Expression Of The Urate Transporter/Channel Is
Developmentally Regulated In Human Kidneys

Deborah P. Hyink, Joshua Z. Rappoport, Patricia D. Wilson, and Ruth G. Abramson

Division of Nephrology, Department of Medicine, Mount Sinai School of Medicine,
One Gustave L. Levy Place, New York, NY, 10029

Abbreviated Title: Renal Expression Of The Human Urate Transporter/Channel

Name and address for mailing proofs:
Ruth G. Abramson, MD
Division of Nephrology
Box 1243
Mount Sinai School of Medicine,
One Gustave L. Levy Place
New York, NY 10029

Telephone: 212-241-0465
Fax: 212-987-0389
e-mail: ruth.abramson@mssm.edu

Copyright 2001 by the American Physiological Society.
ABSTRACT:

Recombinant protein prepared from a cDNA cloned from rat kidney and its human homologue function as urate transporter/channels in lipid bilayers. Using the antibody (anti-uricase) that detected the rat cDNA clone, we now demonstrate that normal human kidneys contain an immunoreactive protein of identical size to that in rat kidney (36-37 kDa), presumably the human urate transporter/channel (hUAT). The amount of hUAT in kidney homogenates increases progressively from 13 weeks of gestation to the early postnatal period. During gestation hUAT expression is confined to the cytoplasm of proximal tubules of stage III/IV nephrons. However, at 1 year of age hUAT is primarily located subapically and within brush-borders of proximal tubules. *Xenopus* oocytes and differentiated A6 cells injected with cRNA and transfected with cDNA of hUAT, respectively, demonstrated a similar pattern: hUAT is not detected in oocytes but is abundantly expressed in cytoplasm and plasma membranes of A6 cells. These data imply that different developmental factors regulate the initiation of cytoplasmic hUAT expression and subsequent insertion into human proximal tubule brush-border membranes.

Key Words:

anti-uricase
fetal kidneys
proximal tubules
*Xenopus* A6 cells
*Xenopus* oocytes
INTRODUCTION:

During the course of evolution the urate oxidase (uricase) gene was inactivated in humans by two nonsense mutations and a mutation that resulted in an aberrant splice acceptor sequence (48, 49). As a consequence, this hepatic peroxisomal enzyme which functions in most mammalian species to catalyze the oxidation of the relatively insoluble urate to the highly soluble compound allantoin (10, 19, 32) is not expressed in humans. In the absence of functional uricase, as in birds, reptiles, and some non-human primates, urate represents the end product of the intracellular degradation of the purines adenine and guanine (3). In species that contain uricase, urate effluxes from systemic cells and thereafter urate homeostasis is largely maintained by the intrahepatic enzymatic degradation of urate to allantoin and to a limited extent by renal and intestinal urate excretion. In humans, however, urate homeostasis and maintenance of stable plasma urate concentrations is entirely dependent on excretion, primarily renal (3) and to a lesser extent intestinal (40, 41).

Because of its prominent role in eliminating urate from the body, the renal handling of urate in man has been subject to investigation over the past fifty of more years (3, 18, 45, 46). Urate does not bind to plasma proteins (26) and, therefore, it is generally accepted that urate is freely filtered at the glomerulus. Since the amount of urate excreted in adult humans only approximates 5 to 10% of the filtered urate (18, 45), it is evident that the vast fraction of filtered urate must be reabsorbed. Based on data obtained in other mammals (3, 46) reabsorption is presumed to occur within the proximal tubule. When tubular urate secretion was detected in the human nephron (17, 36), and after a variety of pharmacological studies were conducted in man, it was suggested that virtually all filtered urate is reabsorbed, that excreted urate derives from tubular secretion and that secretion, like reabsorption also occurs within the proximal tubule (18, 45). It is of interest that the percent of the filtered load of urate that is excreted in pre-term and term infants is significantly greater than in adults (34-75% vs. 5-10%) and is inversely related to the
gestational age of the infant (42, 43). Indeed, fractional urate excretion does not attain the low levels that are observed in adults until approximately one year postpartum (35). Since fractional excretion falls as the filtered load of urate increases, due to a rise in both GFR (35) and plasma urate concentration (44), the reabsorptive capacity of the kidney is presumed to increase and/or the ability to secrete urate diminish with maturation. However, the absolute amount of urate excreted daily actually increases from infancy to adolescence (44). Insofar as excreted urate derives primarily from secretion (18, 45), then the latter observation suggests that the absolute amount of urate secretion increases rather than declines between infancy and adulthood. Thus, the renal handling of urate clearly undergoes developmental changes during gestation and early childhood, but neither the actual changes in transport nor the mechanism(s) responsible for these changes have been defined, in large part due to the complex, bidirectional flux of urate within the proximal tubule.

In a single study in which the mechanism of urate transport has been assessed in renal cortical membrane vesicles prepared from surgically obtained adult human kidney, electroneutral urate/anion exchange and electrogenic urate transport were observed (38). Of note, these same modalities of urate transport have been described in renal cortical membrane vesicles prepared from rat (1, 2, 22, 23), rabbit (24) and dog (5, 15, 16, 21). Although the molecular basis of the urate/anion exchanger remains unknown, we have recently cloned a unique cDNA from a rat renal expression library, demonstrated that the 36-37 kDa recombinant protein prepared from this cDNA functions as a highly selective urate transporter/channel in a lipid bilayer system, and documented that this protein has a number of functional characteristics that suggest that it is the molecular representation of the rat electrogenic urate transporter (27, 29). Of note, this clone was identified with a rabbit polyclonal antibody to affinity purified pig liver uricase (25). Importantly, this same antibody had identified a 36 kDa protein that was affinity purified from rat renal cortices, selectively inhibited electrogenic urate transport in rat renal cortical membrane vesicles,
and, in immunocytochemical studies of adult rat kidney was selectively reactive in apical membranes of proximal tubules, the site of urate transport (25). Finally, anti-uricase also blocked activity of the recombinant urate transporter/channel (27, 29), a phenomenon that could be ascribed to the presence of a local block of homology to porcine uricase within the amino acid sequence of the channel (27). A human homologue with 84% homology (73% identity) to the rat amino acid sequence of the urate transporter/channel has also been identified (31). Moreover, recombinant protein prepared from the human cDNA is both reactive to anti-uricase and functions as a selective urate channel in a lipid bilayer system (31) with a number of functional characteristics identical to that of the rat protein (28). Of note, the human amino acid sequence, like that of the rat, contains a local block of amino acids with homology to uricase.

