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Cloning and Molecular Dissection of the 8.8 kb Pig Uroplakin II Promoter Using Transgenic Mice and RT4 Cells

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Abstract Uroplakin II (UPII) gene expression is highly tissue and cell specific, with mRNA present in the suprabasal cell layers of the bladder and urethra. Previous reports described the mouse UPII (mUPII) promoter as primarily urothelium selective. However, ectopic expression of a transgene under the 3.6 kb mUPII promoter was also detected in brain, kidney, and testis in some transgenic mouse lines. Here, we have cloned an 8.8 kb pig UPII (pUPII) promoter region and investigated which cells within the bladder and urethra express a transgene consisting of the pUPII promoter fused to human erythropoietin (hEPO) or a luciferase gene. pUPII-luciferase expression vectors with various deletions of the promoter region were introduced into mouse fibroblast (NIH3T3), Chinese hamster ovary (CHO), and human bladder transitional carcinoma (RT4). A 2.1 kb pUPII promoter fragment displayed high levels of luciferase activity in transiently transfected RT4 cells, whereas the 8.8 kb pUPII promoter region displayed only low levels of activity. The pUPII-hEPO expression vector was injected into the pronucleus of zygotes to make transgenic mice. To elucidate the in vivo molecular mechanisms controlling the tissue- and cell-specific expression of the pUPII promoter gene, transgenic mice containing 2.1 and 8.8 kb pUPII promoter fragments linked to the genomic hEPO gene were generated. An erythropoietin (EPO) assay showed that all nine transgenic lines carrying the 8.8 kb construct expressed recombinant human erythropoietin (rhEPO) only in their urethra and bladder, whereas two transgenic lines carrying the 2.1 kb pUPII promoter displayed hEPO expression in several organs including bladder, kidney, spleen, heart, and brain. These studies demonstrate that the 2.1 kb promoter contains the DNA elements necessary for high levels of expression, but lacks critical sequences necessary for tissue-specific expression. We compared binding sites in the 2.1 and 8.8 kb promoter sequences and found five peroxisome proliferator responsive elements (PPREs) in the 8.8 kb promoter. Our data demonstrated that proliferator-activated receptor (PPAR)-γ activator treatment in RT4 cells induced the elevated expression of hEPO mRNA under the control of the 8.8 kb pUPII promoter, but not the 2.1 kb promoter. Collectively, our data suggested that all the major trans-regulatory elements required for bladder- and urethra-specific transcription are located in the 8.8 kb upstream region and that it may enhance tissue-specific protein production and be of interest to clinicians who are searching for therapeutic modalities with high efficacy and low toxicity. © J. Cell. Biochem. 99: 462–477, 2006.

Key words: transgenic; erythropoietin (EPO); uroplakin II; promoter assay; mouse; RT4 cells

The development of transgenic animal technologies now allows valuable modifications to the genome of virtually any animal [Brinster et al., 1988; Gordon, 1989]. Such technology can improve agronomic traits and lead to the introduction of new and lower cost biopharmaceutical materials and medical procedures. Among several animal bioreactor systems developed, mammary gland expression has been widely used for production of pharmaceutical proteins [Wilmut et al., 1991; Houdebine,

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2000]. However, purification of recombinant proteins from milk presents additional challenges because of the presence of lipid micelles and fat globules in milk [Wilkins and Velander, 1992]. Also, when producing proteins in the tissues of transgenic animals it is important that the tissue expressing the protein is able to execute complex post-translational modifications. This process is different from protein to protein and might also vary from tissue to tissue. To date, recombinant human erythropoietin (rhEPO) transgenic mice have shown deleterious side effects, including premature death and splenomegaly [Kwon et al., 2006]. Furthermore, recently produced transgenic animals have not proved useful for commercial production of rhEPO, because of the low yield of the protein from the milk [Rodriguez et al., 1995; Aguirre et al., 1998; Mikus et al., 2001; Kwon et al., 2006]. Based on these experiences, erythropoietin (EPO) is considered a very difficult cytokine/growth factor to produce in transgenic animals.

Uroplakins (UPs) are a group of integral membrane proteins that have been identified recently as the major proteins of urothelial plaques [Wu et al., 1995]. These plaques, first described by Porter and Bonnevlle [1963], cover a large portion of the apical surface of the mammalian urothelium. The urothelium is a highly specialized transitional epithelium that lines most of the urinary tract, including the bladder. It consists of basal, intermediate, and superficial cell layers, which function to provide a permeability barrier. The terminally differentiated superficial cells are characterized by the presence of multiple plaques of asymmetric unit membranes (AUM) in the outer leaflet of the apical membrane. The characteristic AUM plaques are formed by the interaction of at least four species of conserved transmembrane proteins, known collectively as the UPs. UPIa and UPIb are members of the tetraspanin family of proteins and form plaques by interacting with the unrelated single transmembrane domain uroplakin II (UPII) and UPIII proteins, respectively [Wu et al., 1995]. In normal human urothelium, expression of the UPIa, UPII, and UPIII genes is restricted to superficial cells, whereas UPIb transcripts are also present in intermediate cells, implying that expression of the UPIb gene is less differentiation restricted [Lobban et al., 1998; Olsburgh et al., 2003].

The tissue-specific expression of desired gene products in the bladder tissue has been considered for gene therapy and development of animal bioreactors. Bladder is an attractive organ for the production of pharmaceutical proteins because urine is easily collected during the lifetime of transgenic animals, irrespective of their sex [Kerr et al., 1998; Ryoo et al., 2001]. A previous study used a 3.6 kb 5′ flanking region of the mouse UPII (mUPII) gene to induce secretion of a pharmaceutical protein, hGH, into the urine of transgenic mice [Kerr et al., 1998]. The concentration of mUPII/hGH reached 0.1–0.5 μg/L in mouse urine, which represents 0.02% of total urinary protein. The bladder-specific promoter produced much lower yields of the protein than when a mammary gland-specific promoter was used. This observation suggests that the function of the UPII promoter is not well understood. Consistent with this report, studies of another transgenic mouse line showed that a bacterial reporter gene (lacZ) under control of the 3.6 kb mUPII promoter is expressed in both brain and bladder [Meyer-Puttlitz et al., 1995]. The utility of promoter fragments isolated from the 5′ flanking regions of endogenous mammalian genes to drive transgene expression in vivo is often limited by low expression levels and non-specific expression. Indeed, high levels of expression of the human ApoB gene were obtained in the liver and intestine of transgenic mice with constructs containing 70 kb of ApoB downstream sequence and 22 kb of upstream sequence, but not with smaller constructs [Nielsen et al., 1997]. These results suggest that large upstream regulatory elements may be required for elevated levels of bladder-specific gene expression.

To address some of these issues, we have cloned a large genomic fragment (23 kb) of the pig UPII (pUPII) gene to identify putative distal trans-regulatory elements [Kwon et al., 2002]. Our data demonstrated that a 2.1 kb promoter fragment directs exogenous gene expression in a cell-specific fashion in vitro. Therefore, the present study was designed to test whether 2.1 or 8.8 kb pUPII promoter fragments can drive tissue-specific expression in vivo. In contrast to the in vitro data, the in vivo data demonstrate that the 8.8 kb promoter directs gene expression in a tissue-specific fashion, but the 2.1 kb promoter does not. The differences in expression may be caused by peroxisome proliferator-activated receptor (PPAR)-γ, which is expressed

in the presumptive urothelium of the mouse urogenital sinus and in the mature urothelium of mice, rabbits, and humans [Guan et al., 1997; Jain et al., 1998; Kawakami et al., 2002].

MATERIALS AND METHODS

DNA Sequencing and Sequence Analysis

Approximately 8.8 kb of the pUPII promoter was isolated from a pig Lambda Fix II genomic library (Stratagene, La Jolla, CA), as described in our previous report [Kwon et al., 2002]. The nucleotide sequence of DNA templates was determined using the fluorescent dideoxy terminator method on an ABI automated sequencer (ABI377 DNA sequencer, Applied Biosystems) according to the manufacturer's protocols. Comparative sequence analysis was performed using BLAST [Altschul et al., 1990], SMART [Schultz et al., 1998], PROSITE [Bairoch and Bucher, 1994], NetOGlyc2.0 [Hansen et al., 1998], and PipMaker [Schwartz et al., 2000] programs. Promoter sequence analysis was performed using TFsearch (http://www.cbrc.jp/research/db/TFSEARCH.html) and rVista 2.0 (http://rvista.dcode.org/).

