Supporting Information

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SI Materials and Methods

Construction of hFIX Minigenes with Leyden Mutations. Human FIX minigene expression vectors -416(-20A)FIXm1, -802(-20A)FIXm1, and -802(-20A)FIXm1/1.4 were constructed by inserting a representative hemophilia B Leyden mutation T-20A (shown as -20A in minigenes) created by site-directed mutagenesis into wild-type hFIX minigenes, -416FIXm1, -802FIXm1, and -802FIXm1/1.4, respectively (1, 2). Minigenes -802(+13G)FIXm1 and -802(-26C) FIXm1 were similarly constructed using -802FIXm1. All minigenes constructed were composed of hFIX gene autologous components, including hFIX promoter sequence extended up to nt -416 or to nt -802 in the 5′ end upstream, coding region with the first intron with its middle 4.8 kb region deleted, and the 3′ UTR with the poly(A) signal and its contiguous genomic sequence (300 bp). The nucleotide (nt) numbering system used in this study followed that of the complete hFIX gene sequence previously reported by Yoshitake et al. (3). Before constructing transgenic mice, they were tested in vitro with HepG2 cells for their hFIX expression assayed by hFIX-specific ELISA.

Transient Expression Assay Using HepG2 Cells. The human hepatoma cell line HepG2 (ATCC HB8065) was used for transient expression assay of hFIX minigene constructs. This cell line, which lacks expression of its intrinsic hFIX, was previously characterized and successfully used as a durable in vitro assay system for hFIX minigene expression (1). Transient hFIX expression assays were carried out as previously described with minor modifications (4). Briefly, a mixture of hFIX minigene vector (3 μg) and pCH110 (0.3 μg) was mixed with 5 μl of FuGENE 6 (Roche) and added to 2 ml of transfection medium in a 3.5 cm plate grown with $\approx 0.6 \times 10^5$ cells (15 to 20% confluency). After overnight incubation, medium was changed to fresh hFIX depleted medium supplemented with 2 μl of vitamin K (10 mg/ml, Abott Laboratories). After an additional 2 days of incubation, the conditioned medium and cells were harvested and subjected to quantification of produced hFIX by hFIX-specific ELISA. Resulting values were then normalized as previously described for transfection efficiency according to the intracellular β-galactosidase activity produced by co-transfected pCH110 vector (5). Five independent assays were carried out, and averaged values were shown with standard deviations.

Construction of Transgenic Mice and Longitudinal in Vivo Assays for hFIX Expression. Transgenic mice were constructed according to standard methods (6) at the Bio-medical Research Animal Model Core facility at the University of Michigan as previously described with minor modifications (1). Human FIX minigene vector plasmids were digested with SphI/KpnI and the hFIX minigene-containing fragments released were isolated by electrophoresis on a 0.8% agarose gel, followed by purification using SpinBind DNA extraction units (FMC Corporation). Fertilized eggs of C57BL/6xSJL mice were microinjected with the DNA (1–2 ng/egg), and implanted into foster mother animals (CD-1). Offspring produced were then screened for founder animals with high transgene copy numbers (5–10 copies per genome) using quantitative multiplex PCR analyses of tail tissue DNA samples. Founders were back-crossed with nontransgenic mice (C57BL/6xSJL background) to generate F1 progeny animals. Homozygous F2 animals were generated by crossing among heterozygous F1 littermates and following generations. Zygosity status of animals was determined by quantitative multiplex PCR analysis. Minimally 3 and in some cases up to 12 founder lines for each minigene construct were subjected to age-axis longitudinal analysis for their life spans up to 1–2 years or as otherwise described.

At various ages, starting at one month of age, transgenic mice were individually subjected to blood sample collection (aliquots of approximately 50–100 μl) via tail-tip snipping, and serum obtained was routinely used to quantify circulatory hFIX levels using duplicated ELISA for each age point. Pooled human plasma (George King Bio-Medical) was used to prepare hFIX standard curve for each assay. To normalize experimental fluctuations in ELISA assays over time, overlapped serum samples from the previous age assay group were included in each assay.

All animal experiments were carried out in accordance with the institutional guidelines of the University of Michigan and National Institute of Advanced Industrial Science and Technology.