Based on the above data which indicates that our polyclonal antibody to pig liver uricase selectively interacts with and identifies the rat and human urate transporter/channel, the present studies were conducted to determine if the human kidney contains a protein reactive to this same antibody. After documenting that the adult human kidney cortex, like that of the rat, contains a 36-37 kDa protein that is immunoreactive to this antibody, Western blot analyses were employed to assess the amount of protein expressed in renal cortices at various gestational ages, at several postnatal ages in children and in normal adults and immunohistochemistry was used to localize the distribution of this protein in human kidneys at comparable developmental stages. These studies demonstrate that a protein immunoreactive to anti-uricase, presumed to be the urate transporter/channel, appears relatively late in the course of nephrogenesis of proximal tubules, and that apical and brush-border membrane localization is first apparent even later, within postnatal proximal tubules. To determine if a developmental pattern might be similar in other species, the expression of the human urate transporter/channel was compared subsequent to microinjection of Xenopus oocytes and transfection of differentiated frog renal cells (A6) with the cRNA and cDNA of the channel, respectively.
As in the human kidney, plasma membrane localization of the human urate/transporter channel is only evident in differentiated A6 cells derived from the amphibian kidney.
MATERIALS AND METHODS:

Tissue Samples:

Normal human fetal kidneys of 13 to 36 weeks gestational age were obtained from the Anatomic Gift Foundation (Woodbine, GA). Normal human kidneys from neonates, children and adults, flushed with Eurocollins or University of Wisconsin salt solution at 4°C, were obtained from the National Disease Research Interchange (Philadelphia, PA).

Western Blot Analysis of Homogenates of Renal Cortex:

Homogenates of kidneys of human fetuses (13 - 36 weeks gestational age), neonates and children (newborn - 16 yrs) and adults (> 18 yrs) were obtained by finely mincing slices of renal cortex with scissors in ice cold lysis buffer containing protease inhibitors (20 mM imidazole, pH 7.2, 250 mM sucrose, 1 mM EDTA, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 1 µg/ml aprotinin, 1 µg/ml chymostatin, and 1 mM benzamidine) (Sigma-Aldrich, St. Louis, MO). The minced tissue (1 g/10 ml in the same ice cold lysis buffer) was then homogenized with a Braun homogenizer. Protein concentrations of the homogenates were determined using a BCA protein assay kit (Pierce, Rockford, IL). Aliquots of homogenates were dissolved in Laemmli buffer containing 30 mg/ml DTT, heated to 100°C for 3 min, and then 15 or 30 µg of protein from each sample was resolved on 10% SDS-PAGE gels. Electrophoresed protein was transferred to Hybond membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Membranes were then blocked in PBS with 5% non-fat milk for 1 h, washed 6 times in PBS-Tween-20 (twice for 10 sec, twice for 15 min and twice for 5 min) and then incubated for 1 h at room temperature with our rabbit polyclonal antibody to affinity purified pig liver uricase (anti-uricase) (25) diluted 1/2500 in PBS with 2% bovine serum albumin (BSA). After washing six times in PBS-Tween-20, as above, the membranes were incubated for 45 min with horseradish peroxidase-coupled goat-anti-rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1/10,000 in PBS with 5% non-fat milk. Thereafter the membranes were again washed 6 times with PBS-Tween-20,
as above, and visualized by enhanced chemiluminescence after exposure to Hyperfilm following the manufacturer's protocol (ECL, Amersham). Densitometry was performed using the program UN-SCAN-IT gel Version 4.3 (Silk Scientific, Orem, UT) after scanning the films into Adobe Photoshop 4.0 (Adobe Systems, Inc., San Jose, CA). Multiple exposure times were assessed to assure that the images were not saturated. In each assay a duplicate electrophoresis was simultaneously performed on a gel loaded with an aliquot of homogenate that was identical in volume to that used for immunolabeling. These gels were stained with Coomassie blue to assess the relative amounts of protein that were loaded per lane.

**Immunohistochemistry of Human Kidneys:**

Blocks of human kidneys were fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Fort Washington, PA) in phosphate buffered saline (PBS), pH 7.4 at 4°C for 4 hours. Sections were then washed three times over 24 hours in PBS at 4°C, dehydrated in a graded series of ethanols, cleared with xylene and then embedded in paraffin. Paraffin embedded tissue was sectioned (5 µm) with a Leica RM-2135 microtome (Leica, Nussloch, Germany), mounted on glass slides, dewaxed and then rehydrated through a graded series of ethanols. Tissue sections were initially incubated in 0.3% H2O2 in methanol for 30 min to block endogenous peroxidase activity and then washed twice in PBS, 10 min/wash. This and all subsequent incubations were carried out in a humidified chamber at room temperature. Non-specific antibody binding was then blocked by incubation with 10% normal goat serum in PBS for 20 min in a humidified chamber. Sections were next incubated for 45 min with anti-uricase that was diluted 1/250 in PBS with 2% BSA. Thereafter sections were washed twice in PBS-Tween 20 (0.02%), 5 min/wash. After one additional 5 min wash in PBS the sections were incubated with goat-anti-rabbit biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 45 min. Sections were again washed twice in PBS-Tween 20 and once in PBS, 5 min/wash, and then incubated with avidin-biotin peroxidase (Vectastain Elite, Vector Laboratories) for
45 min. Following one wash in PBS and two washes in Tris buffered saline, pH 7.5 (TBS), 5 min/wash, color development was carried out for 20 min using aminoethylcarbazole (Vector Laboratories) as chromogen. Sections were mounted with Aquapolymount (Polysciences, Warrington, PA), viewed under a Nikon Microphot-FXA microscope equipped with Nomarski optics (Nikon, Tokyo, Japan) and photographed with a Nikon DBX-DB2 camera.

Some sections were also stained with Periodic Acid Schiff (PAS), a stain that detects glycoproteins. These sections were initially processed for anti-uricase immunolabeling as described above, except that horseradish peroxidase conjugated goat-anti rabbit IgG (Kirkegaard and Perry Laboratories), diluted 1/1000 in PBS with 2% BSA, was utilized as the secondary antibody and diaminobenzidine/metal concentrate (Pierce) containing 1% H₂O₂ was the color development substrate. Following color development these sections were washed three times in distilled water, 5 min/wash, and then stained with PAS according to the supplier’s instructions (Chromaview PAS kit, Richard-Allan Scientific, Kalamazoo, MI). The sections were then dehydrated in a graded series of ethanols, cleared with xylene and then mounted in Permount (Fisher Scientific, Morris Plains, NJ). Sections were viewed under a Nikon Microphot microscope equipped with Nomarski optics and photographed. Pseudocolors were assigned using Adobe Photoshop 4.0.

Preparation of cDNA Constructs:

Restriction sites were incorporated at the 5’ and 3’ ends of the full length coding sequence of the human homologue of the human urate transporter/channel, hUAT, by PCR. The PCR product BamHI-hUAT-HindIII was produced with a sense primer that contains the BamHI restriction site sequence at the 5’ end of nucleotides 1-21 of hUAT and an antisense primer containing the HindIII sequence at the 3’ end of nucleotides 950-972 of hUAT (Table I). A second PCR product, Xhol-hUAT-KpnI, was produced using a sense primer that contains the Xhol restriction site sequence at the 5’ end of nucleotides 1-
21 of hUAT in conjunction with an antisense primer that includes the KpnI sequence at the 3’ end of nucleotides 947-969 of hUAT (Table I). This antisense primer omits the hUAT stop codon (Table I). All primers were purchased from Genosys Biotechnologies, Inc. (The Woodlands, Texas). In each case PCR was begun by an initial denaturation at 95°C for 1 min, followed by 35 cycles each containing denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. PCR was performed with AmpliTaq DNA polymerase (PE Biosystems, Foster City, CA). Following a final cycle that prolonged the extension at 72°C for 7 min samples were maintained at 4°C. PCR products were analyzed by electrophoresis on 1% agarose gels, purified using a gel extraction kit (Qiagen Inc., Valencia, CA) and subcloned into pBluescript SK(-) (Stratagene, La Jolla, CA). Minipreps of DNA were obtained with a plasmid miniprep kit (Qiagen Inc.) and evaluated by restriction digestion. Restriction enzymes were all obtained from New England Biolabs (Beverly, MA). The sequence of each hUAT PCR product was verified by automated sequence analysis with an Applied Biosystem Sequencer (ABI 373A) using dye terminator chemistry. Following sequencing, the BamHI-hUAT-HindIII PCR product was subcloned into pcDNA 3.1(-) (Invitrogen, Carlsbad, CA). Maxipreps of both the BamHI-hUAT-HindIII construct in pcDNA and the XhoI-UAT-KpnI construct in pBluescript were prepared using a plasmid maxiprep kit (Qiagen Inc.).

To produce chimeric constructs in which EGFP was appended to the amino or carboxy terminus of hUAT, the coding sequence of EGFP was removed from pEGFP-N1 (Clontech Laboratories, Inc., Palo Alto, CA) by restriction digestion with ApaI and NotI and then subcloned into pcDNA 3.1(-). PCR was utilized to incorporate restriction sites at the amino and carboxy termini of EGFP to permit ligation to hUAT. One PCR product, designated Xmal-EGFP-BamHI, was produced with a sense primer that contains the sequence for the Xmal restriction site at the 5’ end of nucleotides 1-22 of EGFP and an antisense primer that includes the BamHI sequence at the 3’ end of nucleotides 697-717 of EGFP (Table I). A second PCR product, XhoI-EGFP-BamHI, was produced using a
sense primer that contains the sequence for the XhoI restriction site at the 5' end of nucleotides 1-22 of EGFP and the same antisense primer that includes the BamHI sequence at the 3' end of EGFP (Table I). The stop codon for EGFP is omitted in both of these EGFP antisense primers (Table I). A third PCR product, KpnI-EGFP-HindIII, was amplified using a sense primer that contains the KpnI sequence at the 5' end of nucleotides 4-23 of EGFP and an antisense primer that incorporates the HindIII sequence at the 3' end of nucleotides 697-720 of EGFP (Table 1). The start codon of EGFP is omitted in this sense primer. The conditions used to perform these PCRs were identical to those described above for the hUAT constructs. As detailed above, PCR products were electrophoresed, gel purified, and then subcloned into pBluescript SK(-). Minipreps were produced, evaluated by restriction digestion, and sequenced by automated sequence analysis. Following sequence verification, hUAT/EGFP chimeric constructs were prepared. EGFP was added to the amino terminus of hUAT via a triple ligation between the restriction enzyme digested XmaI-EGFP-BamHI and BamHI-hUAT-HindIII inserts and XmaI and HindIII digested pGEMHE vector (a generous gift of Dr. Diomedes Logothetis), or by triple ligation between the restriction enzyme digested XhoI-EGFP-BamHI and BamHI-hUAT-HindIII inserts and XhoI and HindIII digested pcDNA 3.1 (-) vector. (Digestions of hUAT inserts with BamHI were limited to obviate digestion of an internal BamHI restriction site.) EGFP was appended to the carboxy terminus of hUAT via triple ligation between the restriction enzyme digested XhoI-hUAT-KpnI and KpnI-EGFP-HindIII inserts and XhoI and HindIII digested pcDNA 3.1(-) vector. The construct in pGEMHE was used in oocyte microinjections. This vector contains 3' and 5' untranslated regions of a Xenopus β globin gene which has been shown to permit very high expression of a number of exogenous proteins in Xenopus oocytes (30). The constructs in pcDNA were utilized for transfection of A6 cells.
**Cell Culture and Transfection:**

A6 adult *Xenopus laevis* renal tubular epithelial cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cultures were grown in Falcon tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ). A6 cells were cultured in media NCTC-109 prepared with 15% sterile water and supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Life Technologies, Inc., Rockville, MD). Culture medium was changed three times per week. A6 cells were passaged as necessary following treatment with Cell Dissociation Solution (Sigma-Aldrich). Cultures were maintained at 27°C in a humidified incubator with 5% CO₂ and 95% air. To evaluate the cellular expression of hUAT by confocal microscopy, cells were plated on #1-1/2, 22 mm square, acid washed coverslips (Corning Laboratory and Equipment, Corning, NY) in Falcon 6-well culture plates (Becton Dickinson) prior to transfection. At least 24 hours prior to cell plating, coverslips were coated with 0.02% rat tail collagen type I (Becton Dickinson). Transient transfections were performed 24 hours after 5 x 10⁵ cells were plated on coverslips in 6-well culture plates using 0.6 µg DNA per well in the presence of Effectene (Qiagen Inc.), according to the supplier’s protocol. At six hours post transfection the transfection mixture was removed and the cells were rinsed 4 times with complete NCTC-109 medium.

**Microscopy of A6 Cells:**

Transiently transfected cells were fixed for 5 minutes in 4% PFA (Electron Microscopy Science) in PBS, 24-72 hours post-transfection. Following fixation, coverslips were mounted onto slides with Vectashield Mounting Medium (Vector Laboratories) and sealed with clear nail polish. Confocal microscopy was performed with a LEICA TCS-SP confocal laser scanning microscope (Leica Microsystems, Heidelberg, GmbH) equipped with Argon, Krypton, and HeNe lasers. Green images (EGFP) were obtained after excitation at 492 nm. Confocal images were captured in the GlowOvUn
look-up table and were pseudo-colored green prior to being saved as TCS Export TIFF image files. Images were then imported into Photoshop for analysis and processing.

