Age-Specific Regulation of Clotting Factor IX Gene Expression in Normal and Transgenic Mice

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Factor IX (FIX), a circulating serine protease that serves as an essential component of the blood coagulation pathway, has been shown to increase with age in humans. We show here that murine FIX mRNA and activity levels also increase with age. Furthermore, one form of hemophilia B, hemophilia B Leyden, which is caused by mutations within the promoter region of the FIX gene, has a distinct age-dependent phenotype. To determine the source of the age-related increases in FIX gene expression, we have analyzed the regulation of the normal FIX gene promoter and FIX Leyden gene promoter with the +13 mutation during aging by generating transgenic mice that contain the −189 to +21 bp promoter segment ligated to a chloramphenicol acetyltransferase reporter gene. We have established that the normal FIX promoter and the Leyden promoter transgenes are expressed in a tissue-specific manner in vivo. The normal FIX promoter transgene does not show any differences in the pattern of expression with age or sex of the organism, whereas the Leyden promoter transgene showed age-dependent male-specific expression. This is the first demonstration of the FIX Leyden phenotype in a transgenic mouse model.

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The first Leyden patient identified had a T to A transversion at the −20 position13 and, subsequently, patients with point mutations at −21, −5, −6, +6, +8, and +13 have been identified.17 It has been suggested that these mutations disrupt the binding of essential trans-acting DNA regulatory proteins, thereby decreasing FIX mRNA synthesis.14,15 This hypothesis is strengthened by clinical data showing that the severity of the Leyden phenotype can vary depending on the type of mutation (transversion or transition) at the same location in the promoter.16 Five sequence-specific DNA binding proteins have been identified that bind to elