0026-895X/07/7204-826–837$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 72:826–837, 2007

Vol. 72, No. 4
34504/3251334
Printed in U.S.A.

Discovery of Osmosensitive Transcriptional Regulation of Human Cytochrome P450 3As by the Tonicity-Responsive Enhancer Binding Protein (Nuclear Factor of Activated T Cells 5)

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Received January 25, 2007; accepted June 28, 2007

ABSTRACT

We report the discovery of an osmosensitive transcriptional control of human CYP3A4, CYP3A7, and CYP3A5. Ambient hypertonicity (350–450 mOsmol/kg) increased mRNA expressions of the CYP3A by ~10- to 20-fold in human-intestinal C_2bbe1 cells, followed by an increase of CYP3A protein. Hypotonicity, on the other hand, suppressed CYP3A mRNA levels, indicating that physiological isotonic conditions may regulate the basal expression of CYP3A. Similar responses to ambient tonicity were observed in other human-derived cell lines (intestinal LS180 and hepatic HepG2) and human primary colonic cells. The 11-base pair tonicity-responsive enhancer (TonE) is an osmosensitive regulator that is activated by the transcription factor, the nuclear factor of activated T-cells 5 (NFAT5). Luciferase-based reporter assays of 13 consensus TonE motifs within ±10 kilobases (kb) from the transcription start sites of *CYP3A* showed that only the *CYP3A7* intron 2 region (~5 kb downstream from the transcription start site), which contains two TonE motifs (+5076/+5086 and + 5417/+5427), was responsive to hypertonicity stimuli. This observation was confirmed upon cotransfection with an NFAT5 expression vector, small interfering RNA, or dominant-negative NFAT5. Deletion and mutation analyses suggested that the TonE (+5417/+5427) is indispensable for the enhancer activity. NFAT5 binding to the *CYP3A7* intron 2 TonE motif was demonstrated with electrophoretic mobility shift assay and in a native cell context by chromatin immunoprecipitation. We conclude that transcription of human *CYP3A* is influenced by ambient tonicity. The physiological significance of the tonic regulation of CYP3A enzymes remains to be determined.

This work was supported by Canadian Institute for Health Research grant (MT13747, to S.I.); by University Grants Committee of the Hong Kong Special Administrative Region Areas of Excellence Scheme grant AoE/P-10/01, and by Research Grant Council grants HKU 7427/04M (to B.C.B.K); by the Fellowship award from the Japanese Society for Clinical Pharmacology and Therapeutics (to K.K.); by the Pfizer fellowship (to S.U.); by the Ontario Graduate Scholarship (to A.I.C.); and by the RSTRACOMP studentship, Research Institute, Hospital for Sick Children (to A.I.C. and K.P.T).

K.K. and A.I.C. contributed equally to this work.

This work was presented in part at the annual meetings of the Japanese Society for Clinical Pharmacology and Therapeutics (December 2005, Oita, Japan) and the American Society for Clinical Pharmacology and Therapeutics (March 2006, Baltimore, MD).

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.107.034504.

The human cytochrome P450 3A (CYP3A) subfamily represents the most abundant cytochrome P450 drug-metabolizing enzymes in the liver and intestine. Together with membrane-bound transporters, they constitute a crucial component for drug elimination and excretion. Of the three major isoforms (CYP3A4, CYP3A5, and CYP3A7), CYP3A4 is the most abundant adult form, whereas CYP3A7 is the main fetal form. These isozymes collectively metabolize nearly half of all currently used medications.

The human *CYP3A* genes reside in a cluster on chromosome 7 (Nelson et al., 2004), and their expression is characterized by wide interindividual variations. Significant coex-

ABBREVIATIONS: PXR, pregnane X receptor; CAR, constitutive androstane receptor; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NFAT5, the nuclear factor of activated T-cells 5; PCR, polymerase chain reaction; siRNA, small interfering RNA; SMIT, sodium/myoinositol cotransporter; SV, simian virus; TonE, tonicity-responsive enhancer; VDR, vitamin D receptor; bp, base pair(s); kb, kilobase(s); MEM, minimal essential medium; PBS, phosphate-buffered saline; dn, dominant negative.

pression of the major CYP3A isoforms suggests the presence of common regulatory pathways. CYP3A induction by drugs and chemicals is known to be mediated by pregnane X receptor (PXR) (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998), constitutive androstane receptor (CAR) (Xie et al., 2000), the vitamin D receptor (VDR) (Schmiedlin-Ren et al., 1997; Thummel et al., 2001), and hepatocyte nuclear factor-4-α (Tirona et al., 2003). In contrast, the regulation of CYP3A basal expression in the absence of an inducing agent is poorly defined, although these nuclear factors and other regulatory proteins, such as CCAAT/enhancer-binding protein and hepatic nuclear factor-3-γ (Rodriguez-Antona et al., 2003), may also play a role. As we were examining the dietary effects on human CYP3A expression, we made an unexpected observation that osmotic environments seem to influence the expression of CYP3A.

In mammalian cells, ambient tonicity affects the function of a transcription factor called tonicity-responsive enhancer binding protein, which is also known as the nuclear factor of activated T-cells 5 (NFAT5) or the osmotic response element-binding protein. TonE binding protein/NFAT5/osmotic response element-binding protein (NFAT5 hereafter in this article) is a newly discovered fifth member of the NFAT family of transcription factors (López-Rodríguez et al., 1999; Miyakawa et al., 1999; Ko et al., 2000; Stroud et al., 2002), which forms an obligatory homodimer and transactivates its target genes via the tonicity-responsive enhancer (TonE). NFAT5 is the only known mammalian transcription factor that responds to changes in osmolality, in which an increase in ambient tonicity provokes NFAT5 translocation from the cytoplasm to the nuclear compartment (Woo et al., 2000). NFAT5 controls the expression of osmotic stress-response genes such as the sodium/myoinositol cotransporter (SMIT: SLC5A3) (Yamauchi et al., 1993), and aldose reductase (Ko et al., 1997). These proteins synthesize organic osmolytes or transport them into the cell, thereby counterbalancing the ambient hypertonic stimuli (Ho, 2006). Constitutive nuclear localization of NFAT5 has been shown, suggesting a regulatory role under isotonic conditions (Miyakawa et al., 1999; Woo et al., 2000).

NFAT5-mediated gene regulation has been examined extensively in kidney, which faces intense osmotic stresses. Its role in other tissues, except for immune cells, is virtually unknown. Gastrointestinal epithelia are exposed to elevated postprandial osmolality (Ladas et al., 1983; Houpt, 1991; Kalantzi et al., 2006). Moreover, the osmotic microenvironment in the liver is also not static but rather is active and dynamic (Go et al., 2004). Thus the NFAT5 regulation may be present in these tissues. In this regard, it is intriguing that exposures to salt-rich diet in human subjects for several days have been associated with increased presystemic elimination of CYP3A substrates, such as quinidine (Darbar et al., 1997) and verapamil (Darbar et al., 1998).

In this study, we have characterized the tonicity-dependent expression of human CYP3A4, CYP3A7, and CYP3A5. In addition, a series of promoter-reporter gene assays were conducted to identify any active TonE in the *CYP3A* gene cluster. Our findings show that expressions of human CYP3A are highly dependent on ambient tonicity in various cell lines and primary cells. Furthermore, we found that of the multiple consensus TonE motifs within the $\pm$10 kb from transcription start sites of each major *CYP3A*, only the *CYP3A7* intron 2 region harbors an active osmosensitive TonE element, which is responsive to NFAT5.

Materials and Methods

Cell Culture. Cell lines were purchased from the American Type Culture Collection (Manassas, VA), and the human primary colonic cells were purchased from Celprogen (San Pedro, CA). Human C_2bbe1 and mouse CMT93 cells were grown in Dulbecco's minimal essential medium containing 1.5 g/l sodium bicarbonate, 10 mg/l human holo-transferrin, and 10% fetal bovine serum. Human LS180 and HepG2 cells and mouse Hepa-1c1c7 were grown in α-MEM containing 10% fetal bovine serum. Human normal colonic epithelia obtained from 4 white male subjects aged 35 to 55 years (Celprogen) were processed and propagated in the Human Colon Complete growth medium with 10% fetal bovine serum (Celprogen). The primary cultured cells were 95% positive for the epithelial marker, cytokeratin 19, and used for experiments at approximately 70% confluence.

Ambient Osmolality Modification. Ambient tonicity was increased up to 450 mOsmol/kg by adding either NaCl or sucrose to the regular culture media, and cells were incubated for 24 h unless otherwise stated. For example, the addition of 50 mM NaCl to the regular media (300 mOsmol/kg) increases the osmolality by 100 to 400 mOsmol/kg. To decrease ambient osmolality, cells were cultured in water-diluted hypotonic media [200 mOsmol/kg; 1:2 (v/v), water/media]. To account for the differences caused by nutrient dilution in the water-diluted hypotonic condition, PBS was used as a diluent [1:2 (v/v) PBS/media] as a second isotonic reference condition in some experiments. Glycerol was used as a tonicity-neutral control, because it readily crosses the plasma membranes without eliciting osmotic pressure.