**Xenopus Oocyte Studies:**

The cDNA construct in pGEMHE containing the coding sequence of EGFP appended to the 5' end of the coding sequence of hUAT was linearized by restriction digestion with HindIII. The linearized construct was purified using a gel extraction kit (Qiagen Inc.), and then used as a template for *in vitro* transcription utilizing the T7 mMessage mMachine kit (Ambion, Austin, TX). Transcript size was verified by gel electrophoreses on RNA gels (6.7% formaldehyde, 1x MOPS, 1% agarose in PBS). cRNA concentration was determined by spectrophotometric analysis at 260 and 280 nm. Aliquots of cRNA stocks were maintained at -80° C until use.

Stage 5-6 *Xenopus* oocytes were harvested, processed, maintained and microinjected as described previously (7). Oocytes were microinjected with 50 nl containing up to 50 ng of EGFP-hUAT cRNA, 2 ng of cRNA encoding the G protein-gated inwardly rectifying K⁺ (GIRK) channel, subunit 4, linked at the carboxy terminus to EGFP (GIRK4-EGFP) (a generous gift of Drs. Tooraj Mirshahi and Diomedes Logothetis) or water. Oocytes were maintained up to 7 days post-injection at 18° C. At days 2, 4, or 7 oocytes were rinsed in PBS and then fixed overnight at 4° C in 4% PFA in PBS. Following fixation, oocytes were rinsed in PBS and then imbedded in 2.5% agarose in PBS in 7 x 7 x 5 mm base molds (Fisher Scientific). 50 µm oocyte sections were cut with a Vibratome 1000 (Technical Products International/The Vibratome Company, St. Louis, MO) and mounted onto slides into DTG mounting medium (2.5% DABCO (Sigma-Aldrich) and 50 mM Tris pH 8.6 in 90% glycerol). Slides were sealed with clear nail polish and evaluated, as detailed above, by confocal microscopy.
RESULTS:
Western Blot Analysis of hUAT in Homogenates of Human Kidney Cortex:

As depicted in Figure 1 anti-uricase recognizes a 36 kDa protein in homogenates of human kidneys. The electrophoretic mobility of this immunoreactive protein is comparable to that of a protein that is immunoreactive to this antibody in brush-border membranes isolated from rat renal cortex (Fig. 1A) (25). Since this antibody reacts with a 36 kDa recombinant protein prepared from a cDNA cloned from a rat renal library that functions as a urate channel in lipid bilayers (29) and this antibody blocks both the activity of the recombinant channel (27, 29) and electrogenic urate transport in rat renal cortical membrane vesicles (25), it has been presumed that the immunoreactive rat brush-border membrane protein represents the rat urate transporter/channel, UAT (25, 27, 29). Accordingly, the immunoreactive 36 kDa protein in human kidney (Fig. 1) is presumed to represent the human homologue of the urate transporter/channel and, therefore, protein immunoreactive to anti-uricase in the human kidney is hereafter referred to as hUAT. It is important to note that a 36 kDa recombinant protein that has been prepared from the cDNA of the human homologue of the urate transporter/channel is also immunoreactive to anti-uricase and that this protein, like the rat recombinant protein, functions as a selective urate channel in lipid bilayers (31).

To assess the possibility that hUAT is developmentally regulated, the densities of the 36 kDa immunoreactive band to anti-uricase were qualitatively compared in cortical homogenates prepared from kidneys of human fetuses, neonates, children and adults (Fig. 1B and 1D). As demonstrated in Figure 1B, when approximately 30 µg of protein was loaded per lane, a progressive age-dependent increase in the density of the 36 kDa immunoreactive band was evident in homogenates prepared from kidneys of fetuses of increasing gestational age until birth (Fig. 1B, lanes 1 to 6). Based on the density of the protein bands stained with Coomassie blue (Fig. 1C) in the simultaneously electrophoresed gel that was loaded with identical volumes of homogenate, the age
associated increase in immunoreactive protein cannot be ascribed to differences in protein loading. Indeed, the Coomassie blue stained gel reveals that the 13 week fetal kidney homogenate, with a barely perceptible immunoreactive band (Fig. 1B, lane 1), was overloaded with protein with respect to the other homogenates (Fig. 1C). Because the density of immunoreactive staining of homogenates obtained beyond birth were not distinguishable, precluding the possibility of determining if expression of hUAT increases further postnataally, the relative immunoreactivity to anti-uricase was also determined after 15 µg of protein was loaded per lane (Fig. 1D). As depicted, the density of the 36 kDa immunoreactive band was significantly less in the newborn than at 15 months of age (Fig. 1D, lanes 1 and 2); however, beyond this age the magnitude of expression of hUAT appears to be relatively constant (Fig. 1D, lanes 3 to 7). As above, this qualitative assessment of the expression of hUAT in homogenates at various ages (Fig. 1D) is based on the assessment of the relative amounts of protein loaded per lane in a simultaneously electrophoresed gel stained with Coomassie blue (Fig. 1E). As further depicted, Figure 1F shows that when 10 µg of protein were loaded per lane an almost linear increase in hUAT protein was seen from the newborn through 15 months of age, after which there was no additional change. Of note, the identity of the additional immunoreactive band in human kidney homogenates at approximately 23 kDa (Figs. 1A, B and D) is unknown, but is believed to represent a fragment of hUAT that was proteolytically degraded at some time prior to storing the harvested kidneys at -80°C.

**Immunohistochemical Localization of hUAT:**

The nephron sites of localization of hUAT in fixed sections of human kidneys from 13 weeks gestational age through 59 years of age has been evaluated using anti-uricase. The following stages of nephrogenesis, that have been classified by morphological criteria (4), have been utilized to describe the localization of hUAT in fetal kidneys. Stage I (renal vesicle) nephrons represent the earliest recognizable epithelia that are attached laterally by tight junctions, but glomerular and proximal tubular epithelia can not be distinguished.
Stage II nephrons include comma and S-shaped bodies: at this stage distinct glomerular and proximal tubular epithelia are evident. Stage III nephrons encompass glomeruli that have capillary-loops at their vascular pole and proximal tubules with early microvilli detectable at their apical (luminal) surfaces. Stage IV nephrons are nephrons with fully differentiated glomerular and proximal tubule epithelia with the latter having fully developed brush-borders. In fetal kidneys examined at 13 through 32 weeks of gestational age (Fig. 2A - E) immunolabeling was not detected in stage I or II nephrons: labeling was absent in comma and S-shaped bodies within the nephrogenic zone (Fig. 2A, D and E). In contrast, weak expression of hUAT was evident in the cytoplasm of proximal tubules of stage III/IV nephrons located deeper in the cortex as early as 13 weeks of gestational age (Fig. 2A). With increasing gestational age the intensity of immunoreactivity increased within the cytoplasm of proximal tubules (Fig. 2B - E). Of note, this age associated increase in immunoreactive protein within proximal tubules of fetal kidneys parallels the age associated increase in immunoreactive protein in homogenates of renal cortex prepared from fetal kidneys at similar gestational ages (Fig. 1B and D). In addition to the increased expression of hUAT during gestation, a transition from diffuse cytoplasmic immunolabeling to labeling that is predominantly apical and within the brush-border was evident in renal cortices of children at 1 and 4 years of age and in the adult (Fig. 2F - H). It is of note that hUAT immunolabeling is confined to proximal tubules at all ages examined: immunoreactive protein was absent in glomeruli and other tubular segments (Fig. 2A - H). Indeed, at the site of transition of the S3 segment of the proximal tubule into the descending limb of the loop of Henle (Fig. 2C) a rather abrupt loss of expression of hUAT is apparent.

