The Fetoprotein Transcription Factor (FTF) Gene Is Essential to Embryogenesis and Cholesterol Homeostasis and Is Regulated by a DR4 Element*

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The fetoprotein transcription factor (FTF) gene was inactivated in the mouse, with a lacZ gene inserted in-frame into exon 4. LacZ staining of FTF+/− embryos shows that the mFTF gene is activated at initial stages of zygotic transcription. FTF gene activity is ubiquitous at the morula and blastocyst stages and then follows expression patterns indicative of multiple FTF functions in fetal development. FTF+/− embryos die at E6.5–7.5, with features typical of visceral endoderm dysfunction. Adult FTF+/− mice are hypocholesterolemic, and expression of liver FTF at about 40% of the normal level. Overexpression of liver FTF in transgenic mice indicates in vivo that FTF is an activator of CYP7A1. However, CYP7A1 expression is increased in FTF+/−/− liver. Gene expression profiles indicate that higher CYP7A1 expression is caused by attenuated liver cell stress signaling. Diet experiments support a model where FTF is quenched both by activated c-Jun, and by SHP as a stronger feedback mechanism to repress CYP7A1. A DR4 element is conserved in the FTF gene promoter and activated by LXR-RXR and TR-RXR, qualifying the FTF gene as a direct metabolic sensor. Liver FTF increases in rats treated with thyroid hormone or a high cholesterol diet. The FTF DR4 element tightens functional links between FTF and LXRα in cholesterol homeostasis and can explain transient surges of FTF gene activities during development and FTF levels lower than predicted in FTF+/−/− liver. The FTF-lacZ mouse establishes a central role for FTF in developmental, nutritive, and metabolic functions from early embryogenesis through adulthood.

Development of the mammalian embryo relies upon nutritive functions fulfilled by the visceral endoderm and then by the liver (1). A part of these functions is accomplished by nutrient carrier proteins of the albumin gene family, a multigene locus expressed in hepatocytes, and subject to precise developmental controls. One albumin-related gene, the α1-fetoprotein (AFP)1 gene, is activated at the onset of liver differentiation and operates tightly coupled with liver growth (2, 3). In 1988, our group circumscribed a proximal AFP promoter element essential to AFP gene activity in hepatocytes, and distinct from promoter components regulating the other albumin loci (4). The AFP-specific activator was then identified as orphan receptor fetoprotein transcription factor (5–7), so named for its first identified target locus (genome database nomenclature,5 NR5A2 in the nuclear receptor nomenclature, Ref. 9; also referred to as LRH1 or CFP). FTF belonged to a primitive class of nuclear receptors and emerged as a critical lead to connect AFP gene activation with early embryonic growth and differentiation processes.

Subsequent studies indicated that developmental FTF functions even preceded its activation of the AFP locus in hepatocytes. In situ hybridization analysis in the mouse at embryonic day 8–9 showed abundant FTF transcripts in the foregut endoderm, before liver morphogenesis (10). Characterization of the FTF gene promoter also revealed a cluster of regulatory motifs conserved in distant species and potential targets of cell lineage specification factors (11). Among these were three proximal binding sites for GATA factors, known to be essential for visceral endoderm function (12, 13). Furthermore, three HNF genes function as a direct repressor of the GATA factors (14). FTF was expected to maintain important nutritive roles in the adult, FTF being maintained at high levels in liver, intestine, and acinar pancreas. A landmark finding was the identification of FTF as one factor that binds and regulates the CYP7A1 gene promoter, the rate-limiting enzyme in the conversion of cholesterol to bile acid (15). The CYP7A1 gene is also represented in the albumin promoter, where a DR4 element (16) is present in the albumin sequences representing a potential cis-regulatory element that is conserved in other species (17). This indicates that FTF could also function in the promoter of the albumin gene by binding a DR4 element.

The abbreviations used are: AFP, α1-fetoprotein; ABC1, ATP-binding cassette protein A1; ASBT, apical sodium-dependent bile salt transporter; BA, bile acid; CAT, chloramphenicol acetyltransferase; CYP7A1, cholesterol 7α-hydroxylase; CYP6B1, sterol 12α-hydroxylase; dpc, day post coitum; DR, direct repeat; E, embryonic day; EMSA, electrophoretic mobility shift assay; ES, embryonic stem cell; FGF1, fibroblast growth factor receptor; Fos-F1, fushi tarazu factor 1; FTF, α1-fetoprotein transcription factor; FRE, FTF-response element; FXR, farnesoid X receptor; HNF, hepatocyte nuclear factor; JNK, c-Jun-N-terminal kinase; LXRα, liver X receptor; LXRβ, LXRβ-response element; mFTF, mouse FTF; mmp3, multidrug resistance protein 3; MT1, metallothion- ein 1; nt, nucleotide; RXR, retinoid X receptor; SF1, steroidogenic factor 1; SHP, small heterodimer partner; T/2, triiodothyronine; TR, thyroid hormone receptor; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin.

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acids (BA) (14–17). A model emerged whereby FTF would act as a "competence" factor driving the BA pathway, subject to negative feedback regulation by interaction of FTF with orphan receptor SHF, activated by the BA receptor FXR and by FTF itself (15, 16, 18, 19). Additional reports associated further FTF action with membrane transporters of the enterohepatic BA recycling system (20, 21) and with pancreatic cholesteryl esterase (22), all of which pointing to an important systemic role for FTF in BA/cholesteryl/BA homeostasis.

Here we document an FTF-lacZ gene knockout in the mouse, delineating FTF expression sites at all developmental stages and confirming crucial developmental and metabolic FTF functions from early embryogenesis to adulthood. We also identify a promoter DR4 regulatory element directly connecting FTF gene regulation with hormonal and metabolic homeostasis.