Construction of UPII Promoter-Luciferase Expression Vectors

An *Kpn*I-*Xho*I fragment (8.8 kb) of pUPII genomic DNA was subcloned into a pGL3 basic vector (Promega) generating pUPII/Luc constructs. To generate truncated pUPII promoter fragments, the −8,786/+59 pUPII Luc construct (pUPII-8.8/Luc) was amplified by PCR with a reverse primer (5′-CTCGAGAGCTGG GCTGGTGCTGG-3′) containing a *Xho*I linker (underlined) and the following forward primers: pUPII-8.8/luc, 5′-GGTACCGGGCTAGGAGTG-GAAT-3′; pUPII-4/luc, 5′-GGTACCCACTATGT CCGGTTTT-3′; pUPII-2.1/luc, 5′-GGTACCTC TGGTCAGAGCTCTG-3′; pUPII-1.4/luc, 5′-GG TACCTTTGGAGAAGCGCTGA-3′; and pU PII-0.6/luc, 5′-GGTACCCAGCATCAATTGGCT-3′. The amplified products were ligated into a pGL3 basic plasmid and confirmed by sequencing.

Transient Transfection and Luciferase Analysis

For reporter assays, cells were transiently co-transfected with one of the pUPII promoter-luciferase constructs described above and pRL-SV40 (Renilla luciferase, Promega) using Effectene (Qiagen). Briefly, NIH3T3, Chinese hamster ovary (CHO), and RT4 cells were plated at a density of 2×10^5 cells per 35-mm dish in DME medium or McCoy's 5A containing 10% FBS (RT4 cells). Transient transfections were performed according to the manufacturer's instructions. Briefly, 1 μg of each pUPII promoter-luciferase plasmid in TE buffer, pH7.4, was diluted with a DNA concentration buffer, Buffer EC, to a total volume of 150 μl and 8 μl of Enhancer was added. The mixture was gently tapped for 5 s and incubated at room temperature for 5 min. The mixture was centrifuged for a few seconds and 5 μl of Effectene transfection reagent was added and mixed by pipeting up and down five times. After incubation for 10 min at room temperature to allow transfection complexes to form, the transfection complexes were added into 1 ml of growth medium. The growth medium containing transfection complexes was immediately added drop-wise onto cells in the 35-mm dishes. At 48 h post-transfection, cell extracts were prepared with 1× lysis buffer, and then 20 μl aliquots of the supernatant were mixed with 100 μl of firefly luciferase assay reagent (Promega) and analyzed on a Microplate Luminometer (Perkin Elmer). Firefly luciferase activity was normalized to Renilla luciferase (pRL-SV40) activity as an internal control. All transfection experiments were repeated at least twice with different plasmid preparations.

Construction of human erythropoietin (hEPO) Expression Vectors Under the Control of the 2.1 and 8.8 kb pUPII Promoters

Human EPO genomic DNA was the kind gift of Sasaki R. (Kyoto University, Japan). Human EPO genomic DNA (2.4 kb) was cloned into the pSK (+) vector (Stratagene) and ligated to 2.7 kb of SV40 Poly A. The expression vector, pBC1 (Invitrogen, Carlsbad, CA), was completely digested with *Sal*I and *Not*I to remove casein promoter DNA and the poly A site, and then ligated to the 8.8 or 2.1 kb pUPII promoter fragments to generate the pUPII expression vectors. The 5.1 kb DNA fragment containing hEPO and SV40 poly A was isolated from pSK (+) by digestion with *Xho*I and *Sal*I, followed by ligation to *Sal*I digested pUPII. The 13.9 or 7.2 kb pUPII/hEPO/SV40 polyA cassette was isolated by *Not*I and *Sal*I digestion and used for microinjection (Fig. 3A).

Production and Screening of Transgenic Mice

Transgenic mice were generated according to our previous protocol [Ryoo et al., 2001]. Briefly, fertilized one-cell embryos were obtained from BDF1 (C57BL/6×DBA hybrid) females. A DNA expression cassette was microinjected into the pronucleus of the zygotes. Injected embryos were cultured for 20 h before transfer to a pseudopregnant ICR female mouse. All mice were raised and maintained under pathogen-free conditions.

Genomic DNA was obtained from the mouse tails and the presence of the transgene was verified by PCR. The primers were: hEPO 5-GT AGAAGTCTGGCAGGGCCT-3′ (forward) and 5′-TCATCTGTCCCCTGTCCTGC-3′ (reverse). Transgenic mice screened by PCR analysis were re-confirmed by Southern blot analysis. Briefly, 10 μg of genomic DNA was digested with *Asc*I and *Nco*I, electrophoresed in a 0.7% agarose gel, and transferred onto a nylon membrane. Hybridization was carried out in an aqueous solution containing 6× SSC, 5× Denhardt's reagent, and 1% SDS at 68°C using a randomly primed 456 bp hEPO probe DNA with an activity of 5×10^6 cpm/ml. Final washes were performed with 1× SSC, 0.1% SDS at 65°C.

RT-PCR and Northern Blot Analysis

Total RNA was isolated from various tissues (bladder, kidney, brain, liver, heart, spleen, and lung) and using the TRIZOL reagent (Gibco BRL). In order to specifically detect the expression of EPO cDNA transcripts, RT-PCR was performed with selective primers in a Peltier Thermal Cycler 200 (MJ Research, Nevada). The primers used for EPO cDNA detection were: 5′-ATGGGGGTGCACGAATGTCC-3′ (forward) and 5′-TCATCTGTCCCCTGTCCTG C-3′ (reverse).

Northern blot analysis was performed using randomly primed hEPO or pUPII cDNA, as previously described [Kwon et al., 2002]. Briefly, samples of 20 μg total RNA isolated from transfected cells with 2.1 or 8.8 kb pUPII/hEPO were denatured in 68% formamide and 2.2 M formaldehyde for 15 min at 65°C, and subjected to electrophoresis on a 1.2% agarose/formaldehyde gel. After UV cross-linking, total RNA samples were transferred to a membrane, and pre-hybridized with hybridization buffer for 3 h. Probe DNA was prepared by labeling with [α^{32}P]dCTP. Hybridization was performed for 24 h at 68°C using the pre-hybridization buffer after the addition of labeled probe DNA. Membranes were washed for 10 min at 37°C using 2 × SSC/0.1% SDS (2 × SSC = 0.3 M NaCl/0.03 M sodium citrate, pH 7.0), and for another 10 min at 65°C using 0.2 × SSC/0.1% SDS. After exposure of the blots to X-ray film, the resulting autoradiograms were quantified with an imaging densitometer using the Molecular Analyst software (Bio-Rad).

Immunohistochemistry

The immunohistochemical staining was conducted according to our previous methods [Choi et al., 2004]. Briefly, paraffinized tissue sections were cleared in Histoclear for approximately 10 min and rehydrated in decreasing concentrations of ethanol. Immunohistochemistry was performed with an ABC kit (Oncogene Science, Inc., Boston, MA) according to standard procedures provided by the manufacturer. Sections were placed in 3% peroxide in methanol and 0.1% pepsin in 0.05N HCl (pH 2.25) for 30 min to reduce background staining. Sections were washed twice (5 min each) in TBS (0.05 M Tris-HCl, pH 7.4, 0.85% NaCl) and blocked with normal horse serum diluted in TBS (1:5; NHS-TBS). Sections were incubated overnight with the primary hEPO polyclonal antibody (R&D system, Minneapolis, MN) diluted at a concentration of 1:500 in NHS-TBS. One drop of horse serum from the ABC Kit was used as a negative control. Excess antibody was removed by washing twice for 5 min each with TBS. A biotinylated secondary IgG was added for 30 min, and then rinsed with three changes of TBS for 5 min each. Sections were incubated with ABC reagent for 30 min, washed extensively with TBS, and rinsed in 1% Triton-X-100-PBS for 30 s. The reaction was developed with a solution of 0.5% diaminobenzidine in 0.05 M Tris-HCl (pH 7.6) containing 0.01% hydrogen peroxide. After development of the color reaction, sections were washed in water, dehydrated, and mounted with a coverslip.