Preparation of Mouse Liver Nuclear Extracts (NEs). Liver NEs were prepared from 1-, 3-, 6-, 12-, 18- and 21-month-old male mice (C57BL/6 x SJL F1, $n = 20$ per each age group) according to Graves et al. with minor modifications (7). For other EMSAs and supershift EMSAs, liver NEs were prepared from mice at 6 months of age. Protein concentrations of NEs were quantified by the Bradford method per manufacturer's instructions (Bio-Rad). The NE preparations were stored in aliquots at −80 °C until use.

Electrophoretic Mobility Shift Assay (EMSA). EMSAs were performed as previously described (1, 8). Liver NEs were prepared from 1-, 3-, 6-, 12-, 18- and 21-month-old male mice (C57BL/6 x SJL F1, $n = 20$ per each age group) according to Graves et al. (7) with minor modifications and were used for assaying of age-related protein binding to the ASE probe. For other EMSAs and supershift EMSAs, liver NEs were prepared from mice at 6 months of age. The NEs of cultured cells were prepared from about 1×10^8 cells by the method of Dignam et al. (9) with slight modifications. Protein concentrations of NEs were quantified by the Bradford method per manufacturer's instructions (Bio-Rad). The NE preparations were stored in aliquots at −80 °C until use.

The ASE probe for EMSAs was prepared as follows. A double-stranded 22-mer oligonucleotide corresponding to the region nt -797 through -776 of the hFIX gene (TTCAGTC*GAGGAAG*GATAGGGT) with an ASE sequence in the middle (shown by underline) was prepared by mixing equimolar corresponding sense and antisense strands of synthetic oligonucleotides. While this probe was used for most EMSA, a set of 20-mer probes with all possible combinations of single nucleotide changes in the core heptamer sequence was also prepared in a similar manner (Tables S1 and S2). These oligonucleotide probes were labeled at the 5′ end with [^{32}P]ATP and T4 polynucleotide kinase to a specific activity of about 1×10^9 cpm/μg, and used in EMSAs. Aliquots of NEs prepared from mouse liver tissues or cultured cells were mixed with 1 μg of sonicated ds poly(dI-dC) in the DNA binding buffer and added to ^{32}P-labeled ds oligonucleotides (0.05–0.1 ng with 20,000–40,000 cpm) in a final volume of 20 μl. The reaction mixtures were incubated at room temperature for 20 min, and electrophoresed on a nondenaturing 4% polyacrylamide gel in glycine buffer (50 mM Tris, 0.38 M glycine, 2 mM EDTA, pH 8.5) for 4 h at 25 mA. Gels were then dried and DNA:protein complexes were visualized as mobility-shifted bands by autoradiography. Bands were quantified by

scanning the dried gels on a Storm 830 Image Analyzer (Molecular Dynamics Inc.).

Competitive EMSA was carried out as previously described using non-radiolabled 20-mer or 22-mer ds oligonucleotide probes as the competitor (1, 13). Supershift EMSA analyses with various Ets family protein antibodies (Santa Cruz Biotechnology Inc) were performed according to the manufacturer's instructions with minor modifications. Briefly, before mixing with labeled probe oligonucleotides, 4 μg of purified antibody was mixed with NEs for 4 h at 4 °C. The rest of the procedure followed the standard EMSA method described above.

Preparative EMSA was carried out using 160 μg (4 times greater than the regular application amount used) of liver NEs and 120,000 cpm of ^{32}P-labeled ds oligonucleotide probes (approximately 0.4 ng) on the 18 $\times$ 18 cm electrophoresis glass plates.

All EMSAs were performed twice or more with separate NE preparations to ensure reproducibility.

Preparation of NEs of 293T Cells Overexpressing Murine Ets1. Murine Ets1 expression vector pCMV-SPORT6 and 293T cell line MGC-18571 were purchased from ATCC. Cell transfection was performed using FuGENE 6 (Roche). After 2 days of posttransfection incubation, cells were harvested and used for preparing NEs according to Dignam et al. (9). Ets1 overexpression was confirmed by anti-Ets1 antibodies (N-276 and C-20, Santa Cruz Biotechnology, Inc.).

Two Dimensional Gel Electrophoresis (2DE) Analysis and Western Blot Analysis. ASE:protein or non-ASE:protein complex bands and corresponding gel slices from the control gels without oligonucleotide were obtained from preparative EMSA gels. Proteins were extracted using a Mixer Mill (MM 300, Retsch GmbH) at 4000 rpm $\times$ 30 sec with zirconium beads at room temperature with isoelectrofocusing (IEF) buffer (6 M urea, 2 M Thiourea, 50 mM DTT, 3% CHAPS, 1% Triton X-100). The sample mixture was then centrifuged at 12,000 rpm for 1 h through a microspin column (Amersham Bioscience, 27–3565-01), and resulting extracts were loaded to an Immobiline DryStrip gel (pH 4–7, 13 cm, Amersham Bioscience). The IEF (the first dimension separation of 2DE) was carried out at 50 V for 4 h, 150 V for 1 h, 150-5000 V linear ramping in 2.5 h, and 5000 V final IEF for 15 h using a Bio-Rad IEF Unit. The second dimension (SDS/PAGE, 10–20% precast gradient gel, BioCraft) was performed at 25 mA/gel for 2 h. SDS/PAGE gel was then stained with SYPRO Ruby dye (Molecular Probes, Inc.). Western blot analyses were then performed using polyclonal rabbit anti-Ets1 antibody (N-276, Santa Cruz Biotechnology.) and ECL Plus detection system (Amersham Biosciences).

Hypophysectomy of Mice. Hypophysectomy procedures were performed according to the methods of Davey et al. (10) with some modifications. Briefly, mice were anesthetized with a combination of ketamine/xylazine and the pedal reflex was monitored to ensure adequate depth of anesthesia. A 1-cm midline incision was made, and the salivary glands, the omohyoid muscle, and the lateral branches of the jugular vein were retracted. A #2 dental burr was used to drill a small hole in the occipito-sphenoidal synchondrosis. A modified Pasteur pipette was attached to a vacuum pump and the gland was quickly extruded. The incision was closed with wound clips. Mice were post surgically given 2 ml lactated Ringer's solution s.c. Glucose (10%) was added to the drinking water for the duration of the study. Two weeks after the surgical procedure, urine samples were examined by denaturing gel electrophoresis for the presence of major urinary protein (MUP) as a sensitive marker for production of growth hormone-dependent liver protein (11). Absence of any detectable MUP at the end of monitoring was defined as a successful hypophysectomy, and animals were used for various hormone treatment experiments.

Hormone Treatments of Hypophysectomized Mice. Successfully hypophysectomized transgenic mice were treated with murine growth hormone (GH), a gift from Dr. A.F. Pavlow at the U.S. National Hormone and Pituitary Program, Harbor-University of California, Los Angeles Medical Center, Torrance, CA. GH (20 μg) dissolved in 200 μl sodium carbonate buffer, pH 8, or PBS were intra-peritoneally injected at 12 h intervals (10, 11). Blood samples were collected intermittently from snipped tail veins. With some hypophysectomized animals, GH was given for 13 or 14 days before stopping, and serum sample collection was continued for another 28 days before terminating the experiment. Serum samples collected were assayed for hFIX concentration levels by hFIX-specific ELISA. With another group of hypophysectomized animals, after termination of the first course of GH administration, more than 22 days rest was given to dissipate effects of residual GH. This was then followed by intraperitoneal administration of DHT (200 μg/injection) or E2 (500 μg/injection) (12) dissolved in sesame oil once daily up to 18 days or as shown in corresponding figure legends. This was followed by the second course of GH administration (20 μg every 12 h) for 8 days and sacrifice for liver tissue collection. In control experiments, PBS without GH or sesame oil without sex hormone was injected.

Northern Blot Analysis. Preparations of total RNA from mouse liver tissue samples and Northern blot analysis were performed as previously described (1, 13, 14). A ^{32}P-labeled SspI/BamHI fragment of the hFIX gene was used as the hybridization probe for specific detection of hFIX mRNA and not mouse FIX. The filter was washed off the hFIX probe and a rehybridization with the RNA18 probe was performed as previously described (1).

1. Kurachi S, Deyashiki Y, Takeshita J, Kurachi K (1999) Genetic mechanisms of age regulation of human blood coagulation factor IX. *Science* 285:739–743.
2. Kurachi S, Pantazatos DP, Kurachi K (1997) The carboxyl-terminal region of factor IX is essential for its secretion. *Biochemistry* 36:4337–4344.
3. Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K (1985) Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). *Biochemistry* 24:3736–3750.
4. Kurachi S, Sze L, Kurachi K (1998) Improved transfection of HepG2 cells using FuGene 6 transfection reagent. *Biochemica* 3:43–44.
5. Kurachi S, Hitomi Y, Furukawa M, Kurachi K (1995) Role of intron I in expression of the human factor IX gene. *J Biol Chem* 270:5276–5281.
6. Hogan B, Constantini F, Lacy E (1994) Manipulating the Mouse Embryo. In *A Laboratory Manual* (Cold Spring Harbor Press, New York).
7. Graves BJ, Johnson PF, McKnight SL (1986) Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. *Cell* 44:565–576.
8. Kurachi S, Furukawa M, Salier JP, Wu CT, Wilson EJ, French FS, Kurachi K (1994) Regulatory mechanism of human factor IX gene: protein binding at the Leyden-specific region. *Biochemistry* 33:1580–1591.
9. Dignam JD, Lebovitz RM, Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 11:1475–1489.
10. Davey HW, Park SH, Grattan DR, McLachlan MJ, Waxman DJ (1999) STAT5b-deficient mice are growth hormone pulse-resistant. Role of STAT5b in sex-specific liver p450 expression. *J Biol Chem* 274:35331–35336.
11. Norstedt G, Palmiter R (1984) Secretory rhythm of growth hormone regulates sexual differentiation of mouse liver. *Cell* 36:805–812.
12. Svechnikov K, Ritzen EM, Holst M (2000) Androgen and estrogen stimulation of ornithine decarboxylase activity in mouse kidney. *J Steroid Biochem Mol Biol* 75:329–333.
13. Zhang K, Kurachi S, Kurachi K (2002) Genetic mechanisms of age regulation of protein C and blood coagulation. *J Biol Chem* 277:4532–4540.
14. Zhang K, Kurachi S, Kurachi K (2002) New function for age-related stability element in conferring strict tissue-specific expression of human factor IX and protein C genes. *Thromb Haemost* 88:537–538.

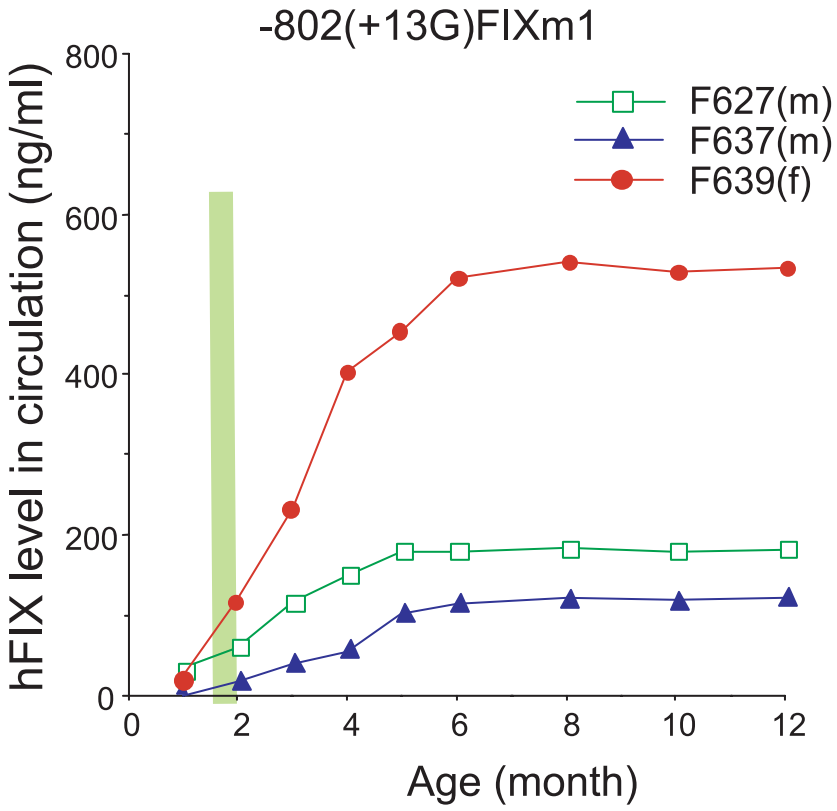

Fig. S1. Age-related longitudinal expression profiles of hFIX in transgenic mice carrying minigene -802(+13G)FIXm1. Human FIX expression profiles in transgenic animals carrying a hFIX minigene -802(+13G)FIXm1 containing a Leyden phenotype mutation A+13G are shown. All experimental procedures as well as animal labels are as described for Fig. 2. Thick green lines represent the pubertal period. Animals composed of 2 males and 1 female showed typical patterns of puberty-onset spontaneous recovery of hFIX expression similar to that observed with animals carrying -802(-20A)hFIXm1, thus recapitulating the spontaneous puberty-onset amelioration seen in hemophilia B Leyden patients.

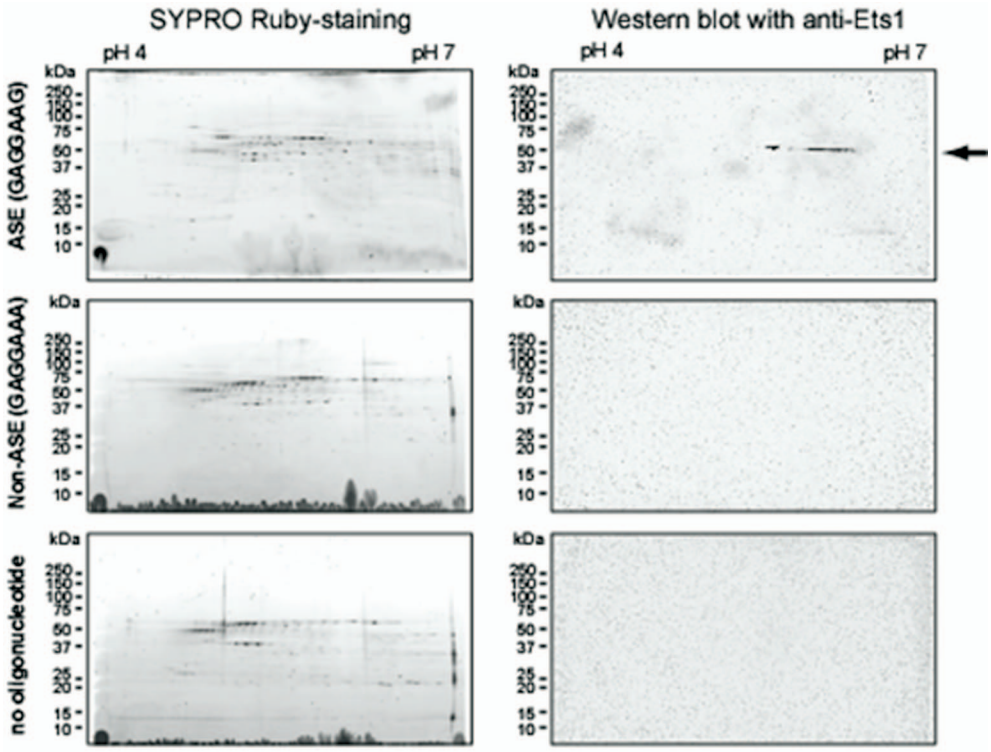

Fig. S2. Identification of the ASE binding protein by preparative EMSAs, 2DE, and Western blot analyses. Matched 2DGE panels (*Left* and *Right*) are shown as pairs. (*Left)* Panels are stained with SYPRO Ruby dye for protein spots; (*Right*) panels are of Western blots with anti-Ets1 antibody. The IEF electrophoresis range is pH 4–7 as shown at the top. Size marker protein positions are shown on the left. (*Top*) Oligonucleotide probe:protein complexes obtained from preparative EMSA with ASE (GAGGAAG) probe were submitted to 2DGE analyses. Arrow on the right side indicates the position of Ets1 protein spots detected by anti-Ets1 antibody. (*Middle*) Similar analyses as in (*Top*) except non-ASE (GAGGAAA) probe was used in preparative EMSA. (*Bottom*) Similar analyses as in the top panels with no oligonucleotide probe used in preparative EMSA.

