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25. Plasmids pKRN and phTLR2 (*9*) were prepared with the Endotoxin-free Plasmid Maxi kit (Qiagen). 293 cells were transfected with the Effectene Transfection reagent (Qiagen). Thirty hours after transfection, an equal volume of diluent or diluent containing sBLP (200 ng/ml) was added. Thirty-six hours later the cells were stained with Annexin V–biotin (Pharmingen) and Streptavidin Tri-Color (Caltag) and analyzed by flow cytometry.
26. THP-1 cells (American Type Culture Collection) were maintained in RPMI 1640, 10% FBS, penicillin, streptomycin, and 2 mM L-glutamine. Six hours after seeding THP-1 cells in RPMI 1640, the indicated compounds diluted in RPMI 1640, 0.05% HSA were added. Six hours later, cell death was quantified by a lactate dehydrogenase release assay (Promega). Where indicated, cells were first treated with 32 nM PMA for 6 hours or cotreated with cycloheximide (50 μg/ml). PMA and cylcoheximide diluents (dimethyl sulfoxide and ethanol) were not cytotoxic.
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34. We thank D. Littman, V. Dixit, C. Scharff, and J. Moss for critical reading of the manuscript, M. Garabedian for discussions, and T. Neubert for mass spectrometry analysis. pCDNA3 and pGFP were a gift of the Littman Lab. A.O.A. was supported by a grant from the Life and Health Insurance Fund. This work was supported by grants from the National Institute of Allergy and Infectious Diseases (AI 37720-04 to A.Z. and AI-38894 to J.D.R.).

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Genetic Mechanisms of Age Regulation of Human Blood Coagulation Factor IX

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Blood coagulation capacity increases with age in healthy individuals. Through extensive longitudinal analyses of human factor IX gene expression in transgenic mice, two essential age-regulatory elements, AE5′ and AE3′, have been identified. These elements are required and together are sufficient for normal age regulation of factor IX expression. AE5′, a PEA-3 related element present in the 5′ upstream region of the gene encoding factor IX, is responsible for age-stable expression of the gene. AE3′, in the middle of the 3′ untranslated region, is responsible for age-associated elevation in messenger RNA levels. In a concerted manner, AE5′ and AE3′ recapitulate natural patterns of the advancing age–associated increase in factor IX gene expression.

Blood coagulation potential in humans, as well as in other mammals, reaches the young adult level around the age of weaning (*1*), followed by a gradual increase during young adulthood and almost doubling by old age (*2*). This age-associated increase in coagulation potential takes place in healthy centenarians (*3*), indicating that the increase is a normal age-associated phenomenon. It may also contribute to the development and progression of age-associated cardiovascular and thrombotic disorders (*4*). This increase in blood coagulation potential coincides with increases in plasma levels of procoagulant factors such as factor IX, whereas plasma levels of anticoagulation factors (such as antithrombin III and protein C) or of factors involved in fibrinolysis are only marginally affected (*5*).

Blood coagulation factor IX (FIX), a plasma protease precursor, occupies a key position in the blood coagulation cascade where the intrinsic and extrinsic pathways merge (*6*). FIX is synthesized in the liver with strict tissue specificity, and its deficiency results in the bleeding disorder hemophilia B. Like human FIX (hFIX), mouse FIX (mFIX) plasma activity increases with age. Mouse FIX plasma activity is also directly correlated with an increase in the level of liver mFIX mRNA (*2*).

To explore the molecular mechanisms that underlie the age-related increase in FIX activity, we constructed a series of hFIX minigene expression vectors (*7*). We first analyzed them in vitro with HepG2 cells, a human hepatoma cell line, and then generated transgenic mice with the hFIX minigene vectors and carried out longitudinal analyses of hFIX expression in vivo for the entire lifespans of founders and successive generations of transgenic mice. The use of hFIX minigene expression vectors, which can produce high levels of plasma hFIX in vitro and in vivo, provided both a reliable animal assay system and multiple unexpected critical insights into the regulatory mechanisms of the hFIX gene. These minigenes consisted of the hFIX homologous components, including promoter sequences of various lengths spanning up to nucleotide (nt) −2231 in the 5′ flanking region, the coding region with a middle-portion truncated first intron, and the

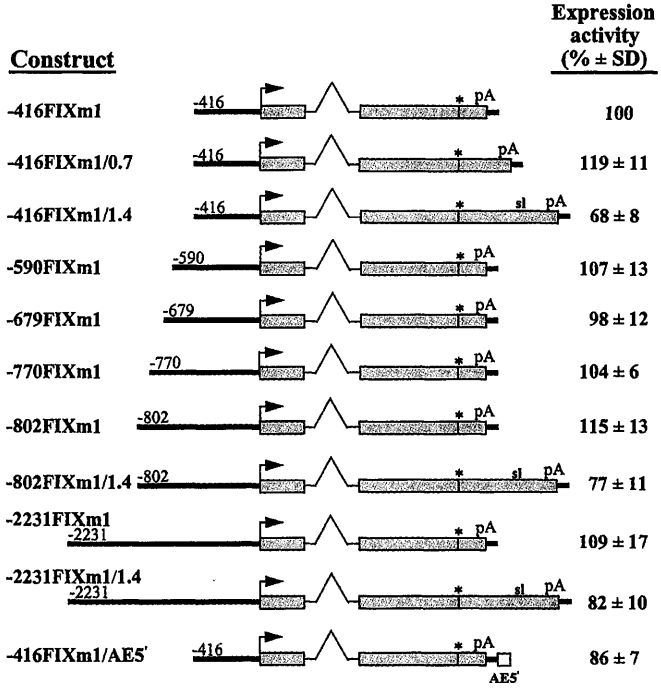

Fig. 1. Human FIX minigene expression constructs and in vitro transient expression activities. The structure is depicted with the pro-moter regions (thick horizontal lines at left) with the 5′ terminal nt number. Transcribed hFIX regions (gray rectangles, with thin peaked lines representing the shortened first intron) are followed by 3′ flanking sequence regions (thick horizontal lines at right). Transient expression activities are expressed relative to that of −416FIXm1 (~50 ng per 10^6 cells per 48 hours in four to five independent assays). Activities were normalized to the size of the minigenes used. Arrow, transcription start site; asterisk, translation stop codon; pA, polyadenylation; sl, potential stem-loop forming dinucleotide repeats.

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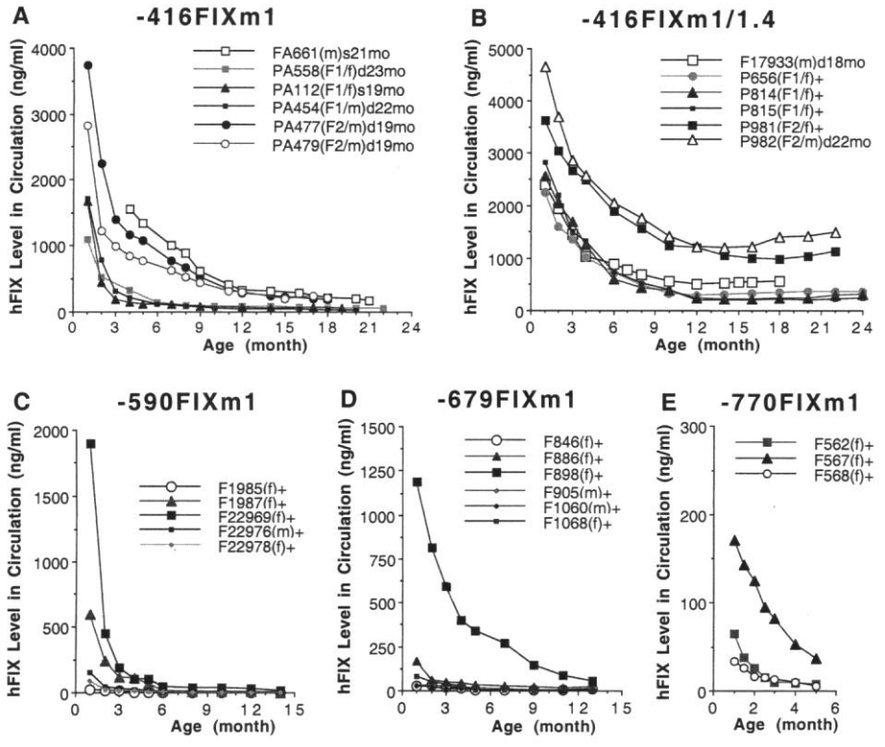
complete or middle-portion deleted 3′ untranslated region (UTR) (Fig. 1). All constructs showed similar high transient hFIX expression in HepG2 cells (~50 ng per 10^6 cells per 48 hours) (*8*). However, all constructs containing the complete 3′ UTR, including a 102–base pair (bp) stretch of AT, GT, and GC dinucleotide repeats (*9*), reproducibly showed expression activity levels 25 to 30% lower than those of corresponding minigenes lacking the region containing repeat sequences (Fig. 1). Dinucleotide repeats similar to those seen in the hFIX 3′ UTR, which can form stable stem-loop (sl) structures in mRNA, have been implicated in controlling mRNA stability in mammals as well as in yeast and plants (*10*). Together, these results suggest a similar negative regulatory activity for this sl structure of the hFIX gene in the HepG2 assay system.



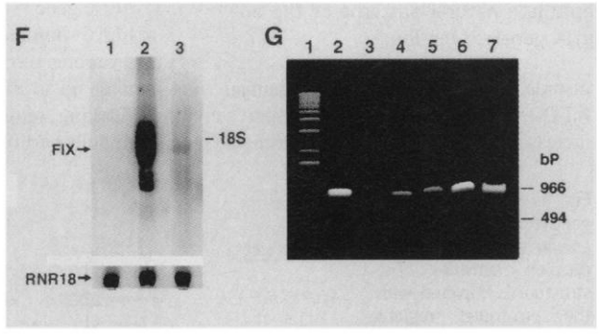


Fig. 2. Longitudinal analyses of hFIX levels in the circulation of transgenic mice carrying hFIX minigenes. (**A**) −416FIXm1. (**B**) −416FIXm1/1.4. (**C**) −590 FIXm1. (**D**) −679FIXm1. (**E**) −770FIXm1. At various time points, blood samples were obtained from snipped tail ends. Human FIX concentrations were determined by duplicated hFIX-specific ELISA; average values are plotted (*11*). The duplicated ELISA values varied less than 11% from the averages. Animals are identified by the following format: (F, founder; P, progeny)(identification number)(progeny generation)(sex)(status: +, alive in good health; d, died; s, sacrificed)(age at death or killing). (**F**) Human FIX mRNA levels in the liver of young (PA412: F_1/f, 1 month of age) and old (PA112; F_1/f, 19 months of age) transgenic animals carrying −416FIXm1. Levels of hFIX mRNA were analyzed by Northern blot analysis of total liver RNA (loaded with 15 μg per lane) (*11*). PA412 and PA112 animals were from the same litter produced by the founder FA661, and expressed 1252 and 1675 ng of circulatory hFIX per milliliter, respectively, at 1 month of age. Animal PA112 was expressing 63.8 ng/ml of serum hFIX when it was killed. The hybridization probe used was the Ssp I–Bam HI fragment of hFIX coding region of −416FIXm1 (upper portion), and equivalent loading of RNA samples was confirmed by rehybridization with RNR18 probe (lower portion). Lane 1, nontransgenic mouse liver RNA; lane 2, transgenic PA412 liver RNA; lane 3, transgenic PA112 liver RNA. (**G**) Quantitative multiplex PCR analysis was carried out to determine the relative transgene levels in tail and liver tissues. Genomic DNA was extracted from snipped tail tissue of a transgenic −416FIXm1 animal (PA112) at 3 weeks and at 19 months of age. Liver DNA was extracted from the same animal (PA112) killed at 19 months of age and a −416FIXm1 animal (PA412) killed at 1 month of age. The positions of the hFIX-specific fragment (966 bp) and the mouse β-globin specific fragment (internal copy number control, 494 bp), which were obtained by multiplex PCR, are shown on the right. Lane 1, kilobase size ladder; lane 2, fragment size control amplified from −416FIXm1 plasmid; lane 3, nontransgenic mouse tail DNA used as template; lane 4, tail DNA of PA112 at 3 weeks of age; lane 5, tail DNA of PA112 at 19 months of age; lane 6, liver DNA of PA412 at 1 month of age; lane 7, liver DNA of PA112 at 19 months of age. Human FIX transgene copy numbers relative to the mouse β-globin gene for 1-month-old versus 19-month-old animals were 1.0 to 1.1 for both tail and liver genomic DNAs. The Multi Analyst program from BioRad was used for quantitation and calculation of ratios.

Circulatory hFIX levels were monitored during longitudinal analyses of transgenic mice carrying various hFIX minigene transgenes (Fig. 2) (*11*). To ensure reproducibility, three to six independent founder lines were generated for each minigene construct, and animals from at least three representative lines were subjected to longitudinal analyses for their entire lifespans. At 1 month of age, the mice carrying −416 FIXm1 minigenes produced hFIX in amounts ranging from as high as that of natural hFIX gene expression (~4 μg/ml) to much lower levels (Fig. 2A). Such variations are primarily due to transgene positional effects. Circulatory hFIX levels, however, declined drastically through puberty and during the subsequent 2- to 3-month period, and then remained stable. This rapid age-dependent decline in the circulatory hFIX level was observed in all animals (n = 69) regardless of founder line, initial prepubertal hFIX level, generation (founders and F_1 or F_2 progeny), sex, or the zygosity status of the transgenes. The decline in hFIX level was correlated with a similar decline in the steady-state level of liver hFIX mRNA (Fig. 2F), which was not due to a loss of the hFIX transgene with age (Fig. 2G). Furthermore, when 4- to 5-month-old mice with much decreased hFIX levels had progeny, their pups showed prepubertal high hFIX expression levels that were equivalent to those of their parents at the same time point (1 month of age).