**EXPERIMENTAL PROCEDURES**

**FTF Gene Inactivation**—A 7.5-kb SpeI mFTF gene fragment encompassing exons 4–6 was retrieved from a mouse 129/SV genomic library screened with rat FTF cDNA (11). The 7.5-kb fragment was used to generate a knockout vector carrying a lacZ gene inserted in-frame into mFTF exon 4. Briefly, a 4-kb lacZ fragment was derived from vector pCI10 (Amersham Biosciences) using PstI, S1 nuclelease, and KpnI, and spliced into pSVK3 (Invitrogen) pretreated with EcoRI, Klenow, and KpnI. Recombinant pSVK3-lacZ was digested with Smal/Xbal and ligated with the 5′-end mFTF fragment obtained with BglII (which cuts exon 4 at position 462 in the sequence shown in Ref. 6) and treated with Klenow. This placed lacZ (with a stop codon and polyadenylation signal) in frame with the N-terminal portion of mFTF exon 4, interrupting the FTF sequence upstream from the first zinc finger of the DNA binding domain (11). An MC1-neo cassette was ligated to the 3′-BglIII mFTF fragment and inserted downstream from lacZ in the opposite polarity, and an MC1-tk cassette was inserted at the 5′-Spel end of the FTF gene fragment. The construct was then used for electrocompetent cells. This knockout vector generated an FTF-lacZ allele would transcribe a 4-kb FTF-lacZ mRNA in all FTF-permissive cells (i.e. from either the mFTF promoter 5′-flanking exon 1, or any other putative promoter elements between exons 1 and 3) (11), and that FTF-lacZ translation products would carry 19, 40, or 101 residues of the mFTF N-terminal domain (from three in-frame initiation codons in exons 1 and 3), with no DNA binding capacity and no other apparent regulatory function (6).

For gene targeting in embryonic stem (ES) cells, mouse WW6 (129Sv) ES cells (23) were grown on STO fibroblasts in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and recombinant LIF (24). ES cells were electroporated with FTF-lacZ plasmid DNA linearized with NotI, and cultured on neomycin-resistant STO fibroblasts for 14 days in selection medium containing 200 μg/ml G418 and 2 μg/ml ganciclovir. Southern colonies were genotyped by Southern blot using a mFTF probe flanking the wild-type FTF allele and a 7.8-kb fragment from the mutant allele.

Four ES clones carrying the lacZ-FTF allele were injected into mouse MF1 blastocysts which were then transferred to pseudopregnant C57BL6/J × CBA mice. The chimeric male progeny (129Sv × MF1) was crossed with MF1 females to establish germ line transmission, and subsequent breeding was maintained in the 129Sv × MF1 genetic background. Mice were housed in filter-topped microisolator cages under 50–70% air changes per hour and 10–14 h dark/light cycle, and fed on Teklad standard chow (TD2018). Neonatal mice were genotyped using tail DNA, by Southern blot as above or by PCR with neo gene primers 5′-GGGATCGGCCATTGAACAGTG-3′ and 5′-CGTAAAGCAAGATGG-3′, all of which pointing to an important systemic role for FTF in BA/cholesteryl/BA homeostasis.

**Northern Blot**—Total liver RNA was purified with guanidine thiocyanate (28), and poly(A)+ RNA was isolated with the Oligotex mRNA Maxi Kit (Qiagen). Poly(A)+ RNA (15 μg) was run on a 1% agarose/10 mm guanidine thiocyanate gel, transferred onto a positively charged nylon membrane (Roche Applied Science), and hybridized with FTF, SHF, CYP7A1, and GAPDH cDNA probes PCR-labeled with digoxigenin-11-dUTP (Roche Applied Science). mRNA bands were detected with a chemiluminescence kit (Roche Applied Science) and quantitated with IMAGEQUANT (Molecular Dynamics).

**Quantitative PCR**—Real-time quantitative PCR (qPCR) was used to determine the relative expression levels of liver FTF, SHF, CYP7A1, c-Jun, HNF1α, and HNF4α mRNAs. Total RNA (5 μg) was reverse transcribed with oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen). Quantitative PCR was carried out with 0.5% of the reverse transcription product in a 20-μl reaction volume containing Platinum SYBR Green qPCR superMix UDG (Invitrogen), using the LightCycler 1.2 instrument and software (Roche Applied Science) and the following sets of primers: FTF forward 5′-GGGATCGGCCATTGAACAGTG-3′ and reverse 5′-GGGGAGTCAGCAGGAGATGC-3′; FTF reverse 5′-AGTGGGACATCGTTTTCTCTGCC-3′ and forward 5′-AGGAGCAGCACGTCCTTAAA-3′. mRNA levels were calculated for fluorescence intensities greater than 2 SD from the mean of the values for the reference genes SHP, CYP7A1, and GAPDH cDNA probes PCR-labeled with digoxigenin-11-dUTP (Roche Applied Science). mRNA bands were detected with a chemiluminescence kit (Roche Applied Science) and quantitated with IMAGEQUANT (Molecular Dynamics).
Electromobility Shift and Scatchard Assays—Saturation electromobility shift analyses were conducted in 2-month-old FTF+/− and FTF+/- mice, as described before (6). Livers were pooled from five or more littersmates per group, and tested in parallel. Five micrograms of total nuclear protein was incubated 30 min on ice with 0.9–9 nm [32P]-labeled FTF probe TTTCAGGACGACA from the AF2 gene promoter (6), and FTF-bound and free double-strand probes were resolved by PAGE and counted with a phosphorimager. Non-specific binding (50-fold excess cold oligo) was subtracted, and net counts were measured to obtain a Scatchard plot. EMSA analysis of the DR4-like sequence at position +8/+21 in the mFTF gene promoter was carried out with mFTF probe –155/+155 (with CAGGAGCAGCTTCGACGTCGTCATTTCTT and GCAGGTGACCTTGCCCAGCGCC and their mutant (lower case letters) version GCTTACGAGACATGGTTTACAGCAGGTCATAC). Point mutations were 79 bp cloned into pBluescript SK-CAT (11). Point mutations were tested for homology by Dnalys by reagent 467825; group values were compared by Student’s t-detailed unpaired t test. Changes in liver FTF levels were assessed in male Fisher rats (75–100 g, 5 per group) fed for 5 days on 2% cholesterol TD86295 diet versus control M/R7001 diet, and in male Sprague-Dawley rats (300 g, 5 per group, fed on regular Teklad chow TD97350) 5 or 24 h following intraperitoneal injection of 375 μg/kg triiodothyronine (Sigma) or saline vehicle.