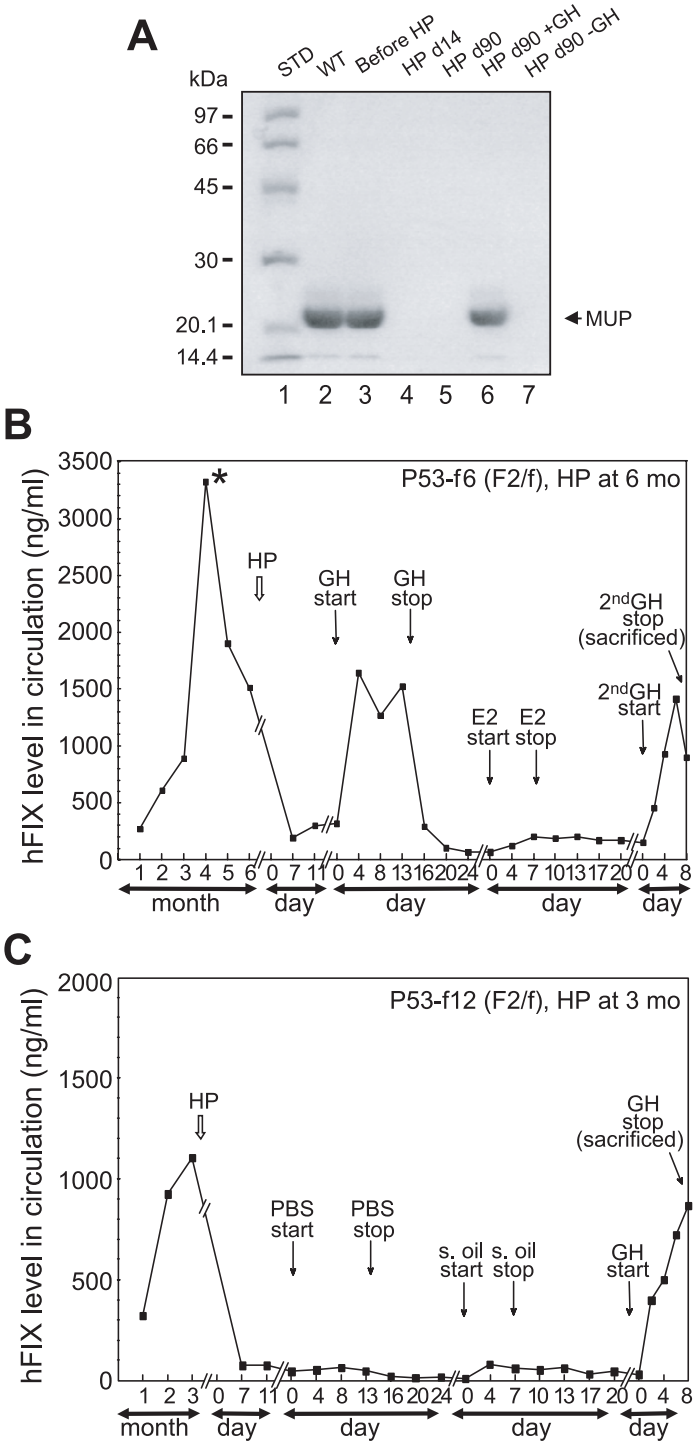

Fig. S3. Effects of hypophysectomy and hormone treatments on transgenic mice with the hemophilia B Leyden phenotype. (*A*) Effects of HP on MUP for a representative Leyden phenotype mouse P44(F2/f) carrying -802(-20A)FIXm1. MUP (approximately 20 kDa) is shown with an arrow on the right and. protein size markers are shown on the left. Aliquots of 2.5 μl urine sample were used for a wild type animal (lane 2) and Leyden phenotype P44(F2/f) animal (lanes 3–7). Lane 1, size markers; lane 2, wild type animal; lane 3, before HP; lane 4, day 14 post-HP; lane 5, day 90 post-HP; lane 6, GH injection (11 days) of day 90 post-HP; lane 7, terminated GH injection (30 days) of day 90 post-HP. (*B*) Effects of a course of HP, GH, E2, and a second series of GH administration, respectively, on hFIX expression in the female animal P53-f6(F2/f) carrying -802(-20A)FIXm1. Arrows indicate time points of treatments including HP, beginnings and ends of GH, E2, and the second series of GH administration, respectively. A greatly elevated hFIX expression peak marked with a star symbol is due to the pregnant condition of this animal at the time point of blood collection. The rest are same as in Fig. 4*A*. Effects of HP and subsequent GH administration on a non-pregnant female animal [P44(F2/f)] also gave similar results (data not shown). This animal was also used for MUP analysis. (*C*) Effects of HP, administrations of PBS containing no GH, sesame oil containing no E2, and a series of GH administration, respectively, on hFIX expression in the control female animal P53-f12(F2/f) carrying -802(-20A) FIXm1. The rest are same as in Fig. 4*A*.