Analysis of Blood Components

Transgenic and control mice were anesthetized with isoflurane and approximately 200 μl whole blood was obtained from the retro-orbital cavity. Blood components were analyzed with a Hamavet 850 automated analyzer (Drew Scientific Group Company, Cumbria, UK).

Quantification of Urine-Derived rhEPO

Urine was collected daily and centrifuged (5 min, 12,000*g*), frozen and stored at −20°C. Before analysis the urine was desalted on Micro Bio-Spin columns (Bio-Rad). Human EPO was measured using an enzyme-linked immunosorbent assay (ELISA, R&D system). Briefly, plates were extensively washed five times with PBS containing 0.05% Tween-20, and maintained at 4°C. Aliquots (50 μl) of standard hEPO solution or urine samples were added to the wells, and incubated for 2 h at room temperature. After extensive washing, the plate was incubated with an HRP-linked anti-human antibody (1:1,000 dilution) for 1 h at 37°C. Plates were re-washed and substrate (*o*-phenylenediamine) added (50 mg in 100 ml of 100 mM sodium acetate, pH 5.6 and 10 ml H_2O_2). The reaction was terminated by the addition of H_2SO_4, and the absorbance at 492 nm measured at room temperature. The presence of hEPO in urine was analyzed with a sensitivity of 0.6 mIU/ml, and a detection range of 2.5–200 mIU/ml.

Establishment of Stable Cell Lines Expressing the 2.1 kb and 8.8 kb pUPII/hEPO Constructs

The human RT4 cell line was maintained in McCoy's 5A modified medium (Gibco BRL). RT4 cells were transfected with 1 μg of the 2.1 or 8.8 kb pUPII/hEPO plasmid containing the Neo gene using Effectene (Qiagen). Individual cell clones were selected for growth in the presence of G418 (1 mg/ml) by limiting dilution. Three individual clones expressing high levels of 2.1 and 8.8 kb pUPII/hEPO were randomly selected.

Treatment of Troglitazone (TZ) and PD153035

RT4 cells non-transfected and stably transfected with 2.1 or 8.8 kb pUPII/hEPO were seeded at 6×10^5 cells/60-mm dish and grown to approximately 70% confluency before treatment with PPAR-γ ligand and inhibitor. The cells were pretreated for 0–24 h with or without 1 μM TZ. After each time point, fresh medium was replaced to remove TZ, and then the cells were further maintained in the presence of 1 μM PD153035 until analysis at day 3. Total RNA was extracted and Northern blot analysis was performed using randomly primed hEPO or pUPII cDNA, and quantified as described above.

Immunoprecipitation

To detect EPO protein, 1 ml of desalted urine was immunoprecipitated with 1 μg of rabbit anti-EPO polyclonal antibody (R&D). Proteins were electrophoresed on 12% SDS–PAGE gels and subsequently transferred to nitrocellulose membranes. Blots were incubated with an anti-mouse hEPO monoclonal antibody, followed by a secondary goat anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) (Calbiochem, CA), and developed with the ECL detection system (Amersham Pharmacia Biotech).

Statistics

All experimental data are presented as mean ± SD. Each experiment was performed at least three times. For statistical analysis (Figs. 6, 7 and Table II), one-way analysis of variance (ANOVA) was performed to determine whether there were differences among the groups, and Fisher's post-test was performed to determine significance between pairs of groups. $P < 0.05$ was considered significant. Statistical tests were performed using StatView software version 5.0 (SAS Institute, Inc., Cary, NC).

RESULTS

Cloning and Sequence Analysis of an 8.8 kb Fragment of the pUPII Promoter

In this study, we cloned and sequenced 8.8 kb of the pUPII promoter. The sequence has been deposited in GenBank (Accession number: AY044189) (Fig. 1). Sequence comparison of the 5′-UTR shows low homology between pig and mouse sequences. Therefore, we searched for conserved regions between the pig and mouse (GenBank Accession No. U14421 and No. AC125129) UPII promoter regions. Five conserved domains, which we call A, B, C, D, and E, were detected by the BLAST [Altschul et al., 1990] and PipMaker [Schwartz et al., 2000] programs (Fig. 2A); no additional conserved regions were detected. These programs find common, ungapped patterns in a set of sequences by the expectation maximization algorithm. The relative locations of the conserved sequences are not similar, A: −2,420 to −2,258 in the pUPII versus 3,438–3,593 in the mUPII; B: −2,265 to −2,104 in the pUPII versus 1,017–1,101 in the mUPII; C: −2,091

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-8786  GGGCTAGGAGTGGAATCAGAGCTGGCCTATGCCACAGCAACGCAGAATCCAAACCACATCTCCGACCTAC
-8717  ACCAGACCGTCACCATAACACAGGATCCTTAACCCACTGAGCAAGGTCAGGGATCAAACCCAAATCCTCA
       ------------------------------------------------------------------------
-6543  CACCCCTCACGATCCTTGGCCCAGGGACATTTTTTGTACCAGCCTTTCAATCCTGACCTTCATATCATCC
-6473  GACACCTCCTTTGTGAAACCCTCCATCCACTTTCTCCTGGTTCCCCTCCTAAGACCCATTCCGCCTTCTT
-6403  CAGCCCCCTCCCTCCATCTGTCCTTTAGATGCCGCATTTCCTAGTATCCTGTCCTGCGCGGNCTCGTCCT
       ------------------------------------------------------------------------
-6178  GCATTAGTTATTACTGTATAACAGCTTATCCCCCAATTTAGTGGCTTATAAAATAAACACTTATTCTGAG
-6108  AATCAGAAACCTAGGCAGGACATAGTTGGGGTCTCATGAAGTTGCACTGAAAATGTCCCCCTGGGCTAAT
-6038  CATACGGAGGACTGACCAGGGCTGGAGGATCTGTTCCAAGCTCATTCATTCACATGGCCGTAGGTTGGAG
       ------------------------------------------------------------------------
-6038  TGGGCTGAGGGGNTAAANTGCCCCTCCTAAAAAACCTGAGCTGCTGCAGTTGGATTCTTAATCCACTGCA
-4940  CCACAAGGGGGAAGGTCAAGAACTGTCTTGCCATCTCTGTATCTTATCACCTAGCATAGTACCCACCATA
-4870  GAGAAGTTGCTCAACAAATGTTTACTGAATGAATAAATGCATGAGCTGGAGTTCCCATTGCGGCTCAGCA
       ------------------------------------------------------------------------
-4499  TGTCTCAGTCATGTTTCCCCCTCAGACTACCTTTCCTGCCCCATCTCTCCCTTTGACATAATTGGAAAAA
-4429  CAAATTCAGAATTTTGTCCCACTACCTTTCTTGCTAGCTCTGTGGCCTTGGGAAAGCTATTTATTGCCTC
-4359  TGAGCCTCTAATTTTCATCTGCACCAAGGATTAATAAAAAGGAGAGGATAAGATGAATTACTTATATTAA
       ------------------------------------------------------------------------
                                                                  A domain
-2458  GGCCCTGGTTCCCTTTTTCCAAGCCATGAGGAAATCCTCAGAGGAACAGAGTGCTGTGGCTTTAAATGAC
-2388  TTCAGCGTTGTCAATGAATCTGCTCGGCTAAAAGAGTTATCCTCTTGCTCCTTCGCTTGTCCTCCCCCTC
                                                                  B domain
-2318  CTCTCAGCTCCCCAAACCCTTCTCGGCTGCTGTGATGGGATAATTAGATGCGAGAGCTCAGCACAGATGA
-2248  TGCTCCAGTTGCCTAGCAACTAATGGTTTCCATGGAGACCGCAAAGCACAGCCTCCAGAGCAGCCAGTGA
-2178  GCAGCTCGGCAGGGCAGGGAGAAGACGCAACTCTCAGCTCCTCCAGAAACCTGGGGAGGGCCAGGAGTGG
                                                C domain
-2108  GGAAGAAGGGGGGGATCGGAGGGCTTAAAGGCACAGGCCCCTCTTATCCTCTTAAAATCTGGTCAGAGCT
-2038  CTGCCCTCCCCTCCCCTACTCTGTCCCACTCATAATTTCAGATGGAGTTGGGGGCTTAGGAGTGGACCCA
-1968  ACACAACCTACCCTGCAATAAACCCAACCTTCTTTCTGCTTCTGGTTTGTGGCTGAAAATGGNAAAAGAA
-1898  ATCTCCCAAGTGCAAGTGTAAACANCNTCCTGGGTTGGCAATGGGATCTGAAGAGTACTAAGATCCCTCA
-1828  GACCTGGAATTCCACCATTTAGTCTTTCCCTCTCTCCAAAGTTCTCAATGTGCAAAAGATCCTCTTTCAG
-1758  TTTGCAGAGCAATGATAGGATCTTCTAAAAGGAGACAAAAGCCAAGGTGCAGGAAAAATAGAATTCAGTT
-1688  CTTCACCCAAAGGCAGCCTGTCCTGGGAGACAGGGGTGAAACACTTGGTCCTGATCTCCATCAGAGGATC
-1618  CAGAGTGTGTGTGTTTGTTGCTGGGGAGGGGGACACAATATAGAGCATCTGGTGACTCAAAGTATGTGCC
                                                                  D domain
-1548  TCCCAGAGTAGCATCAATCAATGTTACCTGGAAGCTTGTTAGAAATGCAGAATTTCAGGCTTCACCTCAG
-1478  ACCCACTGAATCAGAAACTGCATCTTAACAAGATCCCTCATGATTCATACGCACATTAAATTTGGAGAAG
-1408  CGCTGACCTGAGACCCTCCTCCTCTCTGCTTGGGCCCATAGTTCTACCTTTATTGTCACCTCGTCTCACC
       ------------------------------------------------------------------------
 -265  GGTGTCCCGGAAGAAGGGGGTTTTCCACCCCCCGCTTTAGTCACCCTGCCCCTCTGCAGCTGCCTGAGCC
                                                                  E domain
 -195  ACCAAGACCCAGCCAAGGTCTCCTGCCTTCTGGCCTGAGGGCCAGCTCCCCATCCTGAAAAACCTGTCTG
 -125  GGGGCCTCCCCTGAGGCTGTAGGGCCCAAGGCCTCCCCTGAGGCTGTAGGGCCCAAGGGGCAGGTTGAAC
  -55  AGGATTCCCCTCTGGCCCCTCCTACCCCCAGGACAAAACCAGAGCCCCAGGACAGGGCCTCACTTGCCTC
  +15  AGGAAACCACAGCTTGCCAGCACCCAGCCCAGCACCAGCCCAGCT ATGGCATCCCCATTG
                                                     M  A  S  P  L
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Fig. 1. pUPII (8.8 kb) promoter sequences and identification of five putative PPREs. The transcriptional start site was retrieved from NCBI (www.ncbi.nlm.nih.gov) or by linking to the UCSC Genome Browser (http://genome.ucsc.edu). The arrowhead indicates the putative transcription start site and the numbering of the promoter is with respect to the putative transcription start site (+1). Five PPARγ defined PPRE domains are boxed and five conserved regions are underlined, respectively. Overlapped region of A and B domain is marked by bold. This sequence has been submitted to GenBank (Accession number AY044189).