FTF Transgenic Mice—To assess FTF effect on CYP7A1 expression in vivo, we used a transgenic mouse line expressing FTF under the control of an inducible promoter. This system will be documented in detail elsewhere. Briefly, mFTF cDNA was ligated at its 5’-end with a hemagglutinin (HA) epitope sequence, and the HA-FTF cDNA was cloned in the NruI site of pBluescript SK-CAT (11). The mFTF gene was excised by digestion and religated into the ATM HAB site of the CYP7A1 promoter (12). Transgenic mice were generated as described (13), and were 3 or more per group. Transgenic animals were maintained on a regular corn starch diet, and killed under anesthesia with an overdose of sodium pentobarbital (Nembutal). Livers were harvested, fixed in 95% ethanol for 24 h, and embedded in paraffin. Sections (5 μm) were cut and stained with hematoxylin and eosin. Liver sections were photographed by light microscopy (Zeiss, Axioskop 2), and the number of stained cells and nuclei in the liver and bile ducts was counted for each genotype.

RESULTS

The FTF-lacZ Mouse—A total of 4 × 10⁷ ES cells were transfected with the FTF-lacZ recombinant vector (Fig. 1A). Of 502 ES clones analyzed, 15 displayed homologous recombination at one FTF allele (Fig. 1B). Four of those 15 clones were used to generate heterozygous FTF+/− mice (Fig. 1C). Crosses among FTF+/− mice generated early stage embryos with the expected FTF+/+ and FTF+/- genotypes (Fig. 1D).

FTF Expression Sites Throughout Development—Mice expressing lacZ inserted at the FTF locus allowed us to combine β-galactosidase staining and in situ hybridization to delineate FTF expression sites at all developmental stages. For β-galactosidase analysis, FTF+/− males were mated with FTF−/− females. At 2.5 dpc (morula stage), FTF+/− embryos displayed in vivo lacZ activity was also easily detected at the 4-cell stage (not shown). In the egg cylinder (E5.5), FTF expression segregated in the visceral endoderm (embryonic and extra-embryonic); ectodermal cells were uniformly negative (Fig. 2, G and H). At gastrulation (E6.5, E7.5), strong FTF signals persisted in the endodermal layers, and now marked the node that rose to the mesoderm layer and definitive endoderm (Fig. 2, K and L, and O and P). At mid-gestation (E8–E15), FTF gene activity expanded to endodermal derivatives (yolk sac, liver, pancreas, intestine), with strong transient β-galactosidase staining recorded in extra-endodermal structures, growing bone, testis, lung mesenchyme, sensory ganglia, posterior brain, and thalamus (Fig. 2, C and D and data not shown). At postnatal stages, FTF gene activity was detected in all hepatocytes and pancreas acinar cells (not shown), and it persisted in the intestine (prominently in the crypts of Lieberkuhn) (Fig. 2E) and in the granulosa layer of ovarian follicles (Fig. 2F).

Phenotype of FTF−/− Mice—Crosses among FTF−/− mice yielded no homozygous FTF−/− newborns, indicating an embryonic lethal phenotype. A total of 186 embryos were genotyped between E5.5 and E12.5 (Table 1). At E5.5 and E6.5, FTF−/− embryos were recovered at the expected mendelian ratio of 25%, which then declined abruptly between E7.5 and E9.5, to 9% at E10.5, indicating fetal death prior to liver differentiation. At E5.5, FTF−/− embryos showed no histological abnormalities (Fig. 2, G and H, and I and J). At E6.5 however,
they showed conspicuous anomalies, with a disorganized ectoderm and the amniotic cavity absent or malformed (Fig. 2, K and L, and M and N). At E7.5, FTF\textsuperscript{+/-H11002} embryos appeared as cell clumps typical of uterine resorption; a few embryos had recognizable structures with a constricted extra-embryonic junction and no mesodermal cells outside the junction (Fig. 2, O and P, and Q and R); at E8.5, no structures were identifiable.

The constriction and mesoderm clustering at E7.5 was typical of a defective node organizer or primitive line (31). E6.5 embryos were further analyzed for expression of HNF3\beta, a marker of the node and visceral endoderm (32, 33). FTF\textsuperscript{+/-H11002} embryos expressed HNF3\beta in the visceral endoderm but not in the anterior portion of the primitive line (Fig. 2, S and T, and U and V); this suggests that the node does not form in FTF knockout embryos, and consequently they cannot pass gastrulation.