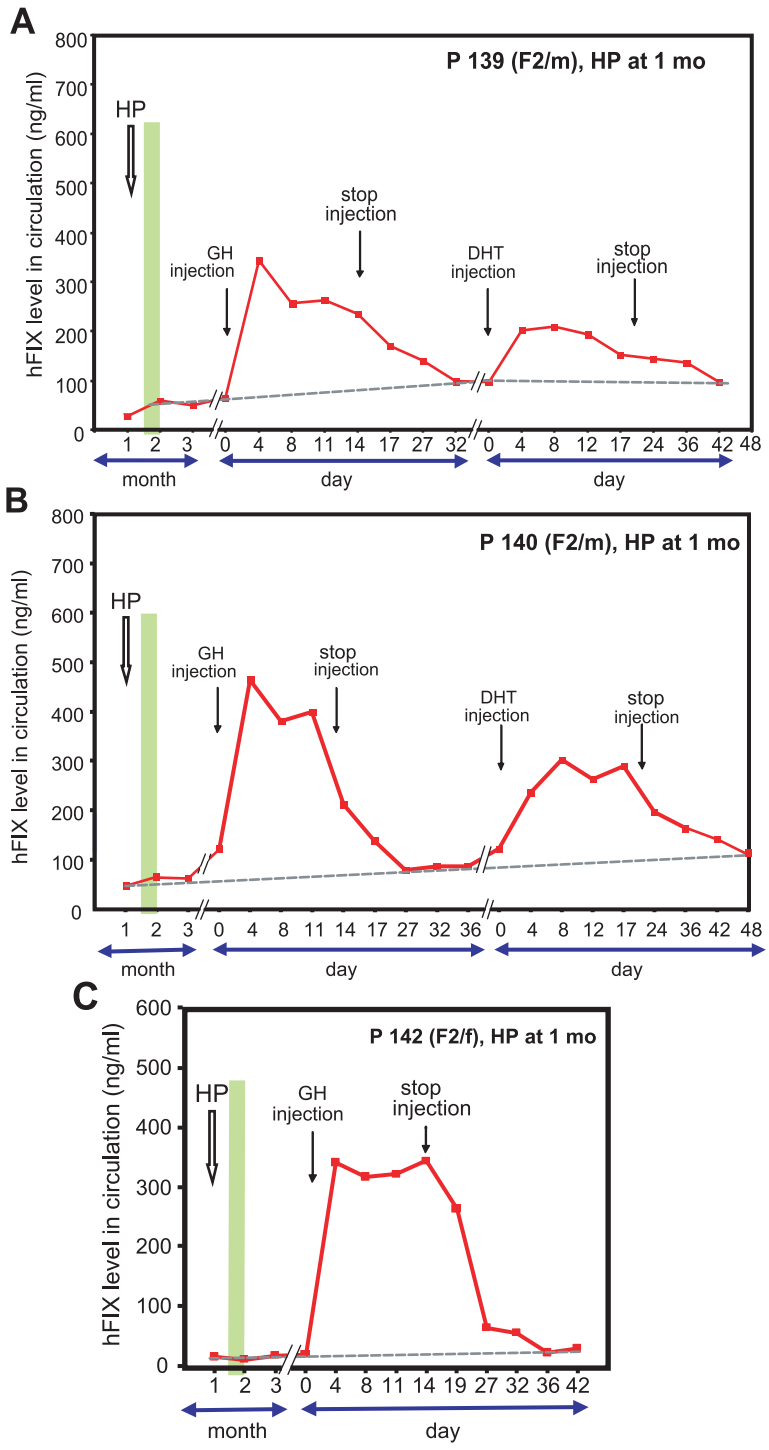

Fig. S4. Effects of hypophysectomy performed before puberty and subsequent administrations of hormones on hFIX expression in transgenic mice carrying -802(-20A)FIXm1. Male animals P139(F2/m) (*A*), P140(F2/m) (*B*), and a female animal P142(F2/f) (*C*) were subjected to HP at one month of age (prepubertal stage) and subsequently administered with GH or DHT. Effects on hFIX expression were monitored by ELISA of serum samples. All procedures were as described in experimental procedures and in Fig. 4 except that hypophysectomy procedures were performed at one month of age. Thick green lines represent the pubertal period. As expected for the Leyden phenotype, these animals showed hFIX expression only at background levels prior or after hypophysectomy with no significant changes. Subsequent GH administrations rapidly increased hFIX expression in both male and female animals as observed with those hypophysectomized at adult ages. Peak hFIX levels in these animals, however, were approximately 60–70% of those observed with animals hypophysectomized at adult ages (Fig. 4*A*). DHT administration also increased hFIX expression in both male animals to lower levels similar to those in male animals hypophysectomized at adult ages (Fig. 4*A*). The female animal P142(F2/f) (*C*) died before E2 treatment. Two animal groups, hypophysectomized either before or after puberty, showed similar responses to GH and DHT treatments. Differences observed between these 2 groups of animals are potentially due to having experienced or not experienced puberty by the time of hypophysectomy.

Table S1. Oligonucleotide probes used for EMSAs

ASE WT	TCAGTCGAGGAAGGATAGGG
ASE m1	TCAGTC**A**AGGAAGGATAGGG
ASE m2	TCAGTC**T**AGGAAGGATAGGG