To verify the cellular localization of hUAT some tubules were examined at higher magnification under oil immersion (Fig. 3). In stage III/IV proximal tubules located deep in the cortex of kidneys of 16 and 36 weeks gestational age fetuses hUAT is clearly diffusely present within the cytoplasm (Fig. 3A and B). Immunolabeling in the kidney of
the newborn was similarly located diffusely within the cytoplasm of proximal tubules (not depicted). In striking contrast, hUAT is strongly expressed at the apical membrane and in the brush-border of proximal tubule cells in postnatal kidneys (Fig. 3C and D). Of interest, in portions of mature proximal tubules (Fig. 3D) hUAT appears to reside within the brush-border while in other portions of these same tubules the brush-border appears to be devoid of hUAT and expression seems to be primarily localized in a compartment that is apical, but just beneath the brush-border. A very similar pattern of distribution of UAT was previously reported in rat kidneys, where UAT was clearly evident within brush-border membranes of S1 and S2 segments of proximal tubules, but was predominantly located apically, beneath the brush-border membrane of S3 segments (25). In this context, the dual location of hUAT in some tubules (Fig. 3D) may reflect the transition between S2 and S3 segments in these human proximal tubules.

To determine whether proximal tubules that are either devoid of immunolabeling or label diffusely for hUAT have a brush-border, hUAT was identified by immunolabeling with anti-uricase while glycoproteins on brush borders (as well as extracellular matrix components in basement membranes) were recognized by staining with Periodic Acid Schiff (PAS). As depicted, a stage III nephron in a kidney at 20 weeks gestational age which lacks a PAS-stained brush-border also failed to label for hUAT (Fig. 4A and C). A more mature stage IV nephron, deeper in the cortex of the same fetal kidney shows distinct proximal tubule PAS stained brush-borders with diffuse cytoplasmic labeling for hUAT, but absence of hUAT within the brush-border membrane (Fig. 4B and D). This observation indicates that the transition from cytoplasmic to apical/brush-border membrane localization of hUAT (Figs. 2 and 3) is not dependent on the simple presence of a brush-border membrane, but rather is likely to depend on the presence of an alternate developmentally regulated, albeit currently unknown process.
Localization of hUAT in Xenopus Oocytes and A6 Cells:

To examine the possibility that the developmental changes in hUAT expression might be similar in undifferentiated and differentiated cells *in vitro*, the expression and targeting of hUAT/EGFP chimeric proteins was evaluated in the undifferentiated *Xenopus laevis* cell, the oocyte, and in the differentiated adult *Xenopus laevis* A6 cell line. These cells were selected since both the *Xenopus* oocyte and A6 cell are commonly used to evaluate the function of transporters and ion channel proteins. As demonstrated in Figure 5, expression of chimeric protein was not detectable in stage 5-6 *Xenopus* oocytes that were microinjected with cRNA encoding a chimeric protein linking EGFP to the amino terminus of hUAT: oocytes microinjected with cRNA encoding EGFP-hUAT (Fig. 5B) were not distinguishable from oocytes microinjected with water (Fig. 5C). No fluorescence was detected above background levels regardless of whether oocytes were fixed and sectioned 2, 4, or 7 days following microinjection. Additionally, gel electrophoresis repeatedly confirmed that intact full-length cRNA EGFP-hUAT transcripts were utilized for microinjection (data not shown). In distinct contrast, significant fluorescence was readily observed at the oocyte periphery (Fig. 5A) when oocytes were microinjected with an EGFP tagged ion channel (GIRK4-EGFP) that has previously been shown to be efficiently expressed and targeted to oocyte plasma membranes (7). These results suggest that *Xenopus* oocytes may be incapable of expressing the hUAT/EGFP chimeric protein, that protein may be expressed but so rapidly degraded that it cannot be targeted to the plasma membrane or the level of expression is so low that it is insufficient to permit detection of EGFP fluorescence above background. Despite the apparent lack of expression of hUAT in oocytes, hUAT/EGFP chimeric proteins were abundantly expressed in mature A6 cells following transfection of cDNA constructs containing EGFP linked at either the amino (Fig. 6A) or carboxy terminus of hUAT (Fig. 6B). Moreover, fluorescence was clearly detected at the periphery of the cell, implying a plasma membrane localization of the chimeric protein. The lack of expression of hUAT in the
undifferentiated oocyte with extensive expression in differentiated A6 cells is reminiscent of the absence of hUAT expression in stage I/II nephrons with abundant expression in stage III/IV proximal tubules of the human kidney (Figs. 2 - 4). These observations in both an in vitro system and the human kidney suggest the possibility that both the regulation of expression and targeting of hUAT to plasma membranes require the presence of some additional factor(s) that is itself developmentally regulated.
DISCUSSION:

We have previously demonstrated that our polyclonal antibody raised against affinity purified pig liver uricase interacts with and specifically identifies the rat urate transporter/channel (25, 27, 29). It is now shown that this same antibody recognizes a protein of identical molecular weight (36-37 kDa) in human kidney tissue (Fig. 1). It is important to note that although uricase is not expressed in humans (48, 49), a cDNA that encodes a protein with 73% amino acid identity to the rat urate transporter/channel (Fig. 7) has been identified in humans which, like the rat protein (27), contains a local block of amino acids with homology to the substrate binding domain of uricase (31). Additionally, recombinant protein prepared from the human cDNA, like that prepared from the rat (27), functions as a selective urate channel in lipid bilayers that is blocked by oxonate, a competitive inhibitor of uricase (14, 28). These observations, in conjunction with the finding that the mRNA for this protein is expressed in human kidney (31), have led to the presumption that the protein that is immunoreactive to anti-uricase in human kidney is the human homologue of the urate transporter/channel, hUAT.