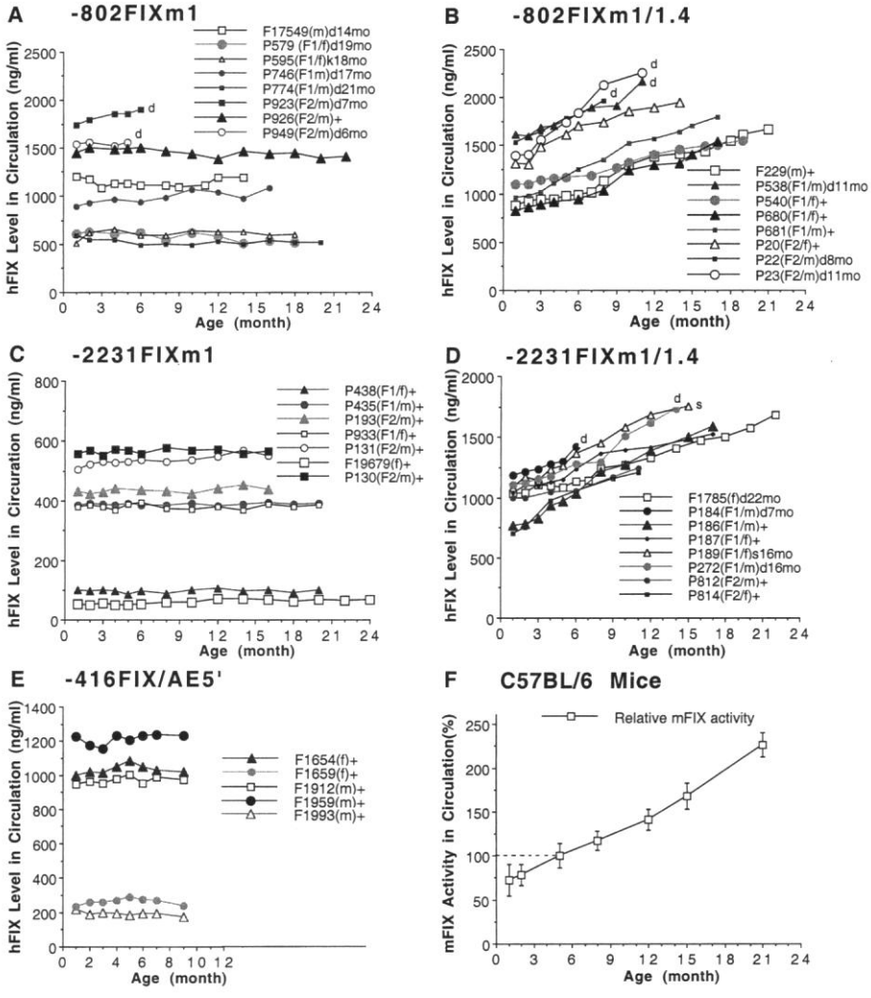
Transgenic mice with −416FIXm1/1.4 minigenes (n = 48) showed prepubertal high levels of hFIX and a subsequent age-dependent decline similar to those mice with −416FIXm1 minigenes, although the decline was less steep and expression levels were stabilized at significantly higher levels than those observed for −416FIXm1 (Fig. 2B). These results indicate that the presence of the complete 3′ UTR containing the extensive dinucleotide repeat structure does not rescue hFIX expression from age-related decline, regardless of founder line, initial prepubertal hFIX level, generation, sex, or the zygosity status of the transgenes. All animals carrying minigenes −590FIXm1 and −679FIXm1 (n = 25 and 26, respectively) also showed an age-related rapid decline in hFIX expression similar to that seen in animals carrying −416FIXm1 (Fig. 2, C and D). Further-

more, hFIX expression levels in three independent founder animals carrying −770 FIXm1 also rapidly decreased over the puberty period in a pattern similar to that produced by the above minigenes (Fig. 2E). Thus, minigenes with the promoter region up to nt −770 still lack an essential structural element or elements required for normal age-associated regulation of hFIX gene expression.

In contrast, striking differences in hFIX expression patterns were observed in animals carrying the minigene −802FIXm1 (Fig. 3A), which is composed of a vector frame identical to −416FIXm1, except that the 5′ end flanking sequence included was extended to nt −802 (Fig. 1) (*12*). All of these animals (n = 62) invariably showed age-stable plasma hFIX levels for their entire lifespans, mostly up to 20 to 24 months of age. Age-stable circulatory hFIX levels were correlated with age-stable mRNA levels (Fig. 3G, lanes 2 and 3). These observations with −802 FIXm1 were further supported by age-stable hFIX expression by mice carrying −2231FIXm1 (Fig. 3C). Together, these results suggest that the critical structural element required for age-stable expression of the hFIX gene resides in the small region spanning nt −770 through −802. We designated this small region "age-regulatory element in the 5′ end" (AE5′). This region contains a sequence element, GAGGAAG (nt −784 to −790) that matches the consensus motif (C/G)AGGA(A/T)G of PEA-3 (*13*), a member of the Ets family of transcriptional factors (*14*). The function of AE5′ is position-independent, as shown by age-stable hFIX expression in animals carrying −416FIXm1/AE5′, in which an AE5′-containing region was moved to the 3′ end outside of the hFIX minigene (Fig. 3E).

Footprint analysis of the region nt −665 through −805 with aged mouse liver nuclear extracts showed a major footprint (nt −784 through −802), a minor footprint (nt −721 through −728), and a deoxyribonuclease (DNase) hypersensitive region (nt −670 through −714) (Fig. 4). With nuclear extracts from 1-month-old animals, no such clear footprints were observed. In agreement with these results, gel electrophoretic mobility shift (bandshift) assays showed an increase in protein binding with the nuclear extracts of aged mice (19 months of age) (Fig. 5A). Bandshifts were competitively reduced with excess amounts of oligonucleotides harboring the PEA-3 motif but not with oligonucleotides harboring a mutant PEA-3 motif sequence (Fig. 5B), thus confirming the presence of a PEA-3 element. However, the possibility of the binding of other nuclear factors closely related to PEA-3, resulting in AE5′ function, cannot be completely ruled out at this stage of study.

The PEA-3 element in the hFIX gene may have been generated through the evolutionary drift of a long interspersed repetitive element (Line 1 or L1) sequence originally recruited via its retro-transposition into the 5′ location. Modern retrotransposable L1 (*15*) does not have the corresponding PEA-3 element. The key PEA-3 element of AE5′ resides within



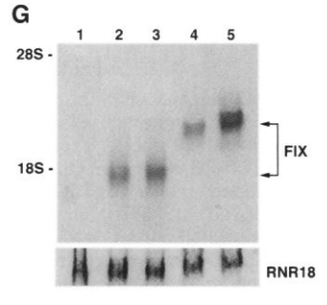


Fig. 3. Longitudinal analyses of hFIX in the circulation of transgenic mice carrying hFIX minigenes. (**A**) −802FIXm1, (**B**) −802FIXm1/1.4, (**C**) −2231 FIXm1, (**D**) −2231FIXm1/1.4, (**E**) −416FIXm1/AE5′. Blood samples were obtained from snipped tail ends, and hFIX concentrations in the circulation were determined as described in Fig. 2. Animal lines are marked according to the format in Fig. 2. Mice that died at much younger ages than their normal life expectancies are marked with a d. (**F**) Age-dependent increase of murine plasma FIX activity (aPTT) in normal nontransgenic C57BL/6 mice (n = 6 to 9 per age group) (*11*). (**G**) Northern blot analyses of transgenic mice carrying −802FIXm1 and −802FIXm1/1.4. The hFIX mRNA levels in the liver of 1-month-old (young) or 22-month-old (aged) mice carrying −802FIXm1 (mouse P327 or P738, respectively) and −802FIXm1/1.4 (mouse P32 and P13, respectively). These animals were from the same litter produced by the founder F17549 for −802FIX m1 and F229 for −802 FIXm1/1.4 [(A) and (B)]. When they were killed, P738 and P13 were expressing 2252 and 1865 ng/ml of hFIX, respectively. Total liver RNA was used for the Northern blot analysis (15-μg aliquot per lane) as described in Fig. 2F. The upper panel shows hFIX mRNA levels probed with the Ssp I–Bam HI fragment of hFIX coding region of −416FIXm1; the lower panel shows RNR18 mRNA levels. Lane 1, nontransgenic mouse liver RNA; lane 2, transgenic P327 liver RNA; lane 3, transgenic P738 liver RNA; lane 4, transgenic P32 liver RNA; lane 5, transgenic P13 liver RNA. A PhosphorImager (Molecular Dynamics) was used for quantitation of mRNA levels (counts), and ratios of young and old were calculated. Young and old animals carrying −802FIXm1 showed no significant differences in the mRNA level (the ratio of old over young was 1.13). In contrast, −802FIXm1/1.4 animals showed a substantial elevation in the mRNA level with older age (the ratio of old over young was 2.05).

the L1-derived sequence retaining a 63 to 70% similarity with the ORF2 region of the modern retrotransposable L1 in the 5′ to 3′ orientation. The mFIX gene also has the L1-derived sequence in its 5′ end region in a similar position as in the hFIX gene and has multiple PEA-3 consensus elements (*16*). The pattern of age regulation of the mFIX gene is very similar to that of the hFIX gene (*2*).

Because transgenes of −416FIXm1, −590FIXm1, −679FIXm1, and −770FIXm1 differ from the minigenes −802FIXm1 and −2231FIXm1 only in their promoters, the hFIX mRNA from all of these minigenes (an intron-spliced form of FIXm1 RNA) should produce identical hFIX protein. Thus, the age-dependent decline in the circulatory hFIX level observed in animals with −416FIXm1, −590FIXm1, −679FIXm1, and −770FIXm1, but not with −802FIXm1 and −2231 FIXm1, must be due to an age-dependent decline in the transcriptional activity of the transgenes. This agrees with the fact that no significant changes in hFIX mRNA levels in the liver were observed with age in animals carrying −802FIXm1 (Fig. 3G, lanes 2 and 3), whereas age-dependent declines in steady-state mRNA levels were observed in those carrying −416FIXm1 (Fig. 2F). Furthermore, the hFIX turnover time from the circulation does not change significantly in vivo with increasing age, from youth (2 months), to middle age (9 to 10 months), to old age (19 to 23 months) (*17*) (Table 1). Unlike in the HepG2 cell assay system results (Fig. 1), the presence or absence of AE5′ in vivo makes a drastic age-dependent difference in hFIX gene expression, demonstrating that in vivo longitudinal analysis is essential for studying age regulation of a gene.

Mice with −802FIXm1/1.4 (n = 48), which contains the complete 3′ UTR, showed an advancing age-associated increase in hFIX levels in the circulation (Fig. 3B). This unexpected age-associated increase in circulatory hFIX levels was directly correlated with an increased level of liver hFIX mRNA (Fig. 3G, lanes 4 and 5) and indicated the presence of another critical age-regulatory element, designated AE3′. AE3′ is located in the middle of the 3′ UTR, where an extensive stretch of dinucleotide repeating structures is contained. In the presence of AE5′, AE3′ confers a crucial age-associated increase in hFIX expression. This conclusion was further supported by the results obtained from mice carrying −2231FIXm1 /1.4 (n = 42) (Fig. 3D). The unique concerted function conferred by the combination of AE5′ and AE3′ was independent of founder line, initial expression levels at 1 month of age, sex, generation, or the zygosity status of animals (*18*).

As shown in in vitro assays with HepG2 cells (Fig. 1), the AE3′-containing region does not function as an enhancer for the promoter but rather exhibits weak down-regulatory effects on hFIX production. AE3′ alone can, to some extent, elevate age-dependent hFIX expression in the absence of AE5′ (Fig. 2, A and B; compare −416FIXm1 with −416FIXm1/1.4), but the combination of AE5′ and AE3′ is needed to recapitulate the physiological advancing age–associated increase of the natural FIX in circulation observed in humans (*2*) as well as mice (Fig. 3F). AE3′ can substantially increase the steady-state hFIX mRNA level (Fig. 3G). Together, these results may suggest that AE3′ probably functions to increase hFIX mRNA stability, which directly correlates with an increase in the circulatory hFIX level. Other possible mechanisms responsible for AE3′ action, however, remain to be explored. Thus, in addi-

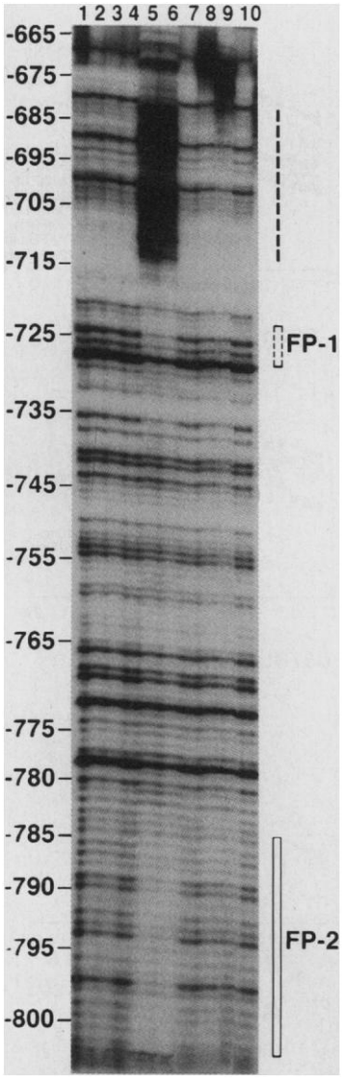


Fig. 4. Footprint analysis of the region nt −665 to nt −805. The fragments used were amplified by PCR with the ^{32}P-labeled 5′ primer ATGGTTAACTGACTTACGAA (nt −833 through −814) and 3′ unlabeled primer GCTCCATTTTGAGTTAATATTTGTGT (nt −657 through −682). The nuclear extracts (NEs) from HepG2 human hepatoma cells and livers of young (1 month of age) and old (19 months of age) mice were prepared as we previously reported (*23*). Various amounts of NEs (0, 100, and 150 μg) were incubated with the labeled fragments [30,000 counts per minute (cpm)] for 1 hour on ice and subjected to DNase 1 digestion (0.5 U) for 2 min at room temperature. Lanes 1, 4, 7, and 10: without NEs; lanes 2 and 3: with 100 μg and 150 μg of HepG2 cell NEs, respectively; lanes 5 and 6: with 100 μg and 150 μg of NEs from old mice, respectively; lanes 8 and 9: with 100 μg and 150 μg of NEs from young mice, respectively. Major and minor footprints and apparent DNase hypersensitivity sites are marked as FP-1, FP-2, and with a vertical dotted line, respectively.

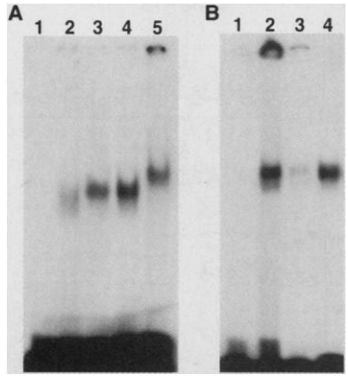


Fig. 5. Gel electrophoretic mobility shift assay using mouse liver NEs from three different age groups. NEs were prepared from 1-, 5-, or 19-month-old mouse livers (as described in Fig. 4). (**A**) Double-stranded oligonucleotides containing a PEA-3 element extending from nt −797 to −776 of the hFIX gene (TTCAGTCGAGGAAGGATAGGGT) were ^{32}P-labeled at the 5′ end to a specific activity of 1.9×10^9 cpm. Aliquots of the radiolabeled oligonucleotide (20,000 cpm) were incubated with 10 μg of NEs in the presence of 1 μg of poly dI-dC in DNA binding buffer for 20 min at room temperature and subjected to polyacrylamide gel electrophoresis (*23*). Lane 1, without NEs; lane 2, with NEs of 1-month-old mice; lane 3, with NEs of 5-month-old mice; lane 4, with NEs of 19-month-old mice; lane 5, with mouse brain NEs (positive control for PEA-3, showing a slightly higher size of shifted band). (**B**) The competition assay for ^{32}P-labeled double-stranded oligonucleotides containing the PEA-3 element is shown. A 100-fold excess of unlabeled oligonucleotide described in (A) or of mutant oligonucleotide (TTCAGTCGGTTGGTGATAGGGT, with mutated sequences underlined) was incubated with 10 μg of 19-month-old mouse liver NEs for 5 min, followed by addition of ^{32}P-labeled oligonucleotides as described in (A). Lane 1, without NEs; lane 2, with NEs; lane 3, with NEs and wild-type competitor; lane 4, with NEs and mutant competitor.

Table 1. The clearance time of hFIX in mice.

Age (months)	Clearance time ($T_{1/2}$ of hFIX)
2	16.8 ± 0.21
9–10	17.4 ± 0.55
19–23	16.9 ± 0.35

tion to the novel function identified for AE5′ (PEA-3 or a closely related element), we have identified another unique structure in the 3′ UTR, AE3′, which is responsible for the 3′ UTR's critical function in age regulation of the hFIX gene. This function of AE3′ presumably is due to the sl structure–forming dinucleotide repeats that are present in the 3′ UTR (*10*), although final confirmation of this hypothesis must be done in longitudinal animal experiments.

The present study established the basic molecular mechanisms of age-associated increases in circulatory hFIX. This will facilitate determination of the overall mechanisms of age-associated increases in blood coagulation potential, the reason why such an increase occurs even in healthy individuals, and its correlation with the aging-associated increase in cardiovascular and thrombotic diseases.

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7. Construction of hFIX minigene expression vectors was carried out with −416FIXm1 as the starting construct (*19*). The nt numbering system used in this study was based on the complete hFIX gene sequence, as we previously reported (*9*). Minigene −416FIXm1/1.4 was constructed from −416FIXm1 by inserting the middle portion of the 3′ UTR (1.2 kb in size), which was generated by polymerase chain reaction (PCR) using 5′ and 3′ primers with Bam HI linker, TAACAGGATCCG-GCCTCTCACTAACTAATCAC (nt +31418 through +31438), and CAACTGGATCCAAGATTCAAGATA-GAAGGAT (nt +32690 through +32671), respectively, and human genomic DNA as the template. The Bam HI fragment obtained by digestion of the PCR product was inserted into the 3′ UTR Bam HI site of −416FIXm1, thus producing −416FIXm1/1.4, which contained the entire 3′ UTR. −416FIXm1/0.7 was constructed by inserting the PCR-amplified 700-bp fragment with Bam HI linker, containing the 3′ contiguous sequence to nt +32117. Minigenes −590FIXm1, −679FIXm1, −770FIXm1, −802FIXm1, and −2231FIXm1 were produced by replacing the 5′ end 433-bp sequence of −416FIXm1 released by Sph I–Nhe I digestion with 607-, 696-, 787-, 819-, and 2248-bp fragments containing the 5′ end hFIX region extended up to nt −590, −679, −770, −802, and −2231, respectively. −416FIX m1/AE5′ was constructed by inserting the Kpn I fragment generated by PCR (nt −802 through nt −417) into the −416FIXm1 vector [the Kpn I site is outside of the FIX gene (Fig. 1)]. The 5′ and 3′ primers used for PCR were CTTGGTACCAGCCATTCAGTCGAGGAAGG (nt −802 through −783) and CTTGGTACCATATGAATC-CTTTCATAGAT (nt −417 through −436), respectively. All constructs were sequenced through PCR-amplified regions as well as fragment ligation sites to confirm correct sequences and orientations. Transient in vitro expression activities of hFIX minigene constructs were assayed using HepG2 cells and an hFIX-specific enzyme-linked immunosorbent assay (ELISA) as previously described (*19*) with some modifications. Cell transfection was carried out by the calcium phosphate–DNA conjugate method or, later, by the use of FuGene 6 (Boehringer-Mannheim). The latter improved transfection method consistently increased transfection efficiency to >20% [S. Kurachi, L. Sze, K. Kurachi, *Biochemica* **3**, 43 (1998)], and all earlier assays were reexamined with FuGene 6. Four to five independent assays were carried out, and the averages were shown with standard deviations.
8. In the HepG2 cell assay system, these hFIX minigenes do not show any down-regulation in the presence of the 5′ upstream region (nt −802 up through nt −1900) (Fig. 1). In contrast, when a CAT reporter gene was used, negative regulatory elements were identified in this region (*20*). The use of a heterologous reporter gene to analyze unrelated genes may produce irrelevant results that are greatly skewed from natural gene regulation, presumably due to the absence of critical structural elements in the heterologous reporter gene that are needed for regulation of the native genes in concert with their own 5′ promoters. Alternatively, structural elements in the heterologous genes may also affect the transcriptional regulation of the genes being studied, thus producing irrelevant results.
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11. Transgenic animals were constructed according to standard methods (*21*) at the Biomedical Research Animal Model Core facility at the University of Michigan, Ann Arbor. Human FIX minigene vector plasmids were digested with Sph I–Kpn I, and the hFIX minigene–containing fragments released were isolated by 0.8% agarose gel electrophoresis, followed by purification with SpinBind DNA extraction units (FMC, Chicago, IL). Fertilized eggs of C57BL/6 × SJL mice were microinjected with the DNA (1 to 2 ng per egg) and implanted into foster mother animals (CD-1). Offspring produced were screened for founder animals with high transgene copy numbers (5 to 10 copies per genome) using quantitative multiplex PCR analyses of tail tissue DNA samples. Two pairs of primers were used: one specific to the 966-bp hFIX transgene fragment corresponding to exons 4 to 8 [nt +6172 to +30867 in the genomic nucleotide numbering (*9*)] and one that produced a 494-bp mouse β-globin gene fragment (endogenous control) [nt +2590 to +3083 (*22*)]. PCR was initiated with 3 min of incubation at 94°C, followed by 25 cycles of 94°C for 30 s, 65°C annealing for 1 min, and 72°C extension for 2 min. Founders were back-crossed with nontransgenic mice (C57BL/6 × SJL) to generate F_1 progeny animals. Homozygous F_2 animals were generated by crossing among heterozygous F_1 littermates and subsequent generations were similarly produced. The zygosity status of animals was determined by quantitative multiplex PCR analysis as described above. At minimum, three founder lines for each minigene construct were subjected to longitudinal analysis for their entire lifespans up to 2 years. At various ages, starting at 1 month of age, transgenic mice were individually subjected to blood sample collection (aliquots of ~100 μl) via tail-tip snipping, and the serum obtained was routinely used to quantify circulatory hFIX levels using duplicated ELISA for each age point (*7*). Pooled human plasma (George King Bio-Medical, Overland Park, KS) was used to prepare a hFIX standard curve for each assay. In order to minimize experimental fluctuations from assay to assay in the longitudinal analysis, overlapped serum samples from the previous assay group were included in each assay. Murine plasma FIX activity in the circulation [activated partial thromboplastin time (aPTT)] of normal nontransgenic mice (C57BL/6) was determined with citrated plasma samples prepared from blood samples obtained by retro-orbital collection as we previously described (*2*). Northern (RNA) blot analyses of the liver RNA samples from animals (15 μg per lane) were carried out as previously described (*19*) using the ^{32}P-labeled Ssp I–Bam HI fragment (the 3′ half of the hFIX coding region −416FIXm1) as a probe and employing stringent washing conditions to eliminate probe hybridization to the mFIX mRNA. To confirm the presence of equivalent amounts of RNA in each lane, the filters were reprobed with the RNR18 cDNA (ribosomal RNA 18*S*). All animal experiments were carried out in accordance with the institutional guidelines of the University of Michigan (Office for Protection from Research Risks no. A3114-01).
12. Liver expression of hFIX observed for minigenes without AE5′ (–770FIXm1 remains to be tested) was high but not as robust as that seen with the natural hFIX gene; expression in other tissues, such as kidney, lung, and muscle, was as high as ~20% of the liver level. In contrast, −802FIXm1 showed virtually complete liver-specific hFIX expression, similar to that for the natural FIX gene (S. Kurachi and K. Kurachi, data not shown).
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17. The effects of age on hFIX clearance from the circulation were tested as follows. Aliquots of plasma-derived hFIX preparation (Haematologic Technologies, Sussex Junction, VT; 5 μg per 0.1 ml of phosphate-buffered saline) were injected via tail vein into normal animals at 2, 9 to 10, and 19 to 23 months of age (n = 3 per age group), which had the same genetic background as the transgenic mice (C57BL/6 × SJL). The hFIX level in circulation was monitored by ELISA of collected blood samples (~50-μl aliquots) at 10 min and 2, 6, 12, 18, 24, 30, 36, and 48 hours after protein injection. As expected, all animals of different age groups showed typical biphasic clearance kinetics (two-compartment distribution and clearance) with a initial rapid clearance phase (α phase), followed by a slower clearance phase (β phase). Half-clearance times (17.8 hours) observed for 2-month-old BALB/c mice were very similar to those observed for C57BL/6 mice.
18. Both males and females with sustained high hFIX levels in circulation (approximately 1500 ng/ml or higher) tended to die at a much earlier age than their expected lifespan (~2 years) (Fig. 3, A, B, and D). These transgenic mice had hFIX in addition to their own mFIX, suggesting the possibility of lethal thrombosis in these animals.
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