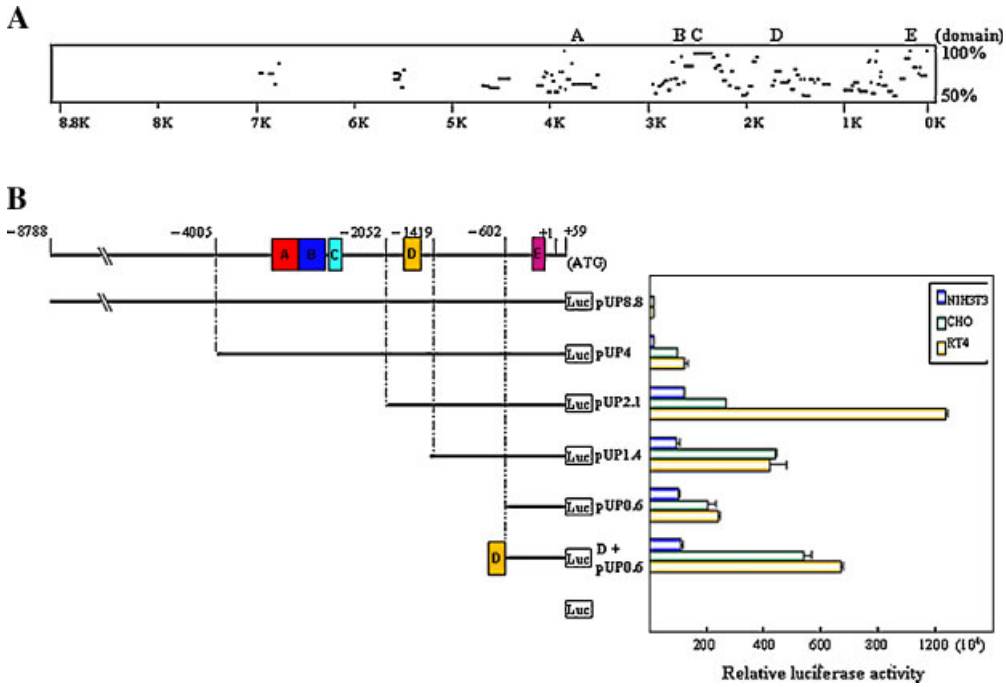

Fig. 2. Alignment of the pig and mouse UPII promoter regions by PipMaker and promoter analysis. **A**: Alignment of the pig and mUPII promoter region by PipMaker. The pig sequence corresponds to nt 1~8,846 of Genbank file AY044189 and the mouse sequence to nt 113,358–122,358 of the AC125129. Consensus nucleotides were shared by the pig and mUPII promoters. Five conserved regions are designated as A, B, C, D, and E domain. Dashes indicate homologous region and the percentage ranging from 50% to 100 % indicates the degree of homologies within two DNA sequences. The resulting alignments are summarized with a ''percent identity plot,'' or ''pip'' for short by PipMaker program (http://bio.cse.psu.edu). **B**: Functional analysis of the 5′ flanking region of the pUPII gene. The 5′ deletion constructs used are depicted schematically on the left. The urothelial cell line RT4 and non-urothelial cell lines CHO and NIH3T3 were co-transfected with luciferase expression vectors containing deletions of the 5′-UTR of the pUPII promoter and a control plasmid, pRL-SV40, expressing Renilla luciferase. Dual-Luciferase activity was assessed after 48 h. Results are expressed relative to the activity of the control vector pGL3 basic. (Bars = Mean ± SEM; n = 6). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to −2,052 in the pUPII versus 3,438–3,593 in the mUPII; D: −1,496 to −1,411 in the pUPII versus 3,438–3,593 in the mUPII; E: −159 to −108 in the pUPII versus 3,438–3,593 in the mUPII. However, the domains show 91%, 87%, 88%, 80%, and 80% conservation between the two genes for the A, B, C, D, and E regions, respectively, suggesting a cis- or trans-acting regulatory role for the conserved sequences. The pUPII promoter is more G/C-rich than the mouse promoter within the first 200 bp upstream of the transcription initiation site (67% vs. 53%). Analysis of the sequences revealed that there are no TATA or CAAT boxes immediately upstream of the putative 5′-UTR. However, this region has other features characteristic of so-called 'housekeeping genes,' such as a high GC content and numerous Sp1 elements, including AP2 and GATA box sites.

Characterization of a Series of pUPII Promoter-Luciferase Constructs in RT4 Cells

To dissect the function of the pUPII promoter, the 5′-UTR was partially deleted using PCR based on the conserved regions and then classified according to their fragment size as follows: (a) pUP8.8 (−8,788 to +59); (b) pUP4 (−4,005 to +59); (c) pUP2.1 (−2,052 to +59); (d) pUP1.4 (−1,419 to +59); (e) pUP0.6 (−602 to +59); and (f) D domain + pUP0.6. These upstream sequences were directly ligated in frame to the firefly luciferase coding sequence (Fig. 2B). Different cell types were used to express the constructs: mouse fibroblast (NIH3T3), CHO, and human bladder transitional carcinoma (RT4). Each cell line was transfected with reporter constructs and an internal control plasmid, pRL-SV40 using Effectene (Qiagen). Luciferase assays were

performed and the results are summarized in Figure 2B. The deletion of the sequences −8,788 and −4,005 bp did not show a significant promoter activity, whereas the 2.1 kb pUPII (pUP2.1) produced approximately 2,000- to 2,400-fold higher luciferase activity in RT4 cells than pGL3 basic. Increased pUP2.1 promoter activity was also observed in CHO (~700-fold) and NIH3T3 cells (~400-fold). These results suggest that the sequence between −2,052 and +59 may contain cis-regulatory elements, which are likely to play an important role in the regulation of pUPII gene expression.