Real-Time PCR. Total RNA was extracted using RNeasy Kit (QIAGEN, Valencia, CA). cDNA was generated using random hexamers and Moloney murine leukemia virus (Invitrogen, Carlsbad, CA). The ABI Prism 7700 Sequence Detection System was used for PCR using TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression probes (Applied Biosystems, Foster City, CA). The $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001) was used to calculate the amplification difference, with Ct value of target genes being normalized to respective GAPDH value. Measurements were done in triplicate and repeated at least three times. Predesigned primers and probe sets are as follows: human GAPDH (Hs99999905_m1), CYP3A7 (Hs00426361_m1), CYP3A4 (Hs00430021_m1), CYP3A5 (Hs00241417_m1), SMIT (Hs00272857_s), NFAT5 (Hs00232437_m1), PXR (Hs00243666_m1), CAR (Hs00231959_m1), and VDR (Hs00172113_m1); and mouse Nfat5 (Mm00467257_m1), Cyp3a11 (Mm00731567_m1), Cyp3a13 (Mm00484110_m1), Cyp3a16 (Mm00655824_m1), and GAPDH (Mm99999915_g1). Primers used in SYBR Green real-time PCR for drug transporter genes are as follows: MDR1 (forward, 5′-cag agg gga tgg tca gtg tt; reverse, 5′-cct gac tca cca cac caa tg); breast cancer resistance protein (forward, 5′-ccc gtt ctg agc ttt ttc ag; reverse, 5′-caa ggg taa ccg cag tca tt); MRP1 (forward, 5′-agg tgg acc tgt ttc gtg ac; reverse, 5′-tcc acc aga agg tga tcc tc); MRP2 (forward, 5′-tga aag gct aca agc gtc ct; reverse, 5′- tcc acc aga agg tga tcc tc); MRP3 (forward, 5′-aca tgc tgc ccc agt taa tc; reverse, 5′-cac act ctg ggg gtc aag tt); MRP4 (forward, 5′-tgt ttg atg cac acc agg at; reverse, 5′-gac aaa cat ggc aca gat gg); OCTN1 (forward, 5′-gac cga gtg gaa tct ggt gt; reverse, 5′-tct tcc tgc caa acc tgt ct); OCTN2 (forward, 5′-ctg gtg gtt cat ccc tga gt; reverse, 5′-agt gga agg cac aac aat cc); OCT1 (forward, 5′-cct gcc tcg tca tga ttt tt; reverse, 5′-acg aat gtg ggg tac agc tc); and GAPDH (forward, 5′-caa tga ccc ctt cat tga cc; reverse, 5′-gac aag ctt ccc gtt ctc ag).

Immunoblotting/Immunohistochemistry. C_2bbe1 cells were lysed in radioimmunoprecipitation assay buffer with protease inhibitor cocktail and centrifuged at 10,000 rpm at 4°C. The supernatants containing 50 μg of protein were used for blotting. Nuclear proteins were extracted with Nuclear Extraction Kit (Panomics, Fremont, CA). Immunoblotting was performed as described previously (Mun-

tane-Relat et al., 1995). The NFAT5 antibody (Affinity BioReagents, Golden, CO) was used at a 1000-fold dilution. The CYP3A4 antibody (Research Diagnostics, Flanders, NJ), which also recognizes CYP3A5 and CYP3A7, was used at a 500-fold dilution. β-Actin (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) was used to ensure equal loading. For immunohistochemical analysis, after 4-h exposure to hypertonicity (NaCl-induced 400 mOsmol/kg), C_2bbe1 cells on glass coverslips were fixed in 4% paraformaldehyde with 0.2% Triton X-100 in PBS and incubated with the above NFAT5 antibody at a 500-fold dilution in 5% donkey serum/PBS for 1 h at room temperature. Secondary antibody, Cy3-conjugated anti-rabbit IgG, was then used for 1 h at room temperature for visualization with fluorescent imaging microscopy.

Expression Plasmids, Small Interfering RNA, and Reporter Constructs. NFAT5 expression plasmid was made from KIAA0827 clone (a gift from Dr. Nagase, Kazusa Institute, Tokyo, Japan) by digestion with NotI and XhoI and ligated into pTARGET (Invitrogen). Human PXR expression plasmid (pEF-hPXR; Tirona et al., 2003) was kindly provided by Dr. Kim (University of Western Ontario, London, ON, Canada). Dominant-negative $NFAT5_{\Delta 1-156}$, which lacks the first 156 amino acids, was derived by in-frame insertion of KIAA0827 cDNA corresponding to amino acid residues 157 to 581 into NotI and BamHI restriction sites of pFLAG-CMV-2 mammalian expression vector (Sigma, St. Louis, MO) as reported previously (Tong et al., 2006). This region of NFAT5 was shown to function in a dominant-negative manner when expressed in transgenic mice (Lam et al., 2004; Wang et al., 2005). Small interfering RNA (siRNA) against NFAT5 was prepared as described by Na et al. (2003); we synthesized $siRNA_{569R}$ (Na et al., 2003) targeting exon 5 of NFAT5, which was reported to show specific silencing of NFAT5 mRNA and protein. The negative control siRNA was an inverted sequence of 569R (inv569R). In separate experiments, we used a mixture of four siRNAs against NFAT5 (Dharmacon SMARTpool; Thermo Fisher Scientific, Lafayette, CO) or control nontargeting mismatched siRNA.

CYP3A7$_{[-9302/+53]}$ and *CYP3A4*$_{[-10466/+53]}$ plasmids (Bertilsson et al., 2001) were kindly provided by Dr. Blomquist (Karolinska Institute, Stockholm, Sweden). The *CYP3A7* promoter fragment encompassing −370/+55 of the transcription start site was cloned by PCR from the *CYP3A7*$_{[-9302/+53]}$ plasmid using cloning primers (forward, 5′-tcc gct agc gca cac tcc agg cat agg taa-3′; reverse, 5′-cat gga tcc tgc tgc tgt ttg ctg ggc tgt-3′). Likewise, the 478-bp *CYP3A4* promoter plasmid from −424 to +54 of the transcription start site was generated from the *CYP3A4*$_{[-10,466/+53]}$ plasmid (forward, 5′-aca gct agc ctg ggt ttg gaa gga tgt gt-3′; reverse, 5′-cat gga tcc tgt tgc tct ttg ctg ggc tat gt-3′). These promoter fragments introduce an NheI and a BamHI restriction site at the 5′- and 3′-ends, respectively, and were inserted into the NheI and BglII sites of the pGL3-Basic luciferase reporter gene vector, thereby destroying the 3′-restriction site (Goodwin et al., 1999) for subsequent reporter constructs. The 737-bp *CYP3A5* promoter fragment (−688/+49 from the transcription start site) (Burk et al., 2004) was generated from C_2bbe1 genomic DNA using cloning primers (forward, 5′-aca gct agc aga tct atc acc aca gag tca gag ggg atg-3′; reverse, 5′-cat gga tcc gct gtt tgc tgg gct gtt tgc ctg g-3′), introducing an NheI–BglII tandem restriction site at the 5′-end and BamHI site at the 3′-end. This was similarly inserted into the NheI and BglII sites of pGL3-Basic vector. The BglII digestion sequence in the tandem restriction site was used for the *CYP3A5* promoter-driven constructs.

The fragments of *CYP3A7* intron 2 for the deletion/mutation assays were made by PCR from genomic DNA of C_2bbe1 cells using the following primers: backbone *CYP3A7*$_{[+4910/+5590]}$ (forward, 5′-tcg gta cca ggc aga atc aca tgc aaa a-3′; reverse, 5′-gaa gat ctt gag caa tct tac gac att cca-3′); *CYP3A7*$_{[+4910/+5204]}$ (forward, 5′-tcg gta cca ggc aga atc aca tgc aaa a-3′; reverse, 5′-gaa gat ctc aac aaa gcc ctc act tag ga-3′); *CYP3A7*$_{[+4910/+5453]}$ (forward, 5′-tcg gta cca ggc aga atc aca tgc aaa a-3′; reverse, 5′-gaa gat ctc tga caa tgg ata acc acc ttta act-3′); *CYP3A7*$_{[+4910/+5453]mutant}$ (forward, 5′-tcg gta cca ggc aga atc aca tgc aaa a-3′; reverse, 5′-gaa gat ctc tga caa tgg ata acc acc ttt aac tTt Tac ttt cca-3′), where *T* indicates mutations; and the backbone reverse *CYP3A7*$_{[+4910/+5590]}$ (reverse, 5′-gaa gat cta ggc aga atc aca tgc aaa a-3′/5′-tcg gta cct gag caa tct tac gac att cca-3′); and *CYP3A7*$_{[+5088/+5590]}$ (forward, 5′-tcg gta cca gct tat ttc cac agg gcc a-3′; reverse, 5′- gaa gat ctt gag caa tct tac gac att cca-3′). These fragments were inserted into KpnI and BglII sites of the *CYP3A* promoter-driven reporter plasmids (see above). The fragment of *CYP3A7*$_{[+4910/+5590]\ 3'\text{-position}}$ was made using primers (forward, 5′-ttc gga tcc agg cag aat cac atg caa aa-3′; reverse, 5′-ctc gtc gac tga gca atc tta cga cat tcc a-3′). They were inserted into the BamHI and SalI sites of the reporter. Other reporter constructs were similarly made and inserted into the appropriate restriction sites of luciferase reporters containing either the respective *CYP3A* or SV40 minimal promoter. All constructs were confirmed by sequencing (TCAG DNA sequencing facility, Hospital for Sick Children).

Transient Transfection and Luciferase-Based Reporter Assay. C_2bbe1 cells were used unless otherwise stated. Cells were seeded onto six-well plates at 0.5×10^6 cells/well. After 48 h, cells were transfected with 0.3 to 0.5 μg of the firefly luciferase reporter plasmids and 0.08 to 0.2 μg of pRL-TK plasmids (Promega, Madison, WI) containing a *Renilla reniformis* luciferase gene by Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen). In some experiments, cells were cotransfected similarly with NFAT5 expression vector, hPXR expression vector (pEF-hPXR; Tirona et al., 2003), siRNA against NFAT5 (569R and inv569R; Na et al., 2003), a combination of four gene-specific siRNAs (SMARTpool NFAT5; Dharmacon), the dominant-negative NFAT5, or empty expression plasmids. At 24 to 48 h after transfection, cells were incubated in various experimental conditions for another 16 to 24 h unless otherwise stated. SMARTpool NFAT5 siRNA experiments were conducted as follows. Overnight-seeded HepG2 cells at 50% confluence were transfected for 48 h with 32.5 nM siRNA against NFAT5 (siNFAT5) or equal molar mismatched siRNA controls. These siRNAs were earlier suspended in liposome carrier Dharmafect 1,2,3,4 transfection reagent (Dharmacon) at 0.1 μl/nM siRNA concentration in serum-free Opti-MEM (Invitrogen). Cells were then treated with different tonicity for 16 h.

Luciferase activities of the cell extracts were determined using the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was calculated as observed relative light units from firefly luciferase normalized to *R. reniformis* luciferase values and expressed as ratios to its minimal promoter construct under isotonic conditions, unless otherwise stated. In some experiments, the ratios were further normalized to those of the respective reporter in a control isotonic condition. All experiments were done in triplicate and repeated at least three times.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared from confluent C_2bbe1 cells treated for 4 h at NaCl-induced hyperosmolality (400 mOsmol/kg) using Nuclear Extraction Kit (Panomics) according to the manufacturer's instruction. Electrophoretic mobility shift assay (EMSA) was performed using the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL) with modifications. A 0.5-pmol sample of the 27-bp 5′-biotinylated probe (+5409/+5435 from CYP3A7 transcriptional start site) was used to detect protein/DNA interaction with 100-, 200-, or 400-fold increase of competitor probes (unbiotinylated) or mutant (tAAaGagA-aG, where capitalized letters represent base changes, and the dash represents a 1-bp deletion from the original "tggaaagttac"). NFAT5 antibody used for supershift was from Affinity BioReagents at a concentration of 2.5 μl per reaction. The binding reaction consists of 10 μg of nuclear extract, 1× binding buffer, 2 μg of poly (dIdC), 3 μg of random primers (Invitrogen), 5 mM $MgCl_2$, 0.05% Nonidet P-40, and 1 pmol of biotinylated probe, with or without the stated amount of competitor or mutant probes, or NFAT5 antibody, at a final volume of 10 μl. Incubation was carried out for 40 min at room temperature for all reactions. The 6% polyacrylamide gel electrophoresis was allowed to run for 2 h before transfer to the nylon membrane followed by UV cross-linking (Ultraviolet Crosslinker; UVP, Upland, CA).

Detection by chemiluminescence was carried out according to manufacturer's instructions.

Chromatin Immunoprecipitation. Chromatin immunoprecipitation (ChIP) assay was done using the ChIP kit (Upstate Biotechnology, Lake Placid, NY). In brief, C_2bbe1 cells were incubated under NaCl-induced hypertonic condition (400 mOsmol/kg) for 16 h, and proteins are cross-linked to DNA by 1% formaldehyde for 10 min at 37°C. The cells were then lysed in SDS and sonicated using a probe sonicator to obtain sheared DNA fragments ranging from 200 to 1000 bp. A 200-μl aliquot was taken for subsequent reverse-linking with 8 μl of 5 M NaCl, and the DNA phenol/chloroform was extracted and ethanol-precipitated. A 1% of fraction was used as the input control. Another aliquot of 200 μl was then diluted with the ChIP dilution buffer and incubated with salmon sperm DNA/protein A agarose beads for 1 h at 4°C to remove nonspecific DNA that initially binds to the beads. The supernatant was incubated with 2 μl (1:500) of rabbit polyclonal IgG NFAT5 antibody, CYP1A1 antibody (both from Santa Cruz Biotechnology) as irrelevant target antibody, or no antibody at 4°C overnight, and then fresh beads (60 μl) were added with agitation at 4°C for 1 h. The beads were washed twice according to the buffer systems supplied in the kit, and the protein/DNA complex is eluted with 250 μl of elution buffer (1% SDS and 0.1 M $NaHCO_3$), and only 200 μl of supernatant is collected after 30 min of shaking in room temperature. This step was repeated twice to obtain 400 μl of eluted samples. The samples were reverse-cross-linked with 20 μl of 5 M NaCl for >4 h at 65°C and treated with proteinase K. DNA of the samples was then phenol/chloroform-extracted and ethanol-precipitated using standard techniques. DNA was resuspended in 100 μl of diethyl pyrocarbonate water for subsequent PCR reaction. All PCR reactions were performed in a 50-μl reaction mix using Mastercycler (Eppendorf, Boulder, CO) with 1% template (1 μl). All PCR conditions were as follows: 95°C for 2 min; 95°C for 45 s, then 60°C for 1 min, and 72°C for 30 s, for 40 cycles; 72°C for 2 min, except for the NFAT5-coding region (annealing temperature at 55°C, 1 min). The *CYP3A7* intron 2 TonE regions were assessed as fragments A and B. Fragment A contains an antisense TonE motif +4688/+4698; and fragment B with a sense TonE +5417/+5427. The primers for fragment A (288 bp) are as follows: forward, 5′-gtc att tgc acc tgc ttg aa; and reverse, 5′-tgc atg tga ttc tgc ctt tg. Those for fragment B (271 bp) were as follows: forward, 5′-aac agg ctt tgt gtg agc aa; and reverse, 5′-atg act tgt tcc tgc cct gt. For positive control, we detected a 194-bp PCR product of the SMIT promoter, which contains an active TonE sequence at −21,622/−21,611 from the start site. This site was originally characterized as TonEp (Rim et al.,1998). The primers used for SMIT-TonEp site are the following: forward, 5′-cgc gaa ggt ccc tag ctc; reverse, 5′-gac cct gcc tgc ccc tac. NFAT5 coding region (exon 14: the third terminal exon lacking a TonE motif) was used as the negative PCR control (López-Rodríguez et al., 2001) with the following primers: forward, 5′ gtt gcc atg cag agt aac tct; and reverse, 5′ cat tgg att ttg att ggg ttg aat atc ctg for an 180-bp product.

Statistical Analysis. Results of mRNA levels are expressed as fold induction compared with isotonic conditions. Luciferase-based reporter activity is shown as ratios to appropriate controls as described above. Data are shown as mean ± S.E.M., unless otherwise stated. When appropriate, data were analyzed by the Student's *t* test or one-way analysis of variance followed by Dunnett's multiple comparison test.

Results

Increased Ambient Hypertonicity Caused a Substantial Increase of CYP3A Expression in the Human Intestinal C_2bbe1 Cells. In C_2bbe1 cells, a subclone of the Caco-2 human colon carcinoma cell line with relatively homogeneous brush-border epithelial characteristics, ambient hypertonicity (400 mOsmol/kg) increased CYP3A mRNA expression by >10-fold in 12 h (Fig. 1a) followed by an increase in protein levels (Fig. 1b). Note that C_2bbe1 cells have wild-type CYP3A7*1A/*1A and CYP3A5*3/*3 genotypes (C. Vyhlidal, personal communication). Relative baseline mRNA levels of each CYP3A in C_2bbe1 cells were roughly 1:3:0.5 (CYP3A4/CYP3A5/CYP3A7). The dose-response of tonicity-induced mRNA levels was also evident in hypertonic conditions created by adding NaCl or sucrose (Fig. 1c). In contrast, the tonicity-neutral membrane-permeable compound glycerol did not have such an effect, which was also reflected in protein expression (Fig. 1d). NFAT5 is accumulated in the nuclear compartment in 4-h posthypertonic treatment with NaCl or sucrose, showing clear demarcation of the nuclei, whereas cytoplasmic NFAT5 remains to be seen in cells with isotonic or glycerol treatment, obscuring cytoplasm-nuclear boundaries (Fig. 1e). In addition, hypertonicity did not significantly prolong CYP3A mRNA decay in the presence of actinomycin D (data not shown), suggesting that hypertonicity-triggered *CYP3A* gene expression cannot be explained by increased mRNA stability. Together with CYP3A, intestinal xenobiotics transporters such as P-glycoprotein (MDR1) and breast cancer resistance protein (ABCG2) constitute a functional unit of drug and toxin absorption barrier. However, in contrast to CYP3A and an osmosensitive gene, SMIT, hypertonicity caused only marginal changes, if any, in expression of these transporters (Fig. 1f).

Osmotic stress (i.e., hypertonicity) provokes the activation of NFAT5, which binds to its cognate response DNA element, TonE, thereby facilitating the transcription of target genes such as SMIT. To investigate NFAT5-dependence of tonicity-induced CYP3A expression, we examined tonicity responses of C_2bbe1 cells transfected with NFAT5 expression plasmids (Fig. 1g). CYP3A and SMIT (positive control) mRNA expressions were increased in parallel with increased NFAT5 levels at isotonic condition (CYP3A4: 2.54 ± 0.47, $p = 0.046$; CYP3A7: 2.32 ± 0.17, $p = 0.03$; CYP3A5: 2.08 ± 0.16, $p = 0.01$; SMIT: 1.68 ± 0.07, $P < 0.01$; fold-increase compared with empty vector transfection, mean ± S.E.M., $n = 4$). This trend was also observed in hypertonic conditions, but statistical significance was not reached because of large variations among cell preparations. On the other hand, hypertonicity/NFAT5-responsiveness in mRNA expression of the known CYP3A regulators, PXR, CAR, and VDR, was unremarkable (Fig. 1g).

To further characterize the involvement of NFAT5 in CYP3A expression, loss-of-function assays were conducted using two different siRNA approaches against NFAT5: a combination of four different siRNAs against NFAT5 (siNFAT5: see *Materials and Methods*), or $siRNA_{569R}$ (Na et al., 2003). Because conditions could not be sufficiently optimized for C_2bbe1 cells in these experiments, we used HepG2 cells. As shown in Fig. 1h, siRNA-mediated knockdown of NFAT5 with siNFAT5 caused significant reduction in CYP3A mRNA levels in both isotonic and NaCl-induced hypertonicity. NFAT5 knockdown using 569R NFAT5 siRNA (Na et al., 2003) showed a similar tendency under hypertonicity conditions (Fig. 1h, inset), although the magnitude of reduction was milder than siNFAT5. In these experiments, $siRNA_{569R}$ caused approximately 50% reduction of NFAT5 mRNA, and siNFAT5 caused ~80% reduction (data not shown). Taken together, these findings indicate that human CYP3A expression is influenced by a tonicity-driven mechanism, possibly involving NFAT5.

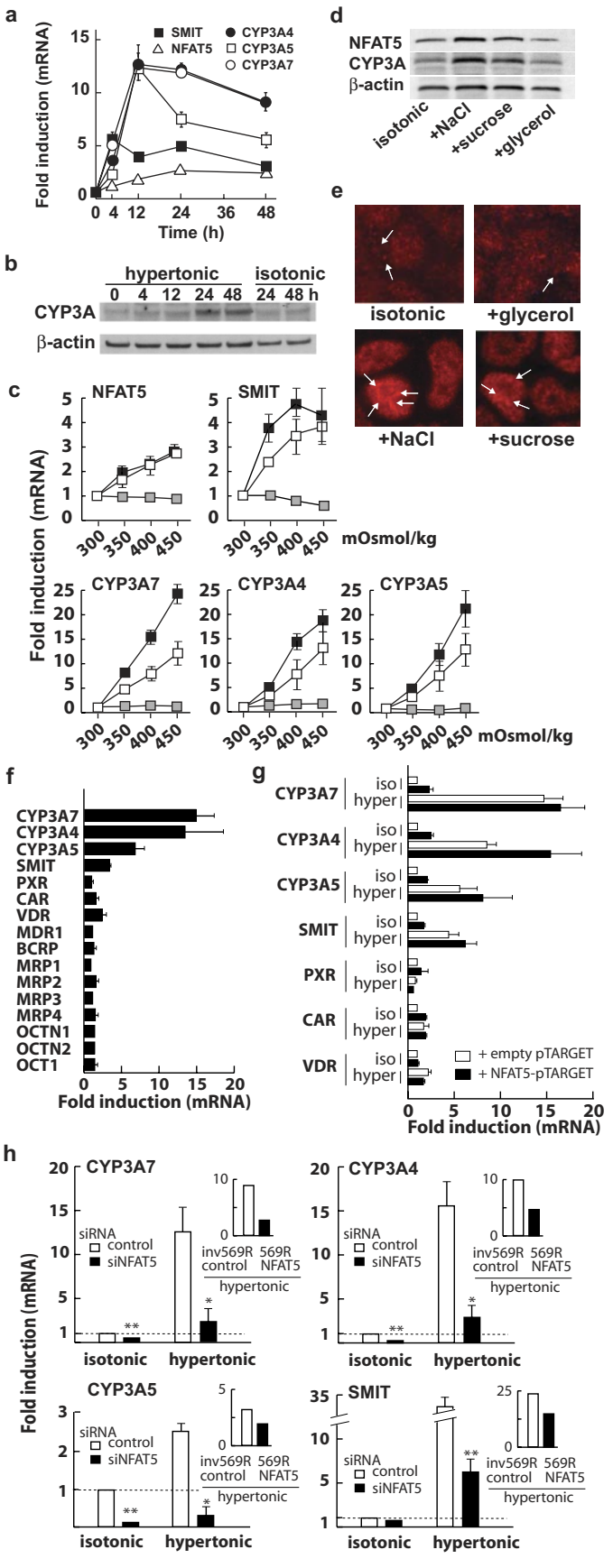

Fig. 1. Ambient hypertonicity induces *CYP3A* expression in C_2bbe1 human intestinal cells. a, time course of gene expression induced by ambient hypertonicity. C_2bbe1 cells were cultured under hypertonic conditions (400 mOsmol/kg), and mRNA levels of CYP3A4 (●), CYP3A7 (○), CYP3A5 (□), SMIT (■), and NFAT5 (△) were measured with real-time PCR. Results are normalized to respective GAPDH levels and expressed as ratios to the value at time 0 of each gene (mean ± S.E.M., $n = 3$). b, time-dependent expression of CYP3A protein in hypertonic (400 mOsmol/kg) or isotonic (300 mOsmol/kg) conditions. Cell lysates were obtained from C_2bbe1 cells incubated for the indicated periods, and immunoblotting was performed. c, dose-response of mRNA levels to increasing ambient osmolality. C_2bbe1 cells were treated for 24 h in media of increasing osmolality using NaCl (□), sucrose (■), or glycerol (▨), and mRNA levels were measured with real-time PCR. GAPDH-standardized results are expressed as ratios to those of respective isotonic condition (mean ± S.E.M., $n = 3$). d, solute-dependent increase of NFAT5 and CYP3A protein expressions in hyperosmotic conditions. Western blotting was performed on cell lysates obtained from C_2bbe1 cells after 24-h incubation under the above-mentioned isotonic or hyperosmolality conditions (400 mOsmol/kg) created with different solutes: NaCl, sucrose, or glycerol. e, immunohistochemical detection of NFAT5 intracellular distribution. After 4-h treatment with iso- or hyperosmolality medium (400 mOsmol/kg), C_2bbe1 cells were fixed, incubated with the NFAT5 antibody, and visualized with Cy3-conjugated anti-rabbit IgG. Arrows show NFAT5 staining, which is confined almost exclusively in the nuclear compartment under NaCl or sucrose treatment but is scattered in cytoplasm in isotonic or glycerol treatment. f, mRNA expression of transcription factors and intestinal xenobiotics transporters upon hypertonicity challenges. C_2bbe1 cells were incubated in hypertonic conditions for 24 h, and mRNA levels were quantified with real-time PCR. GAPDH-standardized results are expressed as ratios to those of respective isotonic condition (mean ± S.E.M., $n = 3$). g, NFAT5/tonicity-responsiveness of mRNA expressions of CYP3A, SMIT, PXR, and CAR. C_2bbe1 cells transfected with NFAT5-pTARGET or pTARGET empty vector were grown in regular isotonic or hypertonic (400 mOsmol/kg) medium for 24 h. mRNA was extracted and measured with real-time PCR. Results are normalized to individual GAPDH and expressed as ratios to those of isotonic conditions with pTARGET empty vector (mean ± S.E.M., $n = 4$). h, effects of NFAT5 knockdown with siRNA on mRNA expressions of CYP3A and SMIT in HepG2 cells. HepG2 cells were treated with siRNA NFAT5 (siNFAT5) or control (mismatched nontarget siRNA) for 48 h and incubated for another 16 h with isotonic or NaCl-induced hypertonic medium. Results are standardized to respective GAPDH levels, and expressed as ratios to the value of each gene in cells transfected with mismatched nontarget siRNAs under isotonic conditions (broken line) and shown as mean ± S.E.M., $n = 3$ (*, $p < 0.05$; **, $p < 0.01$). Inset, similar treatment with siRNA 569R against NFAT5 or control (inv569R), followed by hypertonic treatment for 18 h. Representative figures are shown.

CYP3A Expression Parallels Ambient Tonicity in Human-Derived Cells. NFAT5-mediated gene control is bidirectional (Woo et al., 2000). In other words, levels of target genes increase as ambient tonicity increases, whereas they decrease as ambient tonicity decreases. Changes of CYP3A expression in C_2bbe1 cells followed this pattern (Fig. 2, left), paralleling ambient tonicity changes from hypo- (200 mOsmol/kg) to hypertonicity (450 mOsmol/kg). So were other human cells [primary human colon epithelia, a colon carcinoma-derived cell line (LS180), and a hepatoma-derived cell line (HepG2)] (Fig. 2), suggesting that this is probably a universal phenomenon among human cells. Unlike human CYP3A, however, mouse Cyp3a13 mRNA levels changed inversely to ambient tonicity, whereas *Smit*, an established NFAT5 target gene, showed similar responses in human (Fig. 2) and mouse cells (intestinal CMT93 and hepatic Hepa1c1c7 cells; data not shown). It is unclear whether the tonicity effect on mouse Cyp3a13 represents a system-wide phenomenon in mice or a response specific to Cyp3a13, because other mouse Cyp3a mRNAs were undetectable or were not examined in these cell lines.

Tonicity-Responsive Enhancer (TonE) Is Located in the *CYP3A7* Intron 2 Region. We sought the *CYP3A* gene locus for existence of TonE, which is characterized by an 11-bp consensus sequence (tggaaaNNYNY; N, any nucleo-

tide; Y, any pyrimidine). Analyses of the genome database revealed that there are 85 consensus TonE motifs in the *CYP3A* gene cluster. In our experiments (Figs. 1 and 2), tonicity-dependence was observed in CYP3A4, CYP3A7, and CYP3A5; therefore, we postulated that a functional TonE sequence is located in a relative vicinity of the transcription start site of each *CYP3A* gene cassette. To identify a responsible TonE(s) in the 230-kb-wide *CYP3A* gene locus, our first approach was to screen ±10 kb of the transcription start site of *CYP3A4*, *CYP3A7*, and *CYP3A5*. As shown in Fig. 3a, 11 sense and 2 antisense TonE consensus sequences were located in these regions (*CYP3A4*: 3 sense and 1 antisense; *CYP3A5*: 4 sense; and *CYP3A7*: 4 sense and 1 antisense). Of those, 6 putative TonE consensus motifs were localized in the −10 kb of the 5′-flanking region of each gene; one motif for *CYP3A4* (−7913/−7903 from the *CYP3A4* transcription start site); two for *CYP3A7* (−7900/−7890 and −551/−541); and three for *CYP3A5* (−6341/−6331, −3051/−3041, and −1924/−1914). Some of their localizations were close to xenobiotic-responsive enhancer module and everted repeat 6, which contain known enhancers transactivated by PXR and CAR. However, luciferase reporter assays in C_2bbe1 cells showed that constructs containing these motifs (Fig. 3a; *CYP3A4,* −7979/−7140, −7979/−7831, −7850/−7140, and −638/+53; *CYP3A7,* −7994/−7155, −7994/−7831, −630/+55, and −7856/−7155; and *CYP3A5,* −6543/−5985, −3245/−2940, and −2235/−1862), driven by SV40 promoter or each *CYP3A* promoter, were unresponsive to hypertonicity (data not shown). To further explore these negative findings, we examined tonicity-responsiveness of the two reporter constructs, $CYP3A4_{[-10466/+53]}$ and $CYP3A7_{[-9302/+53]}$ (Bertilsson et al., 2001), which span approximately 9 kb of the 5′-flanking regions of *CYP3A4* or *CYP3A7*, respectively. As shown in Fig. 3b, these reporters were unresponsive to hypertonic stimuli

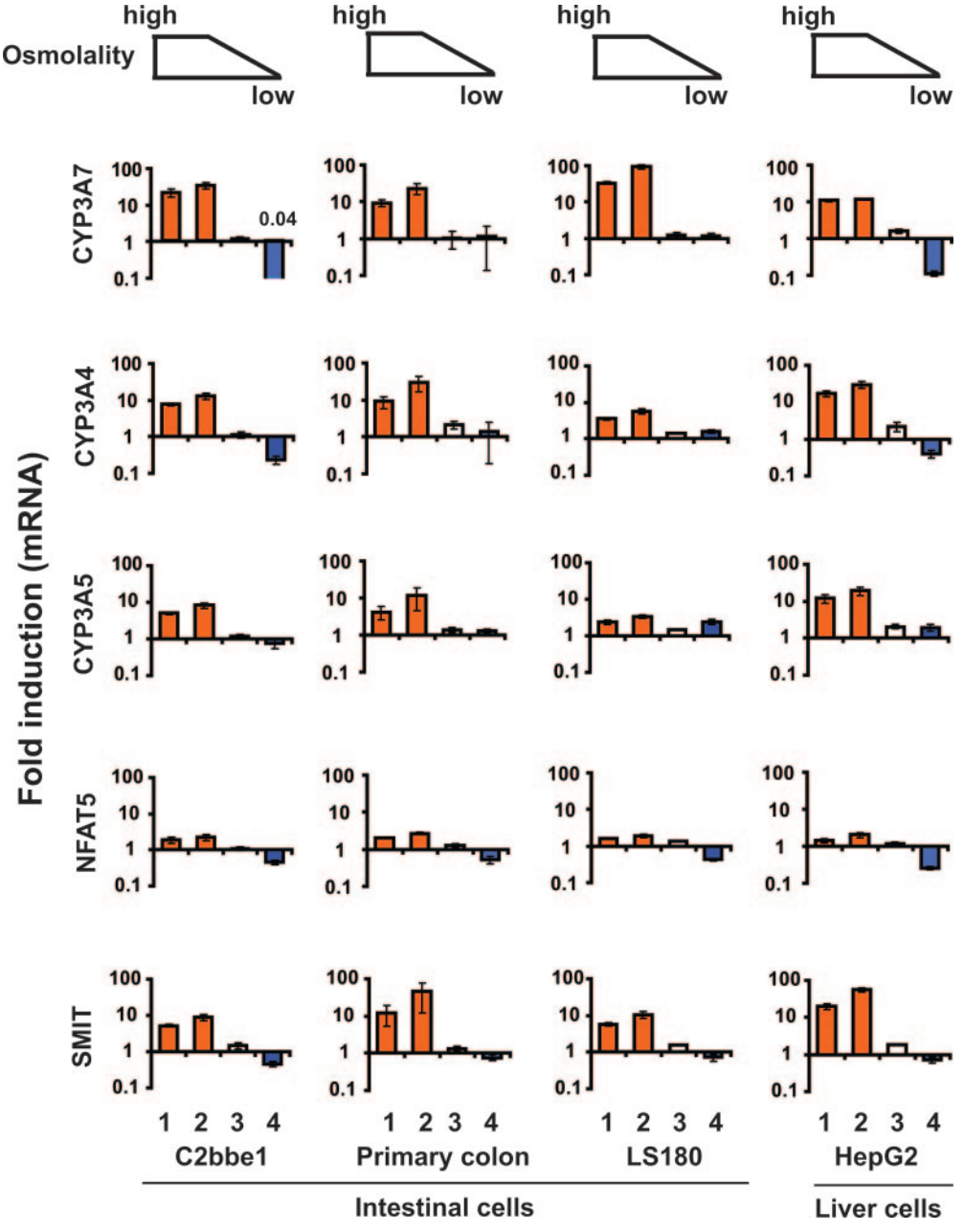

Fig. 2. Ambient tonicity elicits parallel changes in mRNA levels of CYP3A in human cell lines and primary human colonic cells. CYP3A mRNA levels in human-derived cell lines and primary colonic cells in response to ambient hyper- and hypotonicity. Cells were incubated for 24 h in media with osmolality ranging from high (400 mOsmol/kg) to low (200 mOsmol/kg) levels. mRNA was extracted and measured with real-time PCR. Red bar 1, +50 mM NaCl (400 mOsmol/kg); red bar 2, +100 mM sucrose (400 mOsmol/kg); open bar 3, medium/PBS [67%:33% (v/v), isotonic 300 mOsmol/kg]; and blue bar 4, medium/water [67%:33% (v/v), hypotonic 200 mOsmol/kg]. Results are standardized to respective GAPDH levels and expressed as ratios to the value of each gene in control isotonic condition (mean ± S.E.M., $n = 3$).

in hepatic HepG2 cells. In contrast, rifampicin-induced PXR-dependent responses were clearly observed as expected, indicating that the PXR-mediated pathway is intact in these constructs. These reporters transfected in C_2bbe1 cells were not responsive to either rifampicin ± PXR or hypertonicity treatment (data not shown). Taken together, these findings suggest that a tonicity responsive element does not exist at least in the −10-kb 5′-flanking regions of the *CYP3A* genes.