Phenotype of FTF\textsuperscript{+/-H11002} Mice—Heterozygous FTF\textsuperscript{+/-} mice had no apparent defect, their growth and reproduction were normal, they had no visible changes in liver histology, and their plasma levels of AFP, glucose, triglycerides, bilirubin, hepatic enzymes, and amylase were normal (data not shown). However, in each of four pilot experiments comparing adult FTF\textsuperscript{+/+} with FTF\textsuperscript{+/-} littermates (5–8 per group), fasting level of total plasma cholesterol was consistently 14–17% lower in the FTF\textsuperscript{+/-} group (data not shown). Further measurements were made in larger groups of 2-month-old male littermates fed a 2% cholesterol diet or a control diet for 5 days, and confirmed a 10–12% lower serum cholesterol level in FTF\textsuperscript{+/-} mice (Table II). Scatchard assays on several pools of adult FTF\textsuperscript{+/-} versus FTF\textsuperscript{+/-} livers indicated little changes in FTF dissociation constants (\(K_d\) values in Fig. 3), but they suggested that FTF in the haploid group could be lower than 50% of normal (y-axis intercepts \(y_0\) in Fig. 3) (HNF1 and HNF4 appeared unchanged in EMSAs) (Fig. 3). Further qPCR assays on three pools of livers from FTF\textsuperscript{+/-} and FTF\textsuperscript{+/-} littermates indicated an average 94 FTF mRNA copies per cell (assuming 20 pg of RNA per cell) in FTF\textsuperscript{+/-} liver, and an average 35 copies per cell (37% of normal) in FTF\textsuperscript{+/-} livers (data not shown). These initial observations thus indicated a role for FTF in adult cholesterol homeostasis, and suggested FTF-dependent feedback on FTF gene activity.

To gain further insight into hepatic changes incurred in the FTF\textsuperscript{+/-} state, liver microarray analyses were performed. Gene
FIG. 2. FTF expression sites and knockout phenotype. Panels A–F, β-galactosidase staining of FTF+/− embryos or adult tissues. A, morulae (E2.5). B, blastocysts (E3.5, two FTF+/−, one FTF+/+). C, embryo at E11.5 (l, liver bud; r, rib primordium; b, ocular bones; o, otic vesicle; m, mesencephalon). D, ossification zone in a limb at E14.5 (c, cartilage). E, adult intestine. F, adult ovary. Panels G–V: brightfield and darkfield/in situ hybridization analyses of wild-type and FTF-knockout embryos; the results shown were obtained with antisense riboprobes, control sense probes gave no signals. At E5.5, FTF hybridization signals mark the visceral endoderm (G and H), and an FTF-knockout embryo (no signal) shows no apparent abnormalities (I–J). At E6.5, FTF+/+ signals also come from the node (K and L), and FTF−/− embryos exhibit severe dysmorphogenesis (M and N). At E7.5, normal endodermal layers express FTF whereas ectoderm and mesoderm are negative (O and P), and some FTF−/− embryos maintain recognizable structures (Q and R). At E6.5, HNF3β signals mark both the endoderm and node of normal embryos (S and T) but only the endoderm of FTF-knockout embryos (U and V). ee, embryonic visceral endoderm; em, embryonic mesoderm; exec, embryonic ectoderm; exe, extra-embryonic visceral endoderm; exec, extra-embryonic ectoderm; pec, primitive ectoderm; a, amnion; c, chorion; ac, amniotic cavity; pac, proamniotic cavity; epc, ectoplacental cavity; ecc, exocoelomic cavity.
played reduced expression (4.3-fold) of FTF target gene mrp3 profiling using the Affymetrix U74Av2 GeneChip array dis-
the Affymetrix 430A GeneChip array: although not as pro-
Table III). Northern blot analysis confirmed the CYP7A1 and
fold) and a slightly increased SHP mRNA level (array 1 in
(20, 34), but a strong increase in CYP7A1 mRNA levels (8.4-
/H11001
array Chol in Table III) or on a 1% cholic acid diet
versus
fed on a diet containing 2% cholesterol
/H11002
mice. Control transgenic mice showed some increase in total FTF mRNA level compared with wild-type controls, with slightly increased CYP7A1 mRNA, nearly 5-fold higher SHP mRNA, and no change in HNF1α or HNF4α mRNAs (Fig. 5A). Upon zinc injection, total FTF mRNA was raised 5-fold in transgenic liver relative to wild-type liver, and CYP7A1 and SHP mRNA levels were over 6- and 18-fold higher in the transgenic group (Fig. 5A). Northern blot quantitation gave comparable results, and also showed lower amounts of endogenous FTF mRNA following induction of HA-FTF transgene expression by zinc injection (Fig. 5B). These results supported the activating role of FTF on CYP7A1 gene activity and thus pointed to indirect mechanisms for higher CYP7A1 levels in FTF+/+ mice. Gene expression profiles further revealed striking pleiotropic changes in FTF+/+ liver, indicative of broadly attenuated cell stress signal-
ning pathways, including reduced levels of c-Jun (arrays 1–3 in Table III). Apart from CYP7A1, mrp3, and cell signaling
markers, only a few other genes showed consistent changes of expression in FTF+/+ liver (arrays 1–3 in Table III). Liver gene expression profiles were then analyzed in FTF+/− mice fed on a diet containing 2% cholesterol versus a control diet (array Chol in Table III) or on a 1% cholic acid diet versus a control diet (array CA in Table III). As expected, cholesterol feeding reduced the level of mRNAs involved in cholesterol synthesis, and cholic acid increased SHP and decreased CYP7A1 and CYP8B1 mRNA levels (Table III). Notably, the hepatic content of c-Jun mRNA was markedly enhanced by either cholesterol or cholic acid feeding (Table III). CYP7A1, SHP, and c-Jun results were confirmed by qPCR analysis (not shown).