Among the five conserved domains, A, B, and C domains are located between pUP4 and pUP2.1, whereas D domain is located within pUP2.1 and pUP1.4. In transfection assays in RT4 cells, a significant difference in promoter activity was observed between pUP2.1 and pUP1.4. Of note, pUP1.4 compared to pUP2.1 exhibited almost the same level of activation in CHO and NIH3T3 cells, whereas pUP1.4 in RT4 cells resulted in approximately 60% loss of promoter activity (Fig. 2B). To investigate the D domain whether it plays a key role in cell-specific activity, the D domain was inserted into pUP0.6, the minimal promoter, and its activity was measured in each of the cell lines (Fig. 2B). The domain D + pUP0.6 increased promoter activity in RT4 and CHO cells. Collectively, our data suggested that A, B, and C domains act as potential silencer, but the domain D acts as an enhancer in the cell type-independent manner for pUPII gene expression.

Generation of Transgenic Mice

Transgenic mice were generated using the microinjection technique. A total of 350 out of 373 embryos injected with 2.1 kb pUPII/hEPO and 700 of 758 embryos injected with 8.8 kb pUPII/hEPO were transferred to 17 and 30 recipients, respectively. A total of 51 and 98 pups, respectively, were obtained. Among them, two 2.1 kb pUPII/hEPO mice (3.9%) and nine 8.8 kb pUPII/hEPO mice (9.2%) contained at least one copy of the transgene, as revealed by PCR and Southern blot hybridization (Table I and Fig. 3B,C). Each transgenic animal was mated to wild-type mice. Subsequent generations (F2 and F3) were obtained by interbreeding transgenic animals. The transgene was stable in the germ line for three generations, as reflected by hEPO synthesis in the transgenic mice. Interestingly, tail biopsy analyses indicate that the ability to synthesize hEPO was independent of the number of transgene copies (data not shown).

The 8.8 kb pUPII Promoter, But Not the 2.1 kb Promoter, Directs Tissue- and Cell-Specific Activity In Vivo

The luciferase gene under the control of the 2.1 kb pUPII promoter is expressed at high levels in RT4 cells. To test whether this promoter region also possesses intrinsic tissue-specific regulatory activity, nine lines of 8.8 kb transgenic mice and two lines of 2.1 kb transgenic mice were analyzed. The expression of the hEPO transgene was assessed by RT-PCR analysis of total RNA extracted from several organs, including bladder, from the founder mice. As illustrated in Figure 4, hEPO mRNA from 8.8 kb transgenic mice was only detected in bladder and urethra, whereas 2.1 kb transgenic mice expressed hEPO in several organs including kidney and heart.

In order to further analyze hEPO expression in several tissues, immunohistochemical staining for hEPO was performed on formalin-fixed tissue sections. The 2.1 kb hEPO transgene was highly expressed in the brain, tubular and glomerular cells of the kidney, spleen, hepatocytes, cardiac myocytes, pneumocytes, and urothelium of the urethra and bladder (Fig. 5), but not in the testis, intestine, ovary, placenta, or thymus (data not shown). In contrast to a previously reported transgenic mice model showing premature death and splenomegaly [Kwon et al., 2006], expression of hEPO under the control of the 2.1 kb pUPII promoter did not show the any symptom. The reduced side effects in 2.1 kb transgenic mice may be due to the low levels of hEPO expression in transgenic mouse tissues. Unlike the 2.1 kb transgenic mice,

TABLE I. Efficiency of Transgenic Mouse Production

Mouse strain BDF1 (C57BL/6×DBA)	No. of embryos injected	No. of embryos transferred	No. of recipients	No. of mice born	No. of transgenic mice (%)
2.1 kb pUPII/hEPO	373	350	17	51	2 (3.9%)
8.8 kb pUPII/hEPO	758	700	30	98	9 (9.2%)

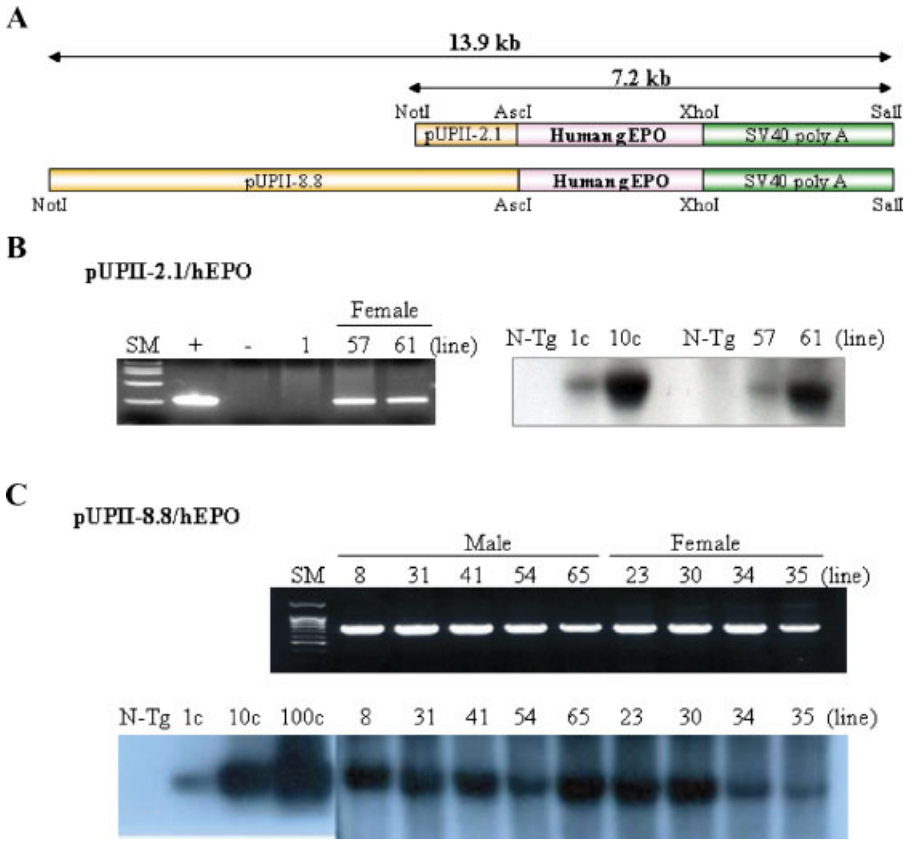

Fig. 3. Schematic representation of hEPO expression vectors and identification of transgenic mice. **A**: Construction of hEPO expression vectors containing 2.1 kb and 8.8 kb promoter regions. See Materials and Methods for details. Transgenic mice were identified by PCR and Southern blotting of samples from animals carrying the 2.1 kb construct (**B**) and the 8.8 kb construct (**C**). B: **Left panel**: PCR. + and − indicate positive and negative controls, respectively. SM, size markers. **Right Panel**: Southern blot. High molecular weight DNA extracted from mice was digested with *AscI*/*XhoI*, and transferred to the membrane. A positive control (1 and 10 copies) was detected when full-length hEPO cDNA was included on the blot. N-Tg, non-transgenic sample. C: **Top panel**, PCR. **Bottom panel**, Southern blot. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

hEPO expression in 8.8 kb transgenic mouse lines was principally limited to the suprabasal cell layers of the urothelium in the bladder and urethra (Fig. 5). These data demonstrate the tissue- and cell-specific regulatory contributions of the 8.8 kb promoter region.

Experiments were additionally performed to investigate the effect of hEPO expression on myeloid hematopoiesis in the transgenic mice since EPO is responsible for the regulation of red blood cell production. Four major blood parameters were examined: platelet numbers, red blood cell number, hemoglobin content, and hematocrit, in the 2.1 and 8.8 kb transgenic mice (Fig. 6). Levels of all components in 2.1 kb pUPII/hEPO transgenic mice ($n = 10$) were significantly increased, compared to those of control mice ($n = 6$) and 8.8 kb pUPII/hEPO transgenic mice ($n = 8$).