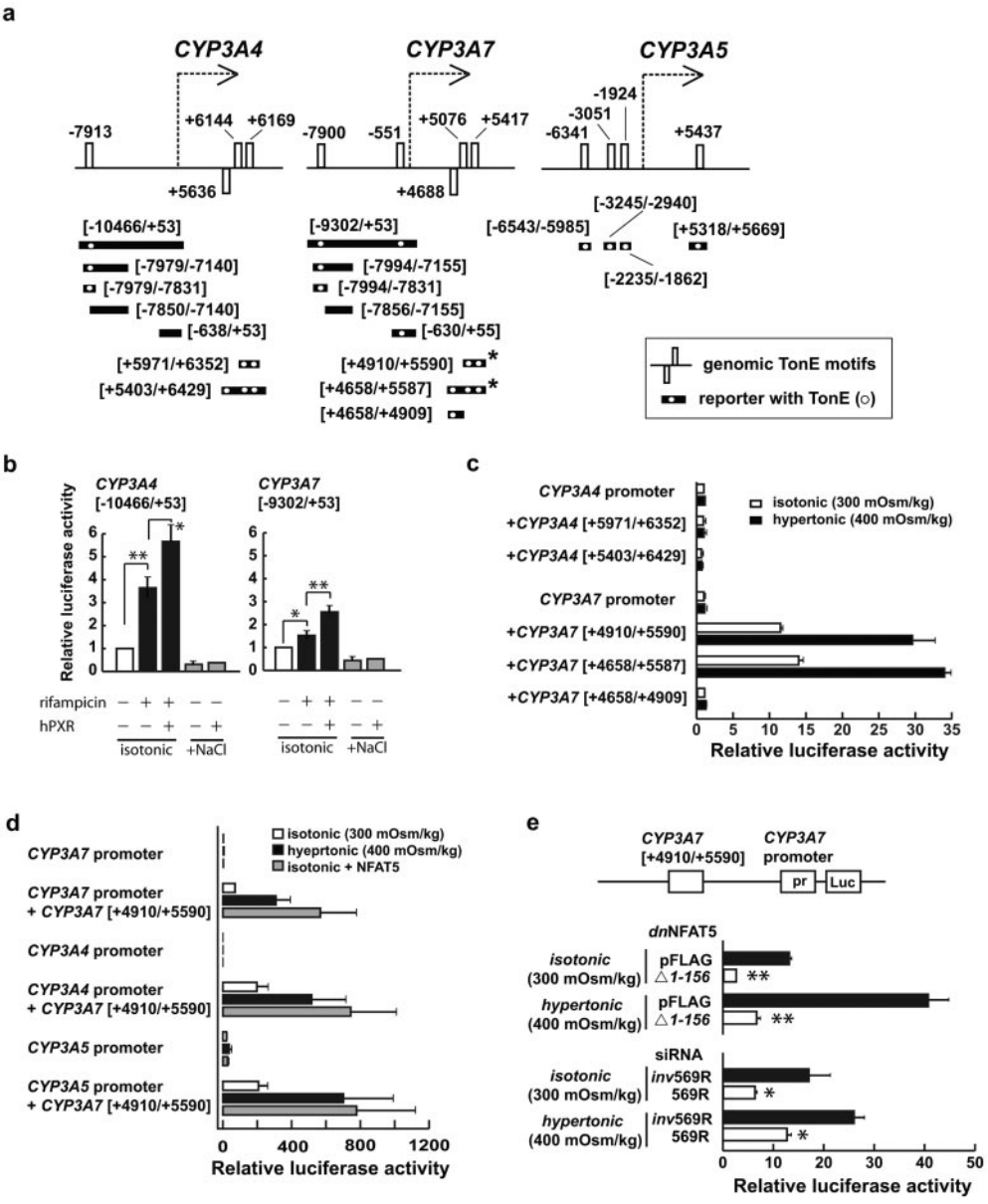

Fig. 3. TonE exists in *CYP3A7* intron 2. a, scheme of the *CYP3A* gene cluster and corresponding reporter constructs, which shows the ±10 kb of the CYP3A transcriptional start sites. Eleven sense and two antisense consensus TonE motifs in the regions are depicted as an open column on either the top (sense) or bottom (antisense) side of the genome (horizontal line) with the starting 5′ (sense TonE) and 3′ base pair positions (antisense TonE) relative to the transcription initiation site of each *CYP3A* gene. The reporter constructs (thick horizontal lines with or without TonE as a white dot) are shown with the 5′-/3′-ends of the sequence. The constructs with an asterisk (*) were responsive to hypertonicity and NFAT5. b, activity of the reporter constructs encompassing 9 to 10 kb of the 5′-flanking regions of *CYP3A4* or *CYP3A7*. Luciferase reporter assays were conducted in hepatic HepG2 cells with cotransfection of human PXR or empty expression vector after 24-h incubation in the presence of 10 μM rifampicin (■) or 50 mM NaCl (▨, hypertonic 400 mOsmol/kg). The normalized reporter responses are shown here as a ratio to the respective reporter activity value in control isotonic conditions (□). Results are expressed as mean ± S.E.M. (*, $p < 0.05$; **, $p < 0.01$; $n = 6$–9). c, reporter assays for constructs harboring regions within the transcription units of *CYP3A4* and *CYP3A7*. Activity of the reporter constructs was measured in C_2bbe1 cells after 24 h of control isotonic (□) or hypertonic conditions (■). Results are expressed as ratios to activity of the respective minimal promoters (mean ± S.E.M.: $n = 3$). d, activity of the reporter construct containing the *CYP3A7* intron 2 region (+4910/+5590) with different *CYP3A* promoters in hypertonic or NFAT5 overexpression conditions. C_2bbe1 cells were cotransfected with respective reporter constructs and either NFAT5 expression plasmids (▨) or empty vector (□ and ■). After 24-h incubation under isotonic (□ or ▨) or hypertonic (■) condition, luciferase activity was measured. In this experiment, results are expressed as values relative to those of the promoterless construct with empty expression plasmid under isotonic condition (mean ± S.E.M., $n = 3$). e, loss-of-function assays on the $CYP3A7_{[+4910/+5590]}$ reporter using dominant-negative (dnNFAT5, top) or siRNA (bottom) against NFAT5. C_2bbe1 cells were cotransfected with the reporter plasmid and either $dnNFAT5_{\Delta 1-156}$ (□) or pFLAG empty vector (■). On another experiments, $siRNA_{569R}$ (□) or an inverted 569R sequence ($siRNA_{inv569R}$, ■) was used. Cells were treated with isotonic or hypertonic medium for 24 h. Results are normalized against the values of the *CYP3A7* minimal promoter reporter under isotonic conditions (means ± S.E.M.; *, $p < 0.05$; **, $p < 0.01$, compared with respective controls; $n = 4$).

We then examined the remaining 7 TonE consensus motifs within 10 kb downstream from the transcription start site of each of those *CYP3A* genes (Fig. 3a): two sense TonE sequences in the *CYP3A4* exon 3/intron 3 (+6144/+6154 and +6169/+6179 from the *CYP3A4* transcription start site); one antisense sequence in the *CYP3A4* intron 2 (+5636/+5646); two sense sequences in the *CYP3A7* intron 2 (+5076/+5086 and + 5417/+5427 from the *CYP3A7* transcription start site); one antisense sequence in the *CYP3A7* intron 2 (+4688/+4788); and one sense sequence in *CYP3A5* exon 3 (+5437/+5447 from the *CYP3A5* transcription start site). Screening with SV40 promoter-driven luciferase reporters in C_2bbe1 cells (Fig. 3a; $CYP3A4_{[+5971/+6352]}$, $CYP3A7_{[+4910/+5590]}$, and $CYP3A5_{[+5318/+5669]}$) showed that only $CYP3A7_{[+4910/+5590]}$ with the *CYP3A7* intron 2 sense TonE sequences resulted in robust responses under isotonic conditions and more so after hypertonicity exposures (data not shown). We then further examined comparable regions of *CYP3A4* and *CYP3A7* approximately 5 to 6 kb downstream of the transcription start sites (Fig. 3a). The $CYP3A4_{[+5971/+6352]}$ reporter driven by the *CYP3A4* promoter, which contains two TonEs at +6144 and +6169, was not responsive (Fig. 3c). Moreover, the $CYP3A4_{[+5403/+6429]}$ construct containing the three TonEs (+5636, +6144, and +6169) did not show activity (Fig. 3c; $+CYP3A4_{[+5403/+6429]}$). In contrast, the $CYP3A7_{[+4910/+5910]}$ with the two sense intronic TonEs (*CYP3A7* +5076 and +5417; see Fig. 3a), driven by the *CYP3A7* promoter, was active under isotonic condition and responded to hypertonicity. However, inclusion of the antisense TonE (*CYP3A7* +4688) did not modify tonicity-responsiveness (Fig. 3c; $+CYP3A7_{[+4658/+5587]}$), and the reporter containing the antisense TonE (*CYP3A7* +4688) alone was inactive (Fig. 3c; $+CYP3A7_{[+4658/+4909]}$). These findings indicate that the *CYP3A7* intron 2 region containing the two sense TonE motifs (+5076/+5086 and +5417/+5427) has a tonicity-responsive enhancer activity.

Transactivation of *CYP3A7* Intron 2 TonE Is Dependent on NFAT5. The *CYP3A7* intron 2 region (+4910/+5590) was placed in each of the *CYP3A* promoters in luciferase constructs and tested for its transactivation activity with and without NFAT5 expression vector cotransfection (Fig. 3d). Results showed that this region is capable of activating all *CYP3A* promoter constructs in response to hypertonicity or NFAT5 overexpression. Loss-of-function assays were also conducted using the $CYP3A7_{[+4910/5590]}$ luciferase reporter cotransfected with dominant-negative NFAT5 (dnNFAT5$_{\Delta 1-156}$; Tong et al., 2006) or empty pFLAG-CMV-2 expression vector (pFLAG). As shown in Fig. 3e, dnNFAT5$_{\Delta 1-156}$ decreased reporter activity in both isotonic and hypertonic conditions ($p < 0.01$; $n = 4$). Likewise, reporter activity was examined with siRNA$_{569R}$ against NFAT5 (Na et al., 2003). siRNA$_{569R}$ significantly reduced reporter activity ($p < 0.05$, $n = 4$; Fig. 3e). In these experiments, siRNA$_{569R}$ caused approximately 50% reduction of NFAT5 mRNA (data not shown). Together, these findings suggest that *CYP3A7* intron 2 region may work in conjunction with other CYP3A promoters and that NFAT5 is required for enhancer activation.

The TonE Motif at +5417 in the *CYP3A7* Intron 2 Region Is Required for Transactivation. Serial deletions of the *CYP3A7* intron 2 fragment were conducted to determine the minimal sequence responsible for enhancer activity (Fig. 4a). Compared with the $CYP3A7_{[+4910/+5590]}$ reporter plasmid with two TonE sense motifs, serial upstream deletions showed a gradual reduction in reporter signals. Constructs without the upstream TonE motif (constructs +5088/+5590 and +5361/+5590) still retained the reporter activity, suggesting that the upstream TonE motif and neighboring regions are dispensable but required for a full response. We found that the reporter +5361/+5590 was the shortest construct that responds to hypertonicity or NFAT5. Further deletion (construct +5428/+5590), which is devoid of both TonE motifs, abolished the response. This observation supports that the downstream motif (+5417/+5427) is required in the NFAT5-mediated transactivation. This notion is further supported by the +4910/+5204 construct, in which the upstream motif without the downstream segment was unresponsive. This indicates that the upstream TonE motif and immediate neighboring regions are insufficient for enhancer activity. Furthermore, mutations in the +5417/+5427 TonE motif (tggaaagt*A*a*A*; the 2 "*A*"s in the mutant represent adenine replacing thymine and cytosine, respectively) drastically reduced tonicity responsiveness (Fig. 4b), reaffirming the requirement of the downstream motif for robust enhancer activity. General enhancers have the ability to transactivate promoter constructs independent of their location. To test whether the *CYP3A7* intron 2 region has such an enhancer characteristics, it was placed downstream of the reporter gene (+4910/+5590 3′-position). Note that the $CYP3A7_{[+4910/+5490]\ 3'\text{position}}$ reporter construct may mimic actual *CYP3A7* promoter–intron 2 DNA configuration to some extent. Results show that this reporter plasmid still retained tonicity/NFAT5 responsiveness, although the magnitude of the response was smaller (Fig. 4b).