**DR4 Regulatory Element in the FTF Gene Promoter**

The FTF gene promoter contains a sequence closely matching a direct AGGTCA repeat with a 4-nt spacer (DR4 motif), at +6 in the mFTF promoter and conserved from fish to man (Fig. 6A). This DR4-like sequence was characterized for its potential regulation by LXR-RXR and TR-RXR heterodimeric receptors. First, electromobility shift assays were conducted with an FTF DR4 probe and rat liver nuclear extracts. Two retarded com-
plexes were specifically competed by low molar excess of cold DR4 oligos from the FTF, CYP7A1, or ABC1 gene promoters (Fig. 6B, lanes 2–4), and not by a mutated FTF DR4 oligo (lane 5). Antibodies against RXR attenuated both complexes (lanes 8 and 12), anti-LXR attenuated the slower complex (lanes 7 and 14), anti-TR attenuated the faster complex (lanes 9 and 13), and anti-FXR had no effect (lane 10). These results indicated

| Genotypes of embryos (E, embryonic day) from interbred FTF+/− mice | Control | Cholesterol |
|---|---|---|
| E5.5 | 10 (77%) | 3 (23%) |
| E6.5 | 22 (76%) | 7 (24%) |
| E7.5 | 6 (24%) | 15 (60%) |
| E8.5 | 6 (23%) | 18 (69%) |
| E9.5 | 7 (24%) | 20 (69%) |
| E10.5 | 6 (33%) | 12 (67%) |
| E11.5 | 8 (29%) | 20 (71%) |
| E12.5 | 6 (33%) | 12 (67%) |

* Number of animals is in parentheses.

Table II

**Fig. 3. EMSA analyses on liver nuclear proteins from FTF+/+ and FTF+/− mice.** Livers were pooled from 5 adult mice in each group, and equal amounts of nuclear proteins were run in EMSA reactions with increasing amounts of FTF probe; free and bound probes were then resolved by electrophoresis and counts were processed by Scatchard plot transformation. Autoradiograms illustrate EMSA reactions using FTF, HNF4, or HNF1 probes with constant amounts of liver nuclear proteins (C, no competitor; set and m, 50-fold molar excess of cold wild-type or mutant oligos; FTF mutant oligo was TGTTCAAt-
AGAaA; split FTF bands are translational isoforms; lower-case letters indicate base changes from wild-type FTF sequence).

profilong using the Affymetrix U74Av2 GeneChip array dis-
played reduced expression (4.3-fold) of FTF target gene mrp3 (20, 34), but a strong increase in CYP7A1 mRNA levels (8.4-
fold) and a slightly increased SHP mRNA level (array 1 in Table III). Northern blot analysis confirmed the CYP7A1 and
SHP results (Fig. 4). Gene profiling was repeated twice using the Affymetrix 430A GeneChip array: although not as pro-
nounced, again mrp3 mRNA level was lower, and CYP7A1 mRNA higher in FTF+/− liver (arrays 2 and 3 in Table III). These results seemed to conflict with the postulated activating role of FTF on CYP7A1 gene activity. To clarify the point, we used transgenic mice (TgF35) carrying an FTF transgene controlled by the inducible **MT1** promoter. qPCR analysis of liver mRNAs was performed 6 h following zinc or saline injection to TgF35 transgenic versus B6C3F1 wild-type mice. Control transgenic mice showed some increase in total FTF mRNA level compared with wild-type controls, with slightly increased CYP7A1 mRNA, nearly 5-fold higher SHP mRNA, and no change in HNF1α or HNF4α mRNAs (Fig. 5A). Upon zinc injection, total FTF mRNA was raised 5-fold in transgenic liver relative to wild-type liver, and CYP7A1 and SHP mRNA levels were over 6- and 18-fold higher in the transgenic group (Fig. 5A). Northern blot quantitation gave comparable results, and also showed lower amounts of endogenous FTF mRNA follow-
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![Image of Table I](https://via.placeholder.com/150)

**Table I**

Genotyping was done by in situ hybridization (E5.5, E6.5, PCR (E7.5, E8.5), or Southern blotting (E9.5 onward). Data are number of embryos (and percent of total) from two or three pregnant mice per time point. At E5.5 and E6.5, FTF+/+ and FTF+/− embryos are combined; in situ hybridization does not distinguish the two genotypes.

| Genotype | Control | Cholesterol |
|---|---|---|
| FTF+/+ | 10 (77%) | 3 (23%) |
| FTF+/− | 22 (76%) | 7 (24%) |
| FTF+/− | 6 (24%) | 15 (60%) |
| FTF+/− | 6 (23%) | 18 (69%) |
| FTF+/− | 7 (24%) | 20 (69%) |
| FTF+/− | 6 (33%) | 12 (67%) |
| FTF+/− | 8 (29%) | 20 (71%) |
| FTF+/− | 6 (33%) | 12 (67%) |

* Number of animals is in parentheses.

![Image of Table II](https://via.placeholder.com/150)

**Table II**

Serum cholesterol levels in wild-type (+/+ ) and FTF-haploid (+/− ) mice

Mice were fed a control diet or a 2% cholesterol diet for 5 days. Results are mean ± S.D.

| Genotype | Control | Cholesterol |
|---|---|---|
| FTF+/+ | 2.37 ± 0.33 (21)* | 3.37 ± 0.60 (26) |
| FTF+/− | 2.14 ± 0.30 (14) | 2.99 ± 0.63 (22) |

* Number of animals is in parentheses.