Of particular interest, immunoreactivity for this presumed human urate transporter, as identified by Western immunoblot analysis, was barely detectable relatively late in gestation in 13 week fetal kidneys after which time it increased quantitatively throughout gestation (Fig. 1). Moreover, the amount of immunoreactive protein continued to increase after birth until maximal levels were reached at approximately 15 months of age (Fig. 1). Subsequently during childhood and adult life the amount of immunoreactive protein remained essentially constant at the level seen in the kidney of the 15 month old (Fig. 1). This developmental pattern of expression is reminiscent of that of aquaporin-1 (13), but differs somewhat from that of both the full-length and truncated versions of the cystic fibrosis transmembrane conductance regulator (CFTR) (12), transport proteins that ultimately reside within brush-border membranes of proximal tubules of the human kidney (8, 11, 34, 39).
In addition to documenting the overall increase in expression of hUAT during renal development, the present study reveals that hUAT expression is confined to stage III/IV proximal tubules (Figs. 2 - 4) and that the magnitude of expression within these tubules increases during gestation (Fig. 2). Insofar as hUAT is only expressed in proximal tubules this finding implies that the level of protein synthesized per cell in maturing proximal tubules must be developmentally regulated. Furthermore, there is a striking relocation of hUAT within proximal tubule cells after birth. During all fetal gestational stages in which the urate transporter was detected (13 - 36 weeks), immunoreactive reaction product was seen diffusely throughout the cytoplasm but not at the cell membranes (Figs. 2 - 4). After birth, the pattern of localization of the transporter was clearly different: by 12 months of age reaction product was predominantly seen associated with the apical plasma membrane and within brush-borders of proximal tubule epithelia (Figs. 2 and 3). This distinct change in the distribution of hUAT strongly suggests that there is an additional level of developmental regulation of hUAT that impacts on the site of location of the transporter within proximal tubule cells.

Of interest, the inability to detect hUAT expression in *Xenopus* oocytes (Fig. 5) is similar to the apparent absence of expression of hUAT in the most immature proximal tubules (stage I and II) of the human kidney (Fig. 2). Additionally, subsequent expression of this same protein within the cytoplasm and at the plasma membrane of differentiated renal *Xenopus* A6 cells (Fig. 6) is reminiscent of the expression of hUAT during the latter stages of nephrogenesis in the more mature proximal tubules of kidneys of fetuses and young children (Figs. 2 and 3). It is of note that aquaporin-1 (13) and CFTR (12), like hUAT, are also not expressed in the most immature proximal tubules within the nephrogenic zone of the human fetal kidney. However, unlike hUAT (Fig. 5), microinjection of the cRNAs of both of these proteins results in expression of functional transporters in oocyte plasma membranes (9, 37). While the basis for the difference in the ability of *Xenopus* oocytes to express hUAT, aquaporin-1 and CFTR is unknown, we
speculate that a developmentally regulated accessory factor(s) for hUAT is expressed relatively late in *Xenopus* and developing human proximal tubules which then allows expression of hUAT in both species. In contrast, if accessory elements are required for expression and membrane insertion of aquaporin-1 and CFTR then it is evident that these must be already present in oocytes, but only appear later in the course of development in much more mature cells of human kidney.

The molecular mechanisms regulating the intracellular trafficking of renal transporters and channels to the cell membrane are poorly understood. The human urate transporter is not the only membrane transporter to be expressed intracellularly in the cytoplasm for a period of time prior to attaining its final restricted membrane localization. The aquaporin-1 water channel (13), cystic fibrosis transmembrane conductance (CFTR) chloride channel (12), the band 3 chloride/bicarbonate exchanger and the sodium/proton antiporter, NHE3, (P. Wilson personal communication) all show similar patterns of developmental regulation in proximal tubules. In all of these cases, protein expression was first seen at 12-13 weeks of gestation in the proximal tubule cytoplasm of human metanephric kidneys. Membrane localization of aquaporin-1 was not seen until later in gestation (13) whereas CFTR, like hUAT, only localized to the plasma membrane after birth (8, 12). It should be noted, however, that this is not the only pattern of developmental regulation of polarized membrane protein localization seen in human renal proximal tubules. For instance, alkaline phosphatase, Na\(^+\)-K\(^+\)-ATPase α1 and β2 subunits, aminopeptidase and H\(^+\)-ATPase are all seen at the apical plasma membrane as soon as they can be detected by immunohistochemistry whether in early, mid or late gestation (6) (P. Wilson unpublished observations). It is interesting to note that cytoplasmic expression of membrane transporters and channels has not been observed during the development of human metanephric collecting tubules. The aquaporin-2 water channel (13) as well as collecting tubule CFTR (12), band-3 and NHE3 (P. Wilson, unpublished observations) are all seen only in plasma membranes of these epithelia.
Taken together, these studies suggest that a specific property of developing proximal tubules leads to the initial cytoplasmic localization of specific membrane proteins, including the human urate transporter. It is clear, however, that the lack of apical membrane distribution is not due to an absence of brush-border microvilli (Fig. 4) and therefore not precisely temporally related to the morphological differentiation of the proximal tubule epithelium. Proteins ultimately destined for the plasma membrane of polarized epithelia are synthesized in the endoplasmic reticulum, translocated to the cis-Golgi and through the medial stacks of the Golgi where they undergo post-translational modification, for instance by glycosylation (33). Once they reach the trans-Golgi, proteins are specifically sorted into vesicles and are delivered to the appropriate polarized membrane (33). It may be hypothesized that during early development, human proximal tubule epithelia may lack the full set of vesicle transport proteins and co-factors, necessary for membrane delivery of the urate transporter. Indeed, it has been suggested that cytoplasmic CFTR is associated with the small GTPase rab-5-negative vesicular fractions while membrane CFTR is associated with rab-5-positive fractions (47). Further studies will be required to determine whether the lack of apparent delivery or retention of the urate transporter to the apical plasma membrane of fetal renal epithelia cells is due to absence or incomplete maturation of specific membrane processing, sorting signals, vesicular factors, or structural anchoring proteins.