NFAT5 Specifically Binds to the *CYP3A7* Intron 2 TonE at +5417. EMSAs showed in Fig. 5 that competition by unlabeled TonE DNA sequence (lanes 2 and 3), lack of effects of mutant TonE (lanes 4 and 5), and shift of NFAT5-TonE complex by NFAT5 antibody (lane 6) indicate specific binding of NFAT5 to the DNA sequence containing the +5417 TonE motif. The mutant TonE sequence shown to have low but detectable activity in the earlier reporter assay (Fig. 4b) was weakly competitive in inhibiting NFAT5-TonE binding in EMSA (data not shown). To further explore NFAT5 binding to the *CYP3A7* intron 2 TonE site at +5417 in a native chromatin context, ChIP assay was performed (Fig. 6). As shown in Fig. 6a, Fragment B represents a DNA region surrounding the active TonE (+5417), whereas fragment A contains a region with an antisense TonE further upstream (+4688) that is inactive in luciferase reporters (Fig. 3C; $CYP3A7_{[+4658/+4909]}$). In our ChIP assay, fragment B containing the TonE at +5417 is clearly detectable and so is the TonE motif located upstream of the *SMIT* promoter (Rim et al., 1998), one of the NFAT5 target genes (Fig. 6b, lane 1). Neither the TonE-absent exon 14 region of *NFAT5* gene (Fig. 6b, lane 4) nor antibody against CYP1A1 (Fig. 6b, lane 2 and 5) produced an amplicon, validating the specificity of NFAT5-TonE binding in our assay. Moreover, fragment A containing the neighboring inactive TonE motif is undetectable (Fig. 6c). Taken together, this is consistent with the notion that NFAT5 binds to the *CYP3A7* intron 2 region at +5417 in a native DNA setting.

Discussion

We have discovered that the expressions of human CYP3A4, CYP3A5, and CYP3A7 are under the influence of ambient tonicity. Moreover, the phenomenon is not restricted to immortalized cell lines, because it is also seen in primary colonic cells (Fig. 2), suggesting that this unexpected link between ambient osmotic environment and human CYP3A expression may represent a process of physiological significance. Although highly speculative, CYP3A may mediate osmolyte production or catabolism of osmolytes to counterbalance increased intracellular concentrations of organic osmolytes upon ambient hypertonicity challenges. Further studies are required to elucidate biological and in vivo significance of this phenomenon. This is important particularly because basal CYP3A expressions in these cells in vitro, including primary cells, are lower than those in vivo.

Our data suggest that the increased CYP3A expression is the result of transcriptional activation mediated by NFAT5. Several transcriptional factors, such as PXR and CAR, have been well established in the *CYP3A* regulation network. It remains to be defined whether the tonicity-mediated CYP3A expression is modified by other transcription factors. Specifically, it awaits further studies to elucidate a hierarchy of

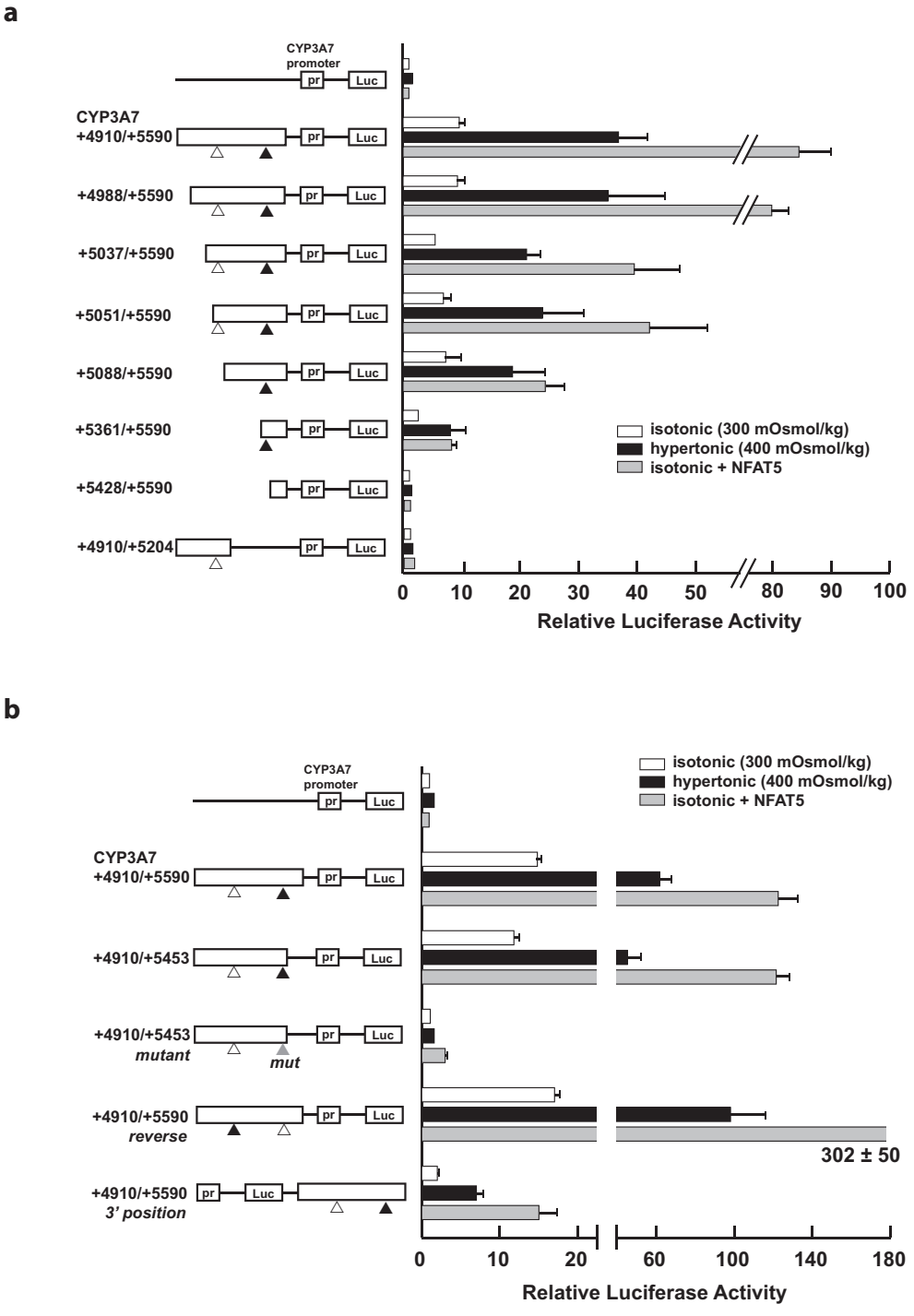

Fig. 4. The *CYP3A7* intron 2 TonE (+5417) is indispensable for the enhancer activity. a, deletion analyses of the *CYP3A7* intron 2 region using the reporter $CYP3A7_{[+4910/+5910]}$, which harbors an upstream (+5076, △) and downstream (+5417, ▲) TonE site. C_2bbe1 cells cotransfected with the reporter constructs and either NFAT5 expression vector or empty plasmid were treated with isotonic or hypertonic conditions for 24 h. The 5′-/3′-ends of the construct sequences are shown on the left as base positions from the *CYP3A7* transcription start site. Luciferase activities are expressed as ratios to those of the minimal promoter in isotonic conditions (means ± S.E.M., $n = 3$). b, mutation and positioning analyses of the *CYP3A7*intron 2 region. The mutant downstream TonE (shaded triangle labeled "*mut*"), the reversed configuration, and the 3′-positioned constructs were similarly tested in C_2bbe1 cells. Results were normalized to that of *CYP3A7* minimal promoter under isotonic conditions (means ± S.E.M., $n = 3$).

these factors in regulation of CYP3A, which determines system characteristics such as additivity, synergism, or antagonism among them. As shown in Fig. 1e, ambient hypertonicity may cause mild alterations in mRNA expression for some of these transcription factors, which may contribute to tonicity-triggered CYP3A induction. However, relatively rapid induction of CYP3A mRNA within 4 h by ambient hypertonicity (Fig. 1a) suggests a direct tonicity-mediated transcriptional induction rather through a secondary mechanism, if any.

Compared with human CYP3A, responses of mouse Cyp3a13 were exactly opposite in the two cell lines tested (CMT93 rectal cells and hepe1c1c7 liver cells), although mouse *Smit*, a known NFAT5 target gene, responded in the same manner as human SMIT. Tonicity responses of other mouse Cyp3a remain elusive, because only Cyp3a13 was detectable in this study. No orthologous *CYP3A* pair exists between mouse and human, suggesting a species-specific independent expansion of the ancestral *CYP3A* gene cassette over the last 75 million years (Nelson et al., 2004). Rodent and human CYP3A share many regulatory factors such as the PXR and CAR, but distinct species differences still exist. Whether mouse Cyp3a in vivo similarly responds to tonicity remains to be examined.