![Image of EMSA](https://via.placeholder.com/150)
that the FTF DR4 sequence can bind LXR-RXR and TR-RXR heterodimers in vitro, like bona fide DR4 elements found in the CYP7A1 and ABC1 gene promoters. Functional assays were then conducted with FTF-CAT reporter 4F-CAT (11), carrying 4 kb of contiguous 5′-flanking mFTF promoter sequence. We started with stable transfection in the well differentiated rat hepatoma 7777.6 subline (4). This stringent system, in which transfected genes are integrated in the genome, has shown much higher sensitivity to mutations affecting basal AFG gene activity, compared with transient expression systems, in which reporter genes function as episomes (4, 5). The activity of 4F-CAT stably transfected in 7777.6 cells was reduced by about 75% upon mutation of the FTF DR4 motif (Fig. 6C), indicating that the DR4 element was used to up-regulate FTF gene activity. Because parental McA-RH7777 cells express functional LXR, TR, and RXR receptors and ligands on the DR4 element, the result suggested that the DR4 element was acting on FTF promoter and that functional high-affinity DR4 motifs are present in the FTF promoter in vivo.

Fig. 4. Northern blot analysis of liver mRNAs from adult FTF+/+ and FTF+/− mice. Equal amounts of poly(A)+ RNA (from liver pools used for microarray 1 (Table III)) were run in duplicates on 1% agarose/10 ml guanidine thiocyanate gels, transferred onto a nylon membrane, and hybridized with digoxigenin-labeled FTF, CYP7A1, SHP, or GAPDH cDNA probes; hybridization signals were quantitated by chemiluminescence. Quantitation of the bands is expressed as fold change in FTF+/− liver relative to FTF+/+ liver, after correction for GADPH levels. FTF ratio 0.4 refers to transcripts originating from wild-type FTF alleles.

TABLE III
Microarray analysis of liver gene expression in adult male FTF+/− mice

Arrays 1, 2, and 3 are three separate analyses comparing FTF-haploid (+/−) and wild-type (+/+ ) livers. Data are fold changes of FTF+/− over FTF+/+ liver. Array Chol is the analysis conducted with FTF+/− mice fed for 5 days on a control diet or on a diet containing 2% cholesterol; fold changes are cholesterol liver over control liver. Array CA is an analysis using FTF+/− mice fed for 5 days a control diet or a diet containing 1% cholic acid: fold changes are cholic acid liver over control liver.

| GenBankTM accession no. | Gene name             | Cholesterol/Bile Acid | FTF+/−/−/FTF+/+ | FTF+/−/−/CA | FTF+/−/−/Chol |
|-------------------------|-----------------------|-----------------------|----------------|-------------|--------------|
| NM_003076               | FTF                   |                      | −3.0          | −1.4        | −1.5         | −1.2         |
| NM_007824               | CYP7A1                | +8.4                  | 2.1           | +2.0        | −1.3         | −5.8         |
| L76587                  | SHP                   | +1.7                  | −1.3          | 1.0         | +1.3         | +2.7         |
| NM_010012               | CYP7B1                | +1.4                  | −1.4          | −1.1        | +1.2         | −4.0         |
| NM_007825               | FXRα                  | +1.6                  | −1.2          | −1.2        | −1.4         | −1.3         |
| NM_009108               | RXRα                  | +1.1                  | +1.2          | 1.0         | −1.2         | +1.2         |
| U77683                  | TRα                   |                      | −1.0          | 1.1         | −1.4         | +1.1         |
| X07750                  |                      |                      | 1.0           | +1.0        | −1.2         | −1.1         |
| NM_013839               | LXRα                  | −1.2                  | −1.2          | +1.2        | −1.1         | −1.1         |
| NM_008261               | HNF4                  | −1.5                  | −1.3          | −1.2        | −1.1         | −1.1         |
| NM_029600               | mpr3                  | −4.3                  | −1.8          | −2.1        | −1.4         | −1.3         |
| NM_145942               | 3-Hydroxy-3-methylglutaryl-CoA synthase 1 | 4.1 | −1.3 | −1.9 | −5.9 | −4.4 |
| NM_020010               | CYP51                 | −1.4                  | −1.4          | −1.2        | −1.2         | −2.5         |
| NM_025436               | Sterol-C4-methyl oxidase-like | +1.3 | +1.2 | +2.5 | +15.7 | −5.2 |
| NM_145360               | Isopentenyl-diphosphate delta isomerase | −1.5 | −1.1 | −1.4 | −24.5 | +1.1 |
| Cell Signaling           |                       |                      |               |             |             |             |
| M53978                  | TNF receptor 1β       | −2.3                  | −1.2          | −1.6        | 1.0          | +1.2         |
| NM_010953               | C/EBPβ                | −2.9                  | −1.7          | −1.9        | +1.1         | −1.5         |
| X61800                  | C/EBPβ                | −4.7                  | −1.7          | −2.0        | −1.1         | −1.1         |
| L28118                  | NFκB 1                | −3.7                  | 1.0           | 0.0         | −1.2         | −1.2         |
| NM_008416               | jun-B                 | −4.0                  | −2.8          | −4.5        | +1.1         | ND           |
| NM_010591               | c-jun                 | ND                    | −4.3          | −2.8        | +4.9         | +6.8         |
| NM_010234               | e-fos                 | ND                    | −25.3         | −46.1       | ND           | ND           |
| M12573                  | Heat shock protein 1B | −4.8                  | −8.4          | −7.0        | +3.2         | +6.3         |
| M33036                  | Intercellular adhesion molecule 1 | −15.6 | −1.6 | −1.4 | −1.2 | −1.1 |
| NM_013602               | Metallothionein 1     | −19.7                 | −5.5          | −5.4        | +2.2         | −4.5         |
| NM_011817               | GAAT467               | −34.0                 | −3.1          | −4.3        | +1.6         | −1.4         |
| NM_008341               | JGBP1                 | −34.4                 | −19.2         | −21.5       | +1.4         | −1.2         |

* U74Av2 GeneChip array (≈ 12,000 transcripts).
* 430A GeneChip array (≈ 20,000 transcripts).
* ND, nondetectable in that sample as determined by GENECHIP software.
that receptor and ligand effects were mediated by the DR4 element. Transfections with control vectors pSG5 or pCMX (lanes 1 and 2 and 11 and 12) were unreactive to ligands (not shown).