The restricted expression of hUAT to proximal tubules at all stages of development of the human renal cortex (Figs. 2 - 4) is identical to the limited pattern of expression of the urate transporter/channel (UAT) that is evident in the adult rat renal cortex (25). The concordance of findings in immunolocalization studies of hUAT and UAT is entirely consistent with functional studies in the two species: the proximal tubule has repeatedly been shown to be responsible for virtually all urate transport in both human and rat kidney (3, 18, 45, 46). The relocalization of the presumed human urate transporter protein in the proximal tubule brush-border membrane after birth would thus be expected
to have important functional consequences since this protein functions as a transporter/channel (28, 31). The excretion of urate by the human kidney undergoes well characterized developmental changes (35, 42-44). Although attention has been focused on the fall in fractional urate excretion which occurs during the first year of life (35), the absolute amount of urate excreted daily increases progressively from infancy to late in childhood (44). Based on the assumption that excreted urate reflects urate that has been secreted in the proximal tubule (18, 45), it seems likely that the rate of tubular secretion is developmentally regulated to increase during early childhood. Importantly, electrogenic urate transport has been detected in renal cortical membrane vesicles derived from adult human kidneys (38) and urate flux on the human electrogenic transporter (38), like that in rat (1, 2) and rabbit (24), carries a negative charge. Since the cell interior is electronegative with respect to the tubular lumen, particularly in the more distal portion of the proximal tubule (20), an apical location of hUAT would favor secretion of urate into the tubular lumen. Insofar as the immunoreactive protein that has been detected with anti-uricase in human kidneys is hUAT (Figs. 1 - 4), and hUAT is the molecular representation of the electrogenic urate transporter, then it seems reasonable to hypothesize that the increase in urate secretory capacity in the postnatal human kidney is mechanistically related, at least in part, to the developmentally regulated insertion of hUAT in proximal tubule brush-border membranes (Figs. 2 and 3) that only occurs in the post-natal period.
ACKNOWLEDGMENTS:

This work was supported in part by National Institutes of Diabetes and Digestive and Kidney Diseases (NIDDK) grants DK44833 (P.D. Wilson) and DK52785 (R.G. Abramson) and NIH NRSA F32 DK09615 (D.P. Hyink).

Confocal laser scanning microscopy was performed at the MSSM-CLSM core facility, supported with funding from NIH shared instrumentation grant (1 S10 RR0 9145) and NSF Major Research Instrumentation grant (DBI-9724504).

The authors express their appreciation to Rebecca Zausmer and Barbara Bloswick for technical assistance.
REFERENCES:


22. Kahn, A. M., S. Branham, and E. J. Weinman. Mechanism of urate and p-


FIGURE LEGENDS:

Figure 1. Expression of UAT in brush-border membranes of rat renal cortex and hUAT in homogenates of normal human kidneys at various gestational and postnatal ages. Immunoblots were probed with rabbit anti-pig liver uricase. A: representative immunoblot of UAT expression in 1 µg of purified brush-border membranes of adult rat renal cortex (lane 1) and hUAT expression in 30 µg of renal cortical homogenate of a 40 yr old human (lane 3). A buffer control is shown in lane 2. B: representative immunoblot of hUAT expression in 30 µg of renal homogenates prepared from normal kidneys of fetuses at 13 weeks (lane 1), 18 weeks (lane 2), 24 weeks (lane 3), 32 weeks (lane 4), and 36 weeks gestational age (lane 5), a newborn (lane 6), and children at 4 months (lane 7), 15 months (lane 8) and 3 yrs of age (lane 9). C: Coomassie stained gel loaded identically to the immunoblot shown in panel B. D: representative immunoblot of hUAT expression in 15 µg of renal homogenates prepared from normal kidneys of a newborn (lane 1), children at 15 months (lane 2), 3 yrs (lane 3), 7 yrs (lane 4), and 16 yrs of age (lane 5) and a 40 yr old adult (lane 6). E: Coomassie stained gel loaded identically to the immunoblot shown in panel D. F. representative immunoblot and accompanying densitometry of hUAT expression in 10 µg of renal homogenates prepared from normal kidneys of a newborn (lane 1) and children at 4 months (lane 2), 15 months (lane 3) and 3 yrs of age (lane 4). G. Coomassie stained gel loaded identically to the immunoblot shown in panel F.

Figure 2. Immunolocalization of hUAT in paraffin embedded sections obtained from normal human kidneys at various gestational and postnatal ages. In each section the outer cortex is located toward the top of the figure. At 13 weeks of gestation (A) weak immunoreactivity for hUAT is seen diffusely in the cytoplasm of proximal tubules (pt) of Stage III/IV nephrons. Immunolabeling is not detected within the nephrogenic zone (nz) or in a glomerulus (g). At 16 weeks of gestation (B) hUAT is detected in the cytoplasm of proximal tubules (pt). No immunolabeling is detected in a glomerulus (g) or other tubule segments. At 19 weeks of gestation (C) somewhat more intense labeling for hUAT is
seen in the cytoplasm of proximal tubules (pt). Glomeruli (g) and other tubule segments remain unlabeled. In this section an abrupt loss of immunolabeling for hUAT is evident at the site of transition (indicated by arrows) between the S3 segment of the proximal tubule and the thin descending limb of the loop of Henle. At 24 weeks of gestation (D) immunolabeling for hUAT is seen in the cytoplasm of proximal tubules (pt). No immunoreactivity is detected in structures within the nephrogenic zone (nz) including an S-shaped nephron (S) or deeper in the cortex in a glomerulus (g). At 32 weeks of gestation (E) more intense immunolabeling for hUAT is detected diffusely within the cytoplasm of proximal tubules (pt). Structures in the nephrogenic zone (nz) remain unlabelled. At 1 yr of age (F) immunolabeling for hUAT is primarily localized to the apical aspects of proximal tubules (pt). Glomeruli (g) and other tubular structures are not immunolabeled. At 4 yrs of age (G) immunolabeling for hUAT is primarily localized to the apical aspects of proximal tubules (pt). Glomeruli (g) and other tubular structures are not immunolabeled. At 59 yrs of age (H) immunolabeling for hUAT is primarily localized to the apical aspects of proximal tubules (pt). Other tubular structures are not immunolabeled. Scale bars equal 50 µm.

**Figure 3.** Higher power images of immunolocalization of hUAT in paraffin embedded sections of normal human kidneys of fetuses and children. At 16 weeks gestational age (A) immunolabeling for hUAT is detected diffusely within the cytoplasm of the proximal tubule. At 36 weeks gestational age (B) more intense immunolabeling for hUAT is detected diffusely within the cytoplasm of the proximal tubule. At 1 yr of age (C) immunolabeling for hUAT is seen primarily in the apical region of proximal tubules. A glomerulus (g) and other tubular structures are not immunolabeled. At 4 yrs of age (D) immunolabeling for hUAT is detected in proximal tubules in both apical brush-border membranes (arrow) and in an apical compartment at the base of the brush-border microvilli (arrowhead). Scale bars equal 10 µm.