Mammalian cellular responses to osmotic stresses are mediated by the osmoregulatory transcription factor called tonicity enhancer binding protein (NFAT5) (reviewed by Ho, 2006). Upon activation by increased osmotic pressure, NFAT5 is translocated to the nucleus and binds to the TonE of target genes. Our NFAT5 siRNA experiments (Fig. 1h) suggest that NFAT5 plays a central role in the tonicity-CYP3A pathway. There are 13 consensus TonE motifs within the ±10 kb from the transcription start sites of *CYP3A* (Fig. 3a). The present study shows that among these DNA elements, only the 0.7-kb region within *CYP3A7* intron 2 has robust enhancer activity, responsive to hypertonicity and NFAT5 overexpression or knockdown (Fig. 3, b–e). Deletion

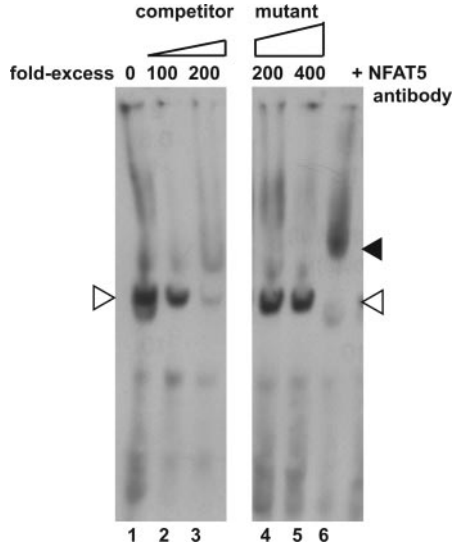

Fig. 5. NFAT5 binds to the *CYP3A7* intron 2 TonE motif (+5417/+5427) in in vitro binding assay. Nuclear extracts from C_2bbe1 cells treated with hypertonic medium for 4 h were incubated with biotinylated DNA probes derived from the *CYP3A7* intron 2 TonE motif (+5417) containing the surrounding region (+5409/+5435). EMSA was conducted with increasing concentrations of unlabeled competitor DNA or mutant DNA probes (see *Materials and Methods*). TonE probe signals in the absence of competing or mutant DNA sequences (lane 1) and those in the presence of increasing concentrations of the competing unlabeled TonE region (lanes 2 and 3) or mutant DNA sequence (lanes 4 and 5) are shown (△). A supershift band driven by NFAT5 antibody is demonstrated (▲, lane 6). A representative figure is shown.

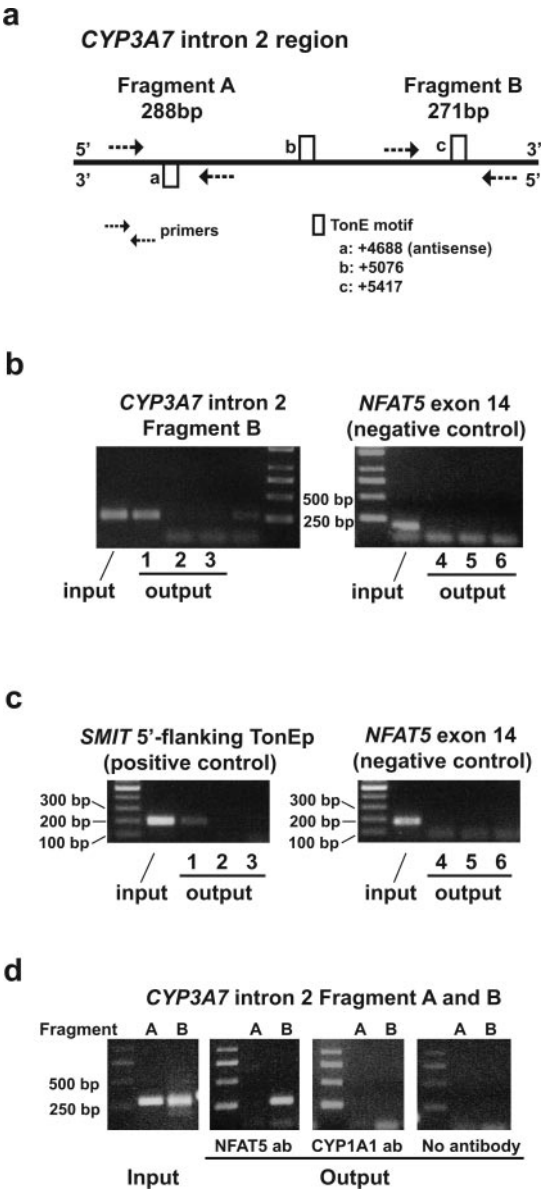

Fig. 6. ChIP assay for NFAT5 binding to the *CYP3A7* intron 2 TonE motif (+5417) in native cell context. a, scheme of the *CYP3A7* intron 2 region shown with TonE motifs. Fragments A and B were amplified in ChIP assay. Fragment A contains an inactive antisense TonE at +4688, and fragment B holds the functional TonE at +5417. b, NFAT5 binding to fragment B containing the TonE motif at +5417. C_2bbe1 cells exposed to NaCl-induced hypertonicity (400 mOsmol/kg) for 16 h were subjected to ChIP analyses using NFAT5 antibody to immunoprecipitate and amplify 271-bp fragment B (see *Materials and Methods*). Output lanes: 1 and 4, NFAT5 antibody; 2 and 5, CYP1A1 antibody; and 3 and 6, no antibody. A 180-bp region within exon 14 of NFAT5 was used as a PCR negative control. c, NFAT5 binding to a TonE site of SMIT, one of the NFAT5 target genes. C_2bbe1 cells were similarly treated, and a 194-bp sequence was amplified after immunoprecipitation using NFAT5 antibody, CYP1A1 antibody, or no antibody. Lane designations are the same as above. d, binding of NFAT5 is specific to TonE motif at +5417 within fragment B. C_2bbe1 cells were treated similarly, and NFAT5-DNA complex was immunoprecipitated using NFAT5 antibody, negative control antibody (CYP1A1 ab), or no antibody. Fragment A with inactive TonE and fragment B with functional TonE were subjected to PCR amplification. a to c, representative results of two to four experiments are shown.

and mutation analyses showed that the *CYP3A7* intron 2 TonE at +5417 is indispensable for the minimal enhancer activity, although a full response requires the neighboring region (Fig. 4, a and b). Using EMSA and ChIP assays, we further showed NFAT5-binding to this TonE motif (Fig. 5 and 6). Altogether, our findings indicate specific binding of NFAT5 to the region surrounding *CYP3A7* intron 2 TonE at +5417 in a native cell context.

The *CYP3A7* intron 2 region containing the responsive TonE site (+5417) placed immediate 3′-side of the luciferase reporter gene ($CYP3A7_{[+4910/+5590]\ 3'position}$) to mimic the natural genomic configuration is responsive to hypertonicity and NFAT5 overexpression (Fig. 4b). An enhancer located in the intron 3 of the human tumor necrosis factor-α gene, which interacts with the human tumor necrosis factor-α promoter, has been characterized in a similar experimental approach (Barthel and Goldfeld, 2003). The magnitude of response of the $CYP3A7_{[+4910/+5590]\ 3'position}$, however, was lower than when placed at 5′ of the *CYP3A7* promoter (Fig. 4b). It is presently unknown whether this quantitative difference is of any biological significance in native DNA context or is simply an experimental limitation in an artificial environment of gene reporter assays.

Our studies show that expressions of CYP3A4, CYP3A5, and CYP3A7 are all dependent on ambient tonicity and that the *CYP3A7* intron 2 region houses the only TonE consensus motif within the ±10 kb from the transcription start sites of *CYP3A*. Based on these findings, we speculate that the *CYP3A7* intronic TonE segment serves as an enhancer for *CYP3A7* and possibly as a long-range enhancer for *CYP3A4* and *CYP3A5,* as seen in some genes including β-globin. Alternatively, there may be unidentified TonEs, or similar enhancers, separately for each CYP3A gene. Involvement of other transcriptional factors in this phenomenon is largely speculative at this point. We think that at least PXR is not involved because overexpression of PXR does not increase tonicity responses of CYP3A mRNA (data not shown) and because xenobiotic-responsive enhancer module-containing 5′-flanking regions of CYP3A4 and CYP3A7 do not respond to tonicity (Fig. 3b). If PXR, CAR, or VDR is involved, lack of tonicity responses in genes such as MDR1 is also difficult to explain. Further studies are required to address these questions.

The implications of our findings are severalfold. First, because NFAT5 shows basal activity under physiological osmolality (Miyakawa et al., 1999; Woo et al., 2000), normal osmotic environment may be one of the baseline stimuli for CYP3A expression. This implies that the tightly regulated constitutive osmotic environment provides consistency and stability to the basal transcriptional drive, thereby sustaining CYP3A basal expression. Second, intestinal CYP3A expression may be affected through this pathway by osmolality/tonicity changes within the intestinal lumen as a result of food or fluid intake. Intestinal lumen faces periodic surges of osmolality related to food intake (Ladas et al., 1983; Houpt, 1991; Kalantzi et al., 2006). Therefore, CYP3A mRNA expression in the intestine may increase after food intake, which then elevates CYP3A protein levels. This may explain clinical observations in which human subjects receiving high-salt diets for 7 to 10 days showed significant increase in presystemic elimination and metabolite formation of orally administered CYP3A substrates quinidine and verapamil (Darbar et al., 1997, 1998). Third, the mechanism of urine volume control by NFAT5, which is exemplified in dehydration (Lam et al., 2004), may be supported by an increased CYP3A5 level because of its role in renal Na^+ and water retention (Kuehl et al., 2001; Givens et al., 2003; Thompson et al., 2004). Given the potential role of kidney-predominant CYP3A5 in salt retention and hypertension by converting corticoids to 6β-hydroxysteroids with mineralocorticoid action, this pathway is likely to be part of a regulation loop for salt homeostasis. The renal NFAT5-CYP3A5 pathway may be crucial to the hypertension pathogenesis theory associated with genetic variation in renal CYP3A5 expression (Kuehl et al., 2001).

In summary, we discovered the ambient tonicity-driven, NFAT5-mediated expression of human CYP3A4, CYP3A5, and CYP3A7. Whether the CYP3A7 intron 2 TonEs are responsible for propagation of the tonicity-NFAT5 signal toward all three isoforms of CYP3A awaits further studies.

Acknowledgments

We thank Dr. Blomquist for providing us with the CYP3A4 and CYP3A7 promoter constructs. We are also grateful to Dr. R. Kim for the human PXR expression plasmid (pEF-hPXR) and Dr. C. Vyhlidal for information of CYP3A genotypes of the cell lines used.

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