The 8.8kb pUPII Promoter, But Not the 2.1 kb Promoter, Is Regulated by PPAR-γ

To address the differences between in vitro and in vivo expression, we compared binding sites in the 2.1 and 8.8 kb promoter sequences the pUPII and identified potential peroxisome proliferator responsive elements (PPREs) at −6,448 to −6,437 bp, −6,091 to −6,080 bp, −4,834 to −4,923 bp, −4,422 to −4,411 bp, and −1,405 to −1,394 bp with respect to the transcription start site (Fig. 1). Therefore, we examined whether the PPAR-γ activator, TZ, can induce the expression of the endogenous UPII gene. RT4 cells were treated with 1 μM TZ for 0–24 h and the medium was changed. Treated cells were then maintained in medium containing 1 μM PD153035, which blocks further PPAR-γ activation via EGF receptors,

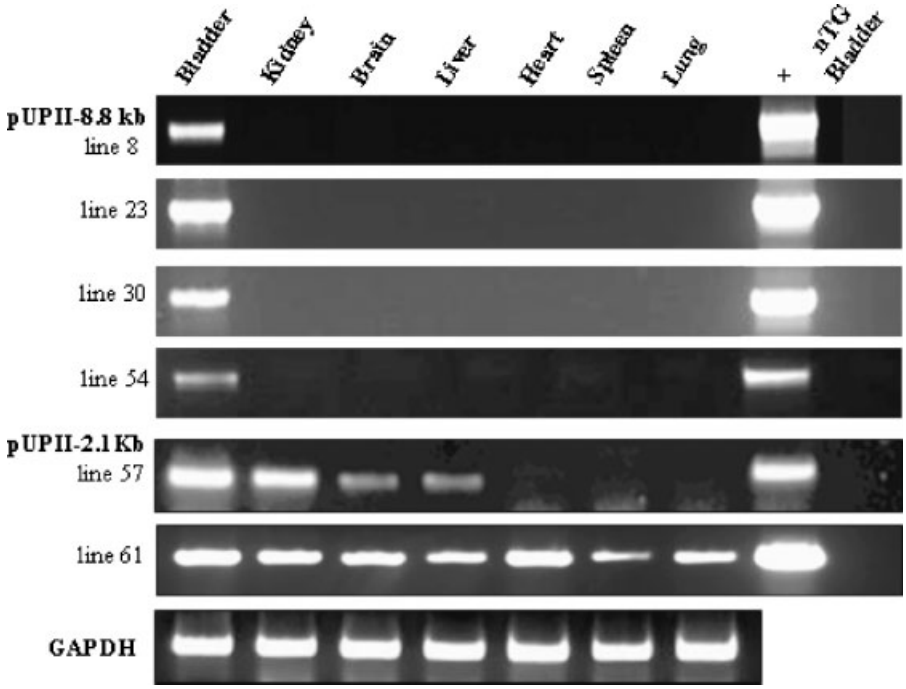

Fig. 4. RT-PCR analysis of hEPO expression in tissues of each transgenic mouse. GAPDH was used as an internal control. + and nTG indicate positive control and non-transgenic controls, respectively.

for 3 days. PPAR-γ induced UPII expression was detected after a 2 h exposure to 1 μM TZ. The maximum induction of endogenous UPII gene expression was obtained in cells cultured in the presence of PD153035 for 3 days after exposure to 1 μM TZ for 24 h (Fig. 7A,B). To determine whether the putative PPRE located in the 8.8 kb promoter was functional, we compared the expression of the 2.1 kb pUPII/hEPO which contains only one putative PPRE binding site and the 8.8 kb pUPII/hEPO which has five putative PPRE binding sites, in RT4 cells. These expression vectors were transfected into RT4 cells and stable cell lines were established. As shown in Figure 7C,D, the induction of hEPO mRNA under the control of the 8.8 kb pUPII promoter by the PPAR-γ agonist was time dependent, with maximum induction of UPII/hEPO mRNA after exposure to 1 μM TZ for 24 h, whereas no UPII-hEPO mRNA induction was found when the 2.1 kb pUPII promoter was used (Fig. 7C,D). This observation indicates that the 8.8 kb pUPII promoter, but not the 2.1 kb promoter, has the ability to respond to PPAR-γ signaling and suggests that using an optimized gene construct could further enhance production of valuable pharmaceutical proteins in target organs of transgenic animals and/or in cell lines.

Evaluation of hEPO Concentration in the Urine of Transgenic Mice

Expression of full-length hEPO in the urine of heterozygous transgenic mice was confirmed by Western blot analysis using an anti-mouse hEPO antibody. The results revealed positive signals at 34 kDa (Fig. 8). The 34-kDa band migrates at a position similar to CHO cell-derived rhEPO, which is generally 34–40 kDa [Skibeli et al., 2001]. Furthermore, quantification of hEPO was performed by ELISA analysis with crude total urine protein. We obtained approximately 200 μl of urine per day from each transgenic mouse. Urine pooled from 8.8 kb and 2.1 kb transgenic mice contained approximately $1{,}032.78 \pm 65.68$ IU and 290.82 ± 45.09 IU/ml hEPO, respectively, as measured by ELISA (Table II).

DISCUSSION

Expression of the endogenous pUPII gene is found exclusively in the urothelium of bladder and urethra (data not shown). RT4 cells, but not

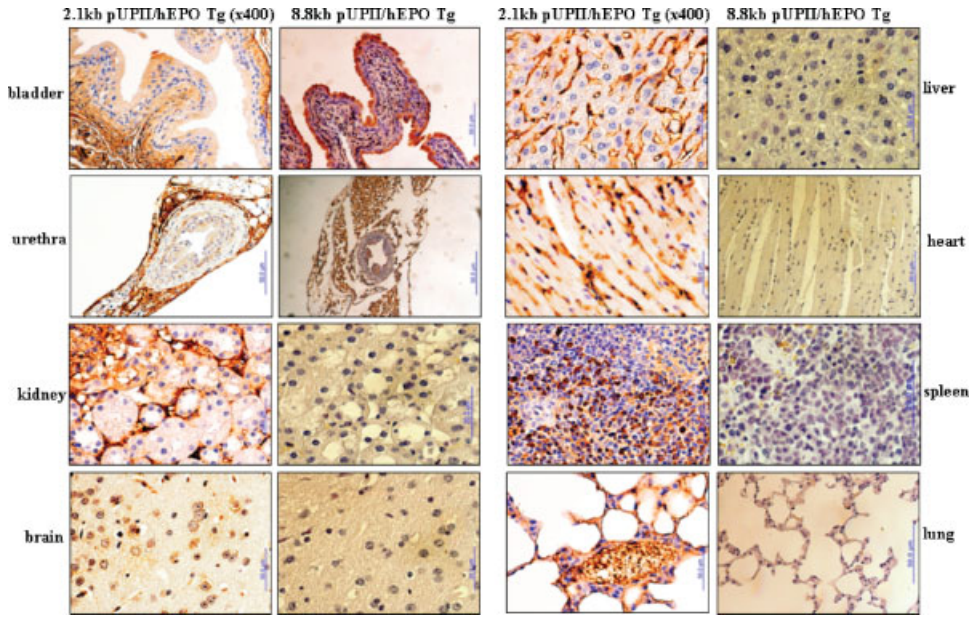

Fig. 5. EPO distribution in organs of 2.1 and 8.8 kb pUPII/hEPO transgenic mice. hEPO expression in 2.1 kb transgenic mice was detected in bladder, urethra, brain, heart, kidney, liver, lung, and spleen, whereas hEPO expression in 8.8 kb transgenic mice was only detected in bladder and urethra. Scale bars are shown in each panel.