**Figure 4.** Immunolocalization of hUAT and histochemical staining with PAS in proximal tubules of stage III and stage IV nephrons within the same paraffin embedded section of a
human fetal kidney at 20 weeks of gestation. The proximal tubule (pt) of a stage III nephron (A) is not immunoreactive to hUAT (absence of brown stain) and does not stain with PAS (absence of bright pink stain). The cytoplasm of the proximal tubule of a stage IV nephron (B) is immunoreactive to hUAT (brown) and the apical brush-border membrane (arrow) stains with PAS (bright pink). To enhance the distinction between hUAT (brown) and PAS (pink), the brown hUAT immunoreaction product was pseudocolored blue to an equal degree in panels C and D. The proximal tubule (pt) of the stage III nephron (C) is not immunoreactive to hUAT (absence of blue stain) and does not stain with PAS (absence of bright pink stain). The cytoplasm of the proximal tubule of the stage IV nephron (D) is immunoreactive to hUAT (blue) whereas the apical brush-border membrane (arrow) stains with PAS (bright pink stain). Neither a brown immunoreaction product to hUAT (A and B) nor a blue pseudocolored reaction product to hUAT (C and D) is detected within the glomerulus (g). Scale bars equal 20 µm.

**Figure 5.** Confocal microscopy of *Xenopus laevis* oocytes that were sectioned 4 days after cRNA micro-injection. A: oocyte injected with 2 ng of cRNA encoding EGFP on the carboxy terminus of GIRK4. B: oocyte injected with 50 ng of cRNA encoding EGFP on the amino terminus of hUAT. C: oocyte injected with water. The location of plasma membranes is indicated by arrows. Scale bars equal 100 µm.

**Figure 6.** Confocal microscopy of *Xenopus* A6 cells expressing chimeric proteins of hUAT/EGFP. A: A6 cell 3 days after transient transfection with a construct containing EGFP on the amino terminus of hUAT. B: A6 cell 3 days after transient transfection with a construct containing EGFP on the carboxy terminus of hUAT. Scale bars equal 10 µm.

**Figure 7.** Comparison of amino acid sequences of the rat (rUAT) and human (hUAT) homologues of the urate transporter/channel. Shaded amino acids are homologous. Underlined amino acids are different in the rat and human sequences. The solid line below amino acids 158 – 169 in the human sequence indicates the block of amino acids with 50% homology to porcine uricase.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI-hUAT (S)</td>
<td>5’ GC GGA TCC ATG GCC TTC AGC GGT TCC CAG</td>
</tr>
<tr>
<td>HindIII-hUAT (AS)</td>
<td>5’ GC AAG CTT CTA TGT CTG CAC ATG GGT CAG C</td>
</tr>
<tr>
<td>XhoI-hUAT (S)</td>
<td>5’ GC CTC GAG ATG GCC TTC AGC GGT TCC CAG</td>
</tr>
<tr>
<td>KpnI-hUAT (AS)</td>
<td>5’ GC GGT ACC TGT CTG CAC ATG GGT CAG CTG C</td>
</tr>
<tr>
<td>Xmal-EGFP (S)</td>
<td>5’ GC CCC GGG ATG GTG AGC AAG GGC GAG GAG C</td>
</tr>
<tr>
<td>BamHI-EGFP (AS)</td>
<td>5’ GC GGA TCC CTT GTA CAG CTC GTC CAT GCC</td>
</tr>
<tr>
<td>XhoI-EGFP (S)</td>
<td>5’ GC CTC GAG ATG GTG AGC AAG GGC GAG GAG C</td>
</tr>
<tr>
<td>KpnI-EGFP (S)</td>
<td>5’ GC GGT ACC GTG AGC AAG GGC GAG GAG CTG</td>
</tr>
<tr>
<td>HindIII-EGFP (AS)</td>
<td>5’ GC AAG CTT TTA CTT GTA CAG CTC GTC CAT GCC</td>
</tr>
</tbody>
</table>

Restriction sites are underlined. (S) designates sense primers, (AS) the antisense primers.
Figure 3
Figure 7

<table>
<thead>
<tr>
<th>rUAT</th>
<th>MAPSTOQEPY</th>
<th>MNVPFPFTGI</th>
<th>IQGGLQGGLQ</th>
<th>ITLQGTVPFF</th>
<th>-RRIAVNFQT</th>
</tr>
</thead>
<tbody>
<tr>
<td>hUAT</td>
<td>MAPSGSQAPY</td>
<td>LSPAPFPFGT</td>
<td>IQGGLQGGLQ</td>
<td>ITVNGTVLSS</td>
<td>GSTRFAVNFQT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>rUAT</th>
<th>GFSGNDIAFHY</th>
<th>FNPRFEPGY</th>
<th>VVCNSTQNGK</th>
<th>WGPEERKMQM</th>
<th>PFKGMPFEDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>hUAT</td>
<td>GFSGNDIAFHY</td>
<td>FNPRFEPGY</td>
<td>VVCNSTQNGS</td>
<td>WGPEERKTHM</td>
<td>PFKGMPFEDL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>rUAT</th>
<th>CFLVQRSFDPK</th>
<th>VMVKNFQVQ</th>
<th>YSHRVPPHLV</th>
<th>DTISVSGCLH</th>
<th>LSINFGTQG</th>
</tr>
</thead>
<tbody>
<tr>
<td>hUAT</td>
<td>CFLVQSDPK</td>
<td>VMV QGPQVQ</td>
<td>YPHRVPHRV</td>
<td>DTISVNGQV</td>
<td>LSISFGPPG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>rUAT</th>
<th>FQPAHAPVPA</th>
<th>QTIIHTVHSI</th>
<th>PGMMLSTPGI</th>
<th>PPMAYPTPAY</th>
<th>TIPFTSPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>hUAT</td>
<td>WVPANAPIT</td>
<td>QTVIIHTQSA</td>
<td>PGQMFSTPAI</td>
<td>PPMYPHPAY</td>
<td>PMPFITTILG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>rUAT</th>
<th>GFYPSKSI</th>
<th>SGVVPDAKR</th>
<th>FHINLRCGDD</th>
<th>IAPHLNPRFN</th>
<th>EKVVVRNTQI</th>
</tr>
</thead>
<tbody>
<tr>
<td>hUAT</td>
<td>GYPSKSL</td>
<td>SGVPDSAOQ</td>
<td>FHINLCSGNN</td>
<td>IAPHLNPRD</td>
<td>ENAVRNTQI</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>rUAT</th>
<th>NNSWGFEERS</th>
<th>LPGRMPFRNG</th>
<th>QSFSVWILCE</th>
<th>QCFCVAVDG</th>
<th>QHEEYYHRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>hUAT</td>
<td>DNSWGEERS</td>
<td>LPRMKPFRVG</td>
<td>QSFSVWILCE</td>
<td>AHCLKVAVDG</td>
<td>QHEEYYHRL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>rUAT</th>
<th>KNLPTINTLE</th>
<th>VAGDIQLTHV</th>
<th>QT 322</th>
</tr>
</thead>
<tbody>
<tr>
<td>hUAT</td>
<td>KNLPTINTLE</td>
<td>VAGDIQLTHV</td>
<td>QT 323</td>
</tr>
</tbody>
</table>