ASE m3	TCAGTCG**T**GGAAGGATAGGG
ASE m4	TCAGTCG**C**GGGAGGATAGGG
ASE m5	TCAGTCG**G**GGAAGGATAGGG
ASE m6	TCAGTCGA**A**GAAGGATAGGG
ASE m7	TCAGTCGA**T**GAAGGATAGGG
ASE m8	TCAGTCGA**C**GAAGGATAGGG
ASE m9	TCAGTCGAG**A**AAGGATAGGG
ASE m10	TCAGTCGAG**T**AAGGATAGGG
ASE m11	TCAGTCGAG**C**AAGGATAGGG
ASE m12	TCAGTCGAGG**T**AGGATAGGG
ASE m13	TCAGTCGAGG**C**AGGATAGGG
ASE m14	TCAGTCGAGG**G**AGGATAGGG
ASE m15	TCAGTCGAGGA**T**GGATAGGG
ASE m16	TCAGTCGAGGA**C**GGATAGGG
ASE m17	TCAGTCGAGGA**G**GGATAGGG
ASE m18	TCAGTCGAGGAA**C**GATAGGG
ASE m19	TCAGTCGAGGAA**A**GATAGGG
ASE m20	TCAGTCGAGGAA**T**GATAGGG

The WT 20-mer double-stranded (ds) oligonucleotide ASE probe is shown on top. Each probe of ASE m1 through ASE m20 contains a single nucleotide mutation in the ASE-like core heptamer sequence placed in the middle of the probe sequence (shown with underline).

Table S2. Protein binding to core heptamer sequences and competition

Core heptamer sequence[a]							Protein binding[c]		
							Liver NE	HepG2 NE	Competition[d]
G/C	A	G	G	A	A	G[b]	+	+	+
A	A	G	G	A	A	G	-	−	
T	A	G	G	A	A	G	-	−	
G	T	G	G	A	A	G	-	−	
G	C	G	G	A	A	G	-	−	
G	G	G	G	A	A	G	-	−	
G	A	A	G	A	A	G	-	−	
G	A	T	G	A	A	G	-	−	
G	A	C	G	A	A	G	-	+	−
G	A	G	A	A	A	G	-	−	
G	A	G	T	A	A	G	-	−	
G	A	G	C	A	A	G	-	+	−
G	A	G	G	T	A	G	-	−	
G	A	G	G	C	A	G	-	−	
G	A	G	G	G	A	G	-	−	
G	A	G	G	A	T	G	+	+	−
G	A	G	G	A	C	G	-	−	
G	A	G	G	A	G	G	-	−	
G	A	G	G	A	A	C	-	+	−
G	A	G	G	A	A	A	+	+	−
G	A	G	G	A	A	T	-	−	

[a]Underline indicate nucleotide sequence different from the functional ASE.
[b]Known functional ASE sequences (1, 2).
[c]Protein binding was assayed by EMSA. +, bound; *, unbound.
[d]Competitive EMSA was done using non-radiolabeled oligonucleotide containing a functional ASE sequence (**TCAGTCGAGGAAGGATAGGG**) as the competitor.