Finally, we tested if FTF would react in vivo to cholesterol-derived LXRs ligands or to TR hormone ligands. We measured liver FTF levels in Fisher rats fed for 5 days on a 2% cholesterol diet, and in Sprague-Dawley rats 5 and 24 h following an intraperitoneal injection of T3 thyroid hormone (serum T3 levels were maximal at 5 h, but still 10-fold over control levels (2.5 nM) at 24 h (data not shown)). With the high cholesterol diet, liver FTF protein level increased about 2-fold, C/EBPβ about 3.5-fold, HNF4α about 1.7-fold, and the FTF gene plus the HA-FTF transgene.

**DISCUSSION**

**FTF in the Embryo**—The FTF/lacZ mouse shows that FTF gene activation is part of prime genomic events surrounding initiation of zygotic transcription at the 2-cell stage. FTF is also ubiquitously expressed in the early embryo, like its homolog in Xenopus (38), and FTF is essential to mouse embryogenesis, like its distant relative Ftz-F1 in Drosophila (39). In contrast, SF1, the closest FTF homolog, is specifically required for development of steroidogenic organs (40). This supports the notion of FTF being a more primitive receptor (41), which also fulfills SF1 functions in lower vertebrates like fish (42). FTF-null mouse embryos provide no signs of essential FTF functions at preimplantation stages. FTF relatives in fly or frog are maternal genes (39, 43), whereas FTF is not expressed in mouse oocytes, which could suggest other ancient functions in lower species. FTF−/− mouse embryos die at E6.5–7.5 with morphological features typical of aborted gastrulation and visceral endoderm dysfunction. This mimics similar phenotypes obtained by inactivating upstream activators (GATA) (12, 13, 44, 45) or downstream targets (HNF4α, HNF3β) (31–33, 46, 47) of the FTF gene, suggesting the possibility of interdependent lethal phenotypes in the GATA-FTF-HNF cascade. GATA6 knockout, for instance, is also lethal at E6.5–7.5 (13), and FTF expression could be expected to be severely curtailed in GATA6−/− embryos. Reciprocally, FTF−/− lethality occurs somewhat earlier than with HNF3β or HNF4α knockouts (32, 33, 46), possibly because the FTF-null state depletes both HNF3β and HNF4α. The FTF/lacZ mouse also shows expression patterns indicative of transient FTF functions in multiple fetal structures outside endodermal derivatives. High FTF gene expression in actively growing cell compartments, such as ossification zones, reinforces the suggestion that the FTF gene may generally react to cell surface growth receptor signaling (11). It also predicts extensive differential use of FTF promoter elements in a developmentally restricted manner. As a case in point, mouse Pdx1 is coexpressed with FTF in fetal pancreas and intestine, but Pdx1 binds to the FTF promoter only in pancreas, and only from E13.5 through E16.5 (48). The FTF DR4 regulatory element, responding to retinoid and thyroid ligands, also opens multiple signaling pathways through which FTF could be transiently up-regulated during fetal development. Finally, FTF physically interacts with the homeodomain protein Ftz (41), a mandatory partner of FTF ancestor Ftz-F1 (39), suggesting that homeodomain proteins may be important FTF coeffectors in developmental events.

**FTF in the Adult**—Converging evidence suggests that FTF basically operates the enterohepatic cholesterol/BA cycle by activating liver BA production and export via CYP7A1 and mrp3 (14–17,34), intestinal BA absorption via ASBT (21), and BA reentry from intestinal cells into portal blood via mrp3 (20). Short term overexpression of hepatic FTF in transgenic TgF35 mice provides compelling in vivo support for the activating effect of FTF on CYP7A1 and SHP. FTF-haploinsufficient mice allowed us to investigate further the net systemic effect of reducing FTF production at both hepatic and intestinal FTF expression sites. FTF+/− mice express higher levels of CYP7A1 mRNA than wild-type mice. Recently, a dual mechanism was brought to light in the feedback repression of CYP7A1. One proceeds via BA activation of FXR leading to SHP induction and FTF quenching (15, 16), while the other involves BA-FXR activation of an FGF-JNK-c-Jun signaling cascade (49–52). Basal CYP7A1 activity is thus higher in FXR−/− (18), SHP−/− (49, 50), or FGFR4+−/− (53) mice, and BA repression of CYP7A1 is eliminated in FXR−/− mice (18) but not in SHP−/− mice (49, 50). Under FTF+/− steady-state conditions, liver SHP expression is not diminished, but c-Jun level and cell signaling are reduced, thus enhancing CYP7A1 activity. This resembles ASBT-deficient mice, in which ileal BA...
recycling is eliminated, also resulting in 3-fold increase in CYP7A1, with no change in SHP (54). Like CYP7A1, the AFp gene is a negative acute phase reactant (5), and has long been known to be repressed by c-Jun via the FTF-binding AFP promoter element (5, 55, 56); furthermore, c-Jun strongly interacts with FTF in GST pull-down assays (57).3 FTF is thus a plausible feedback target for both SHP and c-Jun.

When FTF+/− mice were fed a cholesterol- or cholic acid-enriched diet, liver c-Jun expression was increased (Table III). However, only cholic acid resulted in CYP7A1 repression, showing that c-Jun enhancement per se is not sufficient to mount a feedback response. Robust BA activation of FXR, indicated by higher SHP and lower CYP8B1 in the cholic acid group (array CA, Table III), thus appears required to activate c-Jun into an effective repressor. On the other hand, a pure FXR agonist cannot repress CYP7A1 in the absence of SHP (49, 50) and therefore activation of the FXR-FGF-JNK pathway is also not sufficient to mount a significant repressive response. We conclude that a threshold of repressive c-Jun is contributed by both the absolute level and the activation state of c-Jun. Consistent with this, coexpression of c-Jun in transfection assays enhances the repressive effect of JNK on CYP7A1 (52).