non-urothelial carcinomas, strongly expressed UPII, suggesting a high tissue specificity [Lobban et al., 1998]. The human and mUPII genes have been previously mapped to chromosomes 11q23 and 9, respectively [Ryan et al., 1993], and are recognized to be potentially useful biomarkers of bladder cancer. However, the regulatory elements for tissue-specific expression are poorly understood. Therefore, aims of this study were to clone the urothelium-specific full-length promoter region and to develop transgenic mice to evaluate whether the UPII promoter could control tissue-specific secretion of proteins into the urine. In this study, transgenic mice were used to delineate the regulatory regions of the pUPII promoter involved in bladder-specific expression. To define promoter regions responsible for bladder-specific expression of the pUPII gene, we analyzed the expression of pUPII/hEPO constructs containing either 8.8 or 2.1 kb fragments from the 5′-UTR of pUPII and the hEPO structural gene, as a reporter, in transgenic mice. Human EPO gene expression was analyzed by RT-PCR and immunohistochemistry. In all transgenic F0 mice carrying the 8.8 kb pUPII/hEPO transgene, hEPO mRNA and protein were detected exclusively in the bladder. However, hEPO mRNA and protein in transgenic mice harboring the 2.1 kb pUPII/hEPO construct was detected in brain, intestine, kidney, lung, and liver, as well as bladder and urethra. No hEPO signal was detected in wild-type mice. The results of the present study indicate that RT4 cells contain the necessary transcription factors to activate transcription from the 2.1 kb pUPII promoter, but that the 2.1 kb promoter lacks the cis-acting elements required for bladder-specific expression.

Consistent with expression using a 3.6 kb mUPII promoter [Lin et al., 1995], the ectopic expression of the 2.1 kb pUPII promoter-derived transgene in several tissues may be due to positional effects of transgene integration and/or to some degree of overlapping promoter usage among tissues [Wilson et al., 1990] or the lack of chromatin insulator sequences, which function by blocking communication between enhancer and proximal promoter sequences, followed by militating against variegating positional effects [Geyer, 1997]. The utilization of promoter fragments isolated from the 5′ flanking regions of endogenous mammalian genes is often limited by low level of and non-specific expression of transgene in vivo. Indeed, interaction between the proximal (−378) promoter and the far upstream (−20 kb) enhancer is essential for tissue-specific expression of the human α2(I) collagen gene (COL1A2) in transgenic mice [Tanaka et al., 2004]. As well as this, in mouse

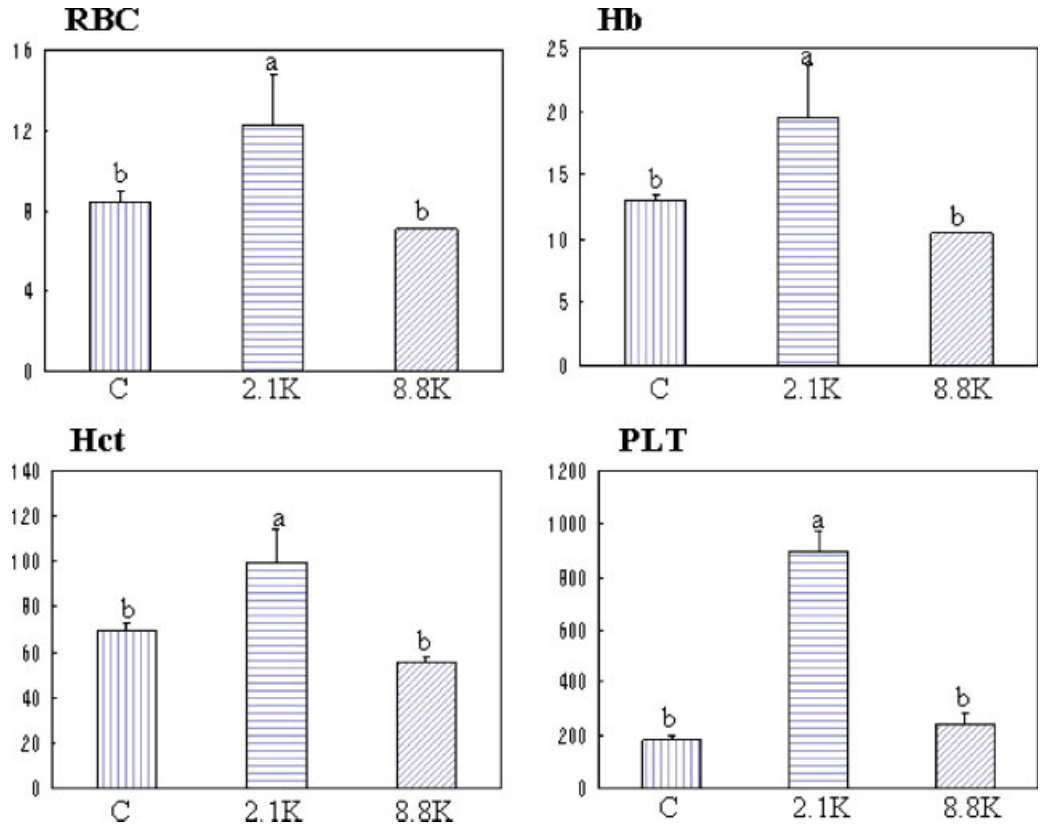

Fig. 6. Effect of hEPO expression on myeloid hematopoiesis in the transgenic mice. Four major blood parameters, platelet numbers, red blood cell number, hemoglobin content, and hematocrit, from 2.1 and 8.8 kb pUPII/hEPO transgenic mice were analyzed. Levels of hematocrit (Hct), red blood cell (RBC), hemoglobin (Hb), and platelet (PLT) count were significantly increased in 2.1 kb pUPII/hEPO transgenic mouse compared to control and 8.8 kb pUPII/hEPO mice. Different letters indicate significantly different levels.

Hoxd complex, series of contiguous genes are coordinately controlled by regulatory sequences located at remote distances. However, in different cellular contexts, Hox genes may have to be insulated from undesirable external regulatory influences to prevent ectopic gene activation, a situation that would likely be detrimental to developing embryos [Kmita et al., 2002]. Therefore, we hypothesized the potential presence of insulators in the 8.8–2.1 kb pUPII promoter, which could block ectopic promoter activation by endogenous DNA sequences at the site of integration.

The study of trans-acting factors within urothelium has been limited. In the present study, an extensive analysis of the pUPII promoter sequence, using the computer database TRANSFAC, version 3.2, revealed the presence of several putative consensus binding sites for various transcription factors likely to be relevant in UPII gene regulation. These DNA consensus sequences included AP, STAT3, HNF, Pax4, LYF1, and PPAR-defined PPRE domains. Among the putative response sequences found in the pUPII promoter region, PPAR-defined PPRE domains are of special interest because these factors are implicated in tissue-specific regulation of gene expression. We found multiple PPARγ-PPRE binding sites in the 8.8 kb pUPII promoter, whereas the 2.1 kb pUPII promoter contains only one PPRE binding site (Fig. 1). PPAR controls gene expression by heterodimerizing with their partner retinoid-X-receptor (RXR) [Kliewer et al., 1992] and binding to PPREs (consensus sequence—AGGTCA-N-AGGTCA) [Dreyer et al., 1992; Kliewer et al., 1992; Palmer et al., 1995]. PPAR-γ expression has been described in the presumptive urothelium of the mouse urogenital sinus and in the mature urothelium of mice, rabbits, and humans [Guan et al., 1997; Jain et al., 1998; Kawakami et al., 2002], and is associated with differentiation [Nakashiro et al., 2001; Kawakami et al., 2002].

