kidneys at 60 (**A**), 75 (**B**), 100 (**C**), and 140 (**D**) days of gestation. During development the nz decreases in size and by 140 days of gestation it has completely disappeared. The cortex (*C*) undergoes dramatic changes with the development of closely packed structures such as the medullary rays (*MR*), proximal and distal tubules, and at the same time the medulla (*M*) becomes densely packed with collecting ducts and loops of Henle. In order to generate an extended view of the kidneys, four separate video images at an original magnification of ×40 were collected. These images were then combined and compressed to form one composite image using the microcomputer imaging device

asone-treated kidney (Fig. 5B, D), there is greater maturity of the corticomedullary area, with a greater number of profiles of proximal convoluted tubules compared with controls, suggesting that proximal tubules have accelerated in development. The staining intensity also appears to have increased. However, no systematic morphometry was performed to quantitate protein expression. At 115 days of gestation (Fig. 5E, F), the AQP_1 protein expression seems stronger than earlier in gestation, and more of the proximal straight tubules are expressing the protein after 3 days of Ang I infusion (Fig. 5F).

Morphology

As shown in Fig. 6, the nephrogenic zone becomes a smaller component of the renal cortex over days 60–100, and has disappeared by 140 days of gestation. At this late stage of development (140 days), the formation of new nephrons has ceased. The cortex contains less interstitial tissue as the glomeruli and tubules proliferate between 60 and 140 days.

Between 60 and 140 days the medulla becomes more complex with the addition of more loops of Henle, vasa recta, and capillary networks, as nephrons develop. At 60 days, the medulla is still rich in interstitial tissue. The medullary rays in the cortex show decreasing interstitial tissue over 60–140 days, as the loops of Henle from the outer cortical glomeruli (the last formed) project into the medulla. Even at 140 days the outer medulla is still interstitial cell rich, as further tubule development occurs in the perinatal period.

Discussion

The ovine AQP_1 cDNA has a high homology to other cloned AQP_1 cDNAs. Moreover, in a separate study we have shown that the mRNA distribution and protein immunoreactivity in the adult kidney of the sheep is similar to that of the rat [24]. The highest concentrations of the mRNA and protein of the adult kidney are in the inner stripe of the outer medulla, followed in descending order by the inner medulla, the outer stripe of the outer medulla, and the cortex [5].

The steady increase of renal AQP_1 mRNA, throughout fetal life and postnatal development in the sheep mirrors the development of the morphology of the renal metanephros. In the sheep, the first division of the ureteric bud, which is the beginning of metanephric development, occurs at day 27 [8, 25]. The metanephric mesenchyme condenses, comma and then S-shaped vesicles are formed; the lower limb of the S becomes the glomerulus, as capillaries invade it, and the upper limb becomes distal tubule, which, at the podocyte folding stage, becomes attached to the ureteric bud/collecting duct [8]. The nephrogenic mesenchyme forms the outermost area of the developing kidney, below which is the nephrogenic zone where the first stages of nephrogenesis occur. As the earliest glomeruli mature, more glomeruli form above them in the nephrogenic zone, so that the "oldest" glomeruli eventually are at the medullary border. The last glomeruli to be formed are at the outermost part of the cortex. The loops of Henle of the glomeruli at the corticomedullary junction extend into the inner medulla, whereas those of the outer cortex have short loops extending only into the inner stripe of the outer medulla. Thus the last section of the kidney to be formed will be the inner stripe of the outer medulla. The increasing complexity of the fetal kidney, particularly of the medulla, is illustrated in Fig. 6. Although nephrogenesis is complete in the ovine fetus by 130–135 days of gestation [8], considerable tubule growth continues into the neonatal period. At birth, there is a sharp three to five fold increase in GFR and an increase in total sodium reabsorption [26–28]. Thus water and sodium reabsorption must also be increased in proximal segments.

The effect of dexamethasone on increased tubule growth, noted earlier [17], was reflected in increased AQP_1 mRNA and protein expression. Synthetic glucocorticoids (betamethasone, 0.3 mg/kg) administered to pregnant rats for 2 days, starting at days E16 to E21, caused significant increases in AQP_1 protein in lungs of both fetuses and mothers [16]. The AQP_1, in the lung, however, was not in alveolar epithelial cells, but in peribronchial blood vessels and visceral pleura. This effect was at least partly at the level of transcription, as shown by a threefold increase in AQP_1 mRNA 12 h after a single injection of betamethasone in day 7 rat pups. The kidneys from day 4 rat pups and adult rats were also studied after betamethasone treatment. Steroid treatment increased AQP_1 protein in the neonatal, but not the adult rats. These experiments are consistent with the hypothesis that in the epithelial cells of the kidney increased AQP_1 expression results from increased tubular maturation, whereas the endothelial cells of peribronchial capillaries and pleural cells may have a tissue-specific upregulation of the *AQP*$_1$ gene. Increased expression of AQP_1 proteins (glycosylated and non-glycosylated) occurred in mouse erythroleukemic cells when differentiated by 1 μM dexamethasone or dimethyl sulfoxide [29]. This indicates that differentiation, by whatever mechanism, can induce increased expression of AQP_1. The mouse gene *Aqp*$_I$ contains two glucocorticoid response elements at –0.5 kilobases from the transcription start site which mediate the effect of dexamethasone. The fact that kidneys responded differently to steroid treatment at different gestational ages is consistent with other reports of time-specific effects. Glucocorticoid treatment alters fibronectin expression in term but not first-trimester human placenta [30]. Glucocorticoid treatment also decreased the expression of the erythropoietin gene in the liver of ovine fetuses at 60 but not at 75 days of gestation [31, 32].

Ang II is known, from in vitro studies, to be a growth factor for adult proximal tubular cells, stimulating hypertrophy and protein synthesis rather than proliferation or hyperplasia, and also causes proliferation of human fetal mesangial cells [33, 34]. Renomedullary interstitial cells in culture hypertrophy and secrete increased extracellular matrix when incubated with Ang II (10^{-6} M) [35], an effect mediated by the AT_{1A} receptor. In the fetal sheep the AT_1 receptor is expressed in presumptive mesangial cells of the glomerulus, and in interstitial cells in the medullary rays and medulla, whilst AT_2 receptors are expressed in the cortical interstitial cells and the epithelial cells of the macula densa [36]. All components of the Ang II-generating system (renin- angiotensinogen, angiotensin converting enzyme) are expressed in ovine fetal kidneys, both meso- and metanephros [25]. The effect of exogenous Ang I on the ovine fetus is to increase blood pressure and GFR, producing a diuresis and natriuresis without change in urine osmolality [19]. These effects are not secondary to changes in fetal plasma cortisol or atrial natriuretic hormone. Thus the increase in AQP_1 mRNA and immunoreactivity in the ovine fetus given Ang I for 3 days could be due to increased growth of tubules and/or maturation of the cells in the straight proximal tubules and descending limbs of loops of Henle. From the immunohistochemistry it seems that maturation (expression in more of the proximal straight tubules) rather than growth contributes mainly to the increased AQP_1 mRNA levels.

In conclusion, the ovine *AQP*$_1$ gene has been cloned; mRNA concentration increases with development, reaching adult values by 6 weeks after birth. Dexamethasone treatment, at mid-gestation, increases AQP_1 mRNA and protein expression, concomitant with increasing the maturity of the corticomedullary zone of the developing kidney. Ang I increased AQP_1 mRNA in the last third of gestation, mainly due to maturation of cells in the proximal straight tubules.

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