Cholic acid does not completely repress CYP7A1 in the absence of SHP (49, 50), suggesting that SHP is a more effective silencer in using both competitive and active repression mechanisms to quench FTF activity (8).

Cell signaling and the FXR-SHP loop provide first-line feedback mechanisms to reduce BA production in normal conditions. Under cholestasis such as produced by ligation of the bile duct, a second-line hepatoprotective mechanism is activated whereby intense cytokine signaling exacerbates the JNK pathway, resulting in enhancement of hepatic FTF level, with a strong increase of liver Mrp3 to expel BA from hepatocytes (34).

FIG. 6. Characterization of a DR4 sequence in the FTF gene promoter. A, the upper diagram locates FTF gene regulatory elements (G, GATA; N, Nkx; E, E-box; H, homeo) (11, 35) and the position of the DR4 motif conserved in human, mouse, and zebrafish FTF genes; mDR4: base changes in DR4-mutant oligo mF (panel B) and in DR4-mutant 4F-CAT (panels C and D). B, EMSA analyses using an FTF DR4 probe and total liver nuclear proteins (3 μg) from adult male Sprague-Dawley rats. Lanes 1–10, pool of 5 normal livers; lanes 11–14, pool of 3 livers taken 24 h following intraperitoneal injection of 375 μg/kg triiodothyronine; lanes C and T3, no competitor; lanes F, 7A, AB, mF, 20-fold molar excess of cold FTF, CYP7A1, and ABC1 DR4-oligos, and FTF DR4-mutant oligo. C, functional assay of FTF reporter construct 4F-CAT and its DR4 mutant, using stable transfection in 7777.6 hepatoma cells. Results are CAT activity in cells transfected with mDR4, relative to CAT activity in cells transfected with 4F-CAT and given a value of 1 (triplicate assays in two sets of transfections with pools of 200–300 cell clones per transfection; bar is 1 S.D.). D, functional assays of 4F-CAT and mDR4 constructs, using transient transfection in HepG2 and Hep3B hepatoma cells. Reporter constructs were cotransfected with a RXRa expression vector, and with LXRx or TRβ expression vectors, and cells were cultured without (−) or with (+) ligands added to the medium (22R(OH)2-DHcy and 9-cis-RA in LXRx-RXR transfections; T3 and 9-cis-RA in TR-RXR transfections). Results are CAT activities from three or more transfections, relative to 4F-CAT activity in control transfections (lanes 1 and 2) using vectors pSG5 or pCMX and given a value of 1 (bars are 1 S.D.).

3 S. Gilbert, S. Roy, and L. Bélanger, unpublished observations.
excess BA feeding (18), cytokine shortcuts to JNK activation may have limited bearing on normal physiology. Nevertheless, it illustrates that FTF-regulated genes respond differently to homeostatic changes. In FTF+/−/ liver, FTF target genes mrp3 and CYP7A1 showed opposite changes in steady-state conditions (arrays 1–3, Table III), and CYP7A1, but not mrp3, was inhibited by cholic acid feeding (array CA, Table III). Likewise, in isolated intestinal cells, bile acids activate mrp3 and deactivate ASBT, via their FTF-regulatory domains (20, 21). Thus, mrp3 and CYP7A1/ASBT respond to bile acids in opposite directions, as a coherent systemic way to reduce cellular toxicity. This differential regulation seems to be provided by the particular arrangement of tandem FTF activation sites in the mrp3 promoter (20), whereas CYP7A1 and mouse ASBT promoters contain a single FTF activation site.

A simplified model of FTF regulation and function is presented in Fig. 8. In essence, FTF activates CYP7A1 and BA production in hepatocytes (steps 1 and 2), and BA export from enterocytes via mrp3 (step 3). FTF activation of CYP7A1 is subject to feedback repression by SHP (step 4) and by JNK-activated c-Jun (step 5). In the FTF+/−/ mouse, BA recycling (step 3) and signaling (steps 6 and 7) is attenuated, resulting in higher CYP7A1 activity, higher cholesterol elimination, and lower plasma cholesterol levels. The model can explain why FXR is essential for BA feedback repression of CYP7A1 (in the absence of FXR, steps 7 and 8 are not operational), while the loss of SHP can be bypassed by BA via step 5, but not by an FXR-specific ligand (step 4 is not operational, and step 6 is not activated). The FTF DR4 element further integrates metabolic functions of FTF and LXRa (step 9) in reducing hepatic cholesterol stores and enhancing cholesterol secretion. LXRa can also indirectly activate genes carrying an FTF response element but devoid of a DR4 element, such as the human CYP7A1 gene. FTF levels somewhat lower than predicted in FTF+/−/ liver, and negative feedback on the endogenous FTF gene following acute induction of transgenic FTF (Fig. 5B) may be explained by reduced signaling to the DR4 element as a result of enhanced CYP7A1 activity and reduced availability of cholesterol-derived LXRa ligands (step 2).

By affecting all FTF-regulated genes in a mildly disruptive way, the FTF+/−/ mouse establishes a new systemic model closely relevant to physiological conditions, and to better control of atherogenic processes. The FTF-inducible transgenic mouse (TgF35) also provides a powerful tool to identify primary FTF gene targets: this work is in progress in our laboratory.

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The Fetoprotein Transcription Factor (FTF) Gene Is Essential to Embryogenesis and Cholesterol Homeostasis and Is Regulated by a DR4 Element

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