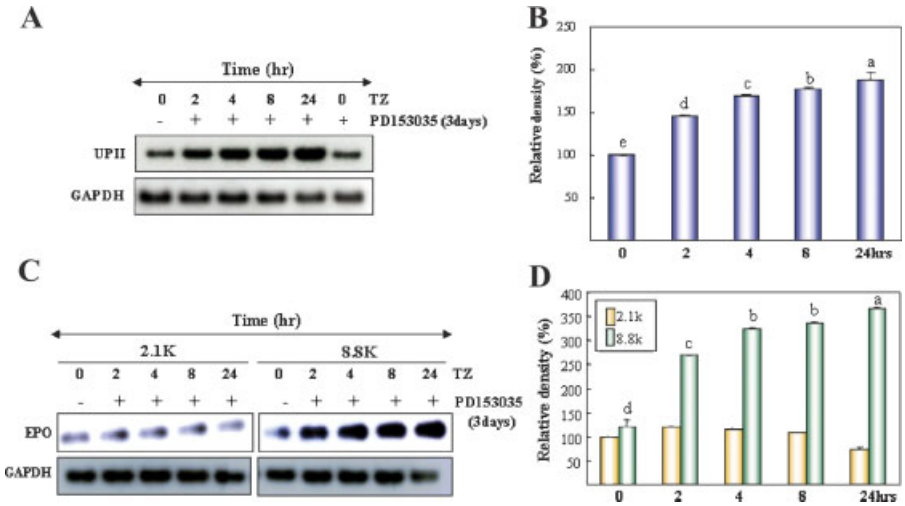

Fig. 7. Effect of hEPO regulation on PD153035 exposure after PPARγ-activation. **A**: Uroplakin mRNA expression in TZ exposed RT4 cells treated with PD153035 for 3 days. RT4 cells were pretreated with or without 1 μM TZ for the lengths of time indicated. After each time point, fresh medium was replaced to remove TZ, and then the cells were further maintained in the presence of 1 μM PD153035 until analysis at day 3. Total RNA was extracted and 20 μg were hybridized with ^{32}P-labeled UPII and GAPDH cDNA probes. **B**: Experimental data of (A) were quantified by relative density and presented as mean ± SD. Different letters indicate significantly different densities. Maximal UPII expression was induced when cells were treated with TZ for 24 h. **C**: Comparison of hEPO mRNA expression under the control of the 2.1 kb or 8.8 kb pUPII promoter gene. RT4 cells stably transfected with 2.1 or 8.8 kb pUPII/hEPO were pretreated with or without 1 μM TZ for the lengths of time indicated. After each time point, fresh medium was replaced to remove TZ, and then the cells were further maintained in the presence of 1 μM PD153035 until analysis at day 3. Total RNA was extracted and 20 μg were hybridized with ^{32}P-labeled EOP and GAPDH cDNA probes. **D**: Experimental data of (C) were quantified by relative density and presented as mean ± SD. Different letters indicate significantly different densities. Note that the 8.8 kb promoter upregulated hEPO mRNA expression, but the 2.1 kb promoter did not. Each experiment was performed at least three times and data subjected to statistical analysis. 2.1 k or 8.8 k indicates the 2.1 kb or 8.8 kb pUPII promoter. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Additionally, activation of PPAR-γ has been shown to suppress the growth of normal and malignant urothelial cells in vitro [Nakashiro et al., 2001]. Only recently, endogenous UPII upregulation by TZ treatment in the presence of EGF has been demonstrated in human NHU cells, but PPRE binding sites had not been identified in the promoter region of UPII [Varley et al., 2004].

The expression of the hEPO genes under the control of the 2.1 or 8.8 kb pUPII promoter in cultured RT4 cells was assessed by addition of the PPAR-γ agonist, TZ (Fig. 7). The expression of UPII mRNA was not induced by the PPAR-γ agonist TZ alone, whereas maximal expression of UPII mRNA was induced in the presence of the selective PPAR-γ antagonist PD153035 after exposure to TZ for 24 h. The effects of TZ and the selective PPAR-γ antagonist PD153035 on hEPO gene expression were mirrored by the induction of the endogenous UPII gene. Treatment of TZ alone in RT4 cells stably transfected with 8.8 kb pUPII/hEPO did not result in an increase in hEPO expression. Consistent with the presence of potential PPREs in the promoter region of the pUPII gene, we demonstrated that PD153035 treatment increases rosiglitazone-induced hEPO expression in RT4 cells stably trasfected with 8.8 kb pUPII/hEPO, but did not affect expression of the 2.1 kb pUPII/hEPO (data not shown). Although we did not perform EMSA or site-directed mutagenesis at this stage to directly validate PPAR-γ binding or functional activity of these predicted PPREs, Northern blot analysis with the 2.1 kb pUPII/hEPO and 8.8 kb pUPII/hEPO constructs suggested that the PPREs located between −8.8 and −2.1 kb are functional. This result provides additional evidence for the presence of PPAR-γ in these cells. In conclusion, our observations demonstrated that the PPAR-γ-mediated effect on UPII gene expression was maximally stimulated by inhibition of autocrine-activated EGF receptor signaling. Additional studies to identify multiple regulatory elements including cis-acting elements and insulator between −8.8 and −2.1 kb upstream of pUPII gene are

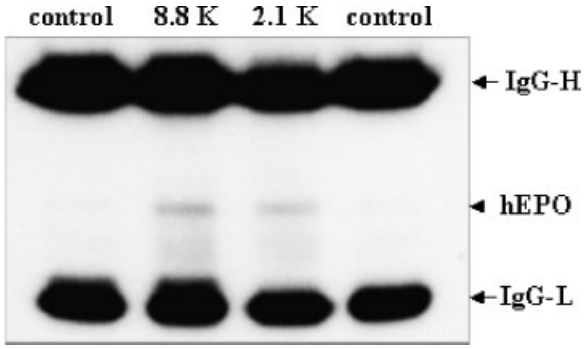

Fig. 8. Immunoprecipitation of hEPO in urine from transgenic mice. After immunoprecipitation, proteins were separated on a 12% SDS–PAGE gel under non-reducing conditions, transferred onto PVDF membrane, and probed with the appropriate antibodies (see Materials and Methods for details). Control; normal mouse urine; 8.8 K, hEPO in a line 23 (8.8 kb pUPII/hEPO) founder mouse urine; 2.1 K, hEPO in a line 61 (2.1 kb pUPII/hEPO) founder mouse urine.

required to understand the mechanisms involved in uroepithelium differentiation and bladder-specific gene expression.

The bladder is an attractive organ for the production of pharmaceutical proteins because urine is easily collected and contains low protein and fat, which simplifies protein purification. Even though previous studies [Lin et al., 1995; Ryoo et al., 2001] have attempted to induce secretion of a pharmaceutical protein into the urine of transgenic mice, only low concentrations of foreign protein were obtained when the 3.6 kb UPII promoter was used. Although the concentration of hEPO produced from the 8.8 kb promoter construct is higher ($1{,}032.78 \pm 65.68$ mIU/μl) than that produced from the 2.1 kb construct (290.82 ± 45.09 mIU/μl) in transgenic mice (Table II), the present observations suggest that it will be necessary to develop an induction system for upregulation of foreign proteins in bladder. To examine the side effects of hEPO expression in the transgenic mice, we analyzed four major blood parameters, platelets, red blood cell number, hemoglobin, and hematocrit, in blood samples from the 2.1 kb and 8.8 kb transgenic mice (Fig. 6). These components in 2.1 kb transgenic mice blood were significantly higher, than either the control or 8.8 kb transgenic mice. The highest increase in 2.1 kb transgenic mice was in platelet levels. The increased platelet concentration in 2.1 kb transgenic mice is inconsistent with a previous report showing that rhEPO does not alter platelet number in human patients [Beguin, 1999]. Early EPO research identified red blood cells as the only component stimulated by EPO, whereas Lappin [2003] reported that EPO has a broad spectrum of regulatory activity in modulating blood composition. However, the precise mechanism by which platelet levels rather than RBCs are increased in 2.1 kb transgenic mice remains to be determined.

TABLE II. Quantitative Analysis of hEPO in Transgenic Mouse Urine

Transgenic mice (line)	ELISA Kit (R&D) (mIU/μl)
8.8 kb (line 23)	$1{,}079.22 \pm 55.47$
8.8 kb (line 31)	986.33 ± 51.79
Mean ± SD	$1{,}032.78 \pm 65.68$
2.1 kb (line 61)	322.7 ± 10.34
2.1 kb (line 57)	258.93 ± 25.38
Mean ± SD	290.82 ± 45.09

In this study, we focused on a detailed analysis of the pUPII promoter, in order to identify putative elements regulating bladder-specific expression in vivo. We generated various fragments from the promoter region and constructed luciferase expression vectors containing up to 8.8 kb of the 5′ flanking region of the pUPII gene. Although our transgenic animals did not produce hEPO at the high levels needed for commercial production of hEPO, all the major cis- and trans-regulatory elements required for bladder- and urethra-specific transcription were located in the 8.8 kb upstream region. Therefore, our findings indicate that this promoter could be modified to enhance protein production of pharmaceutically relevant proteins that could then be purified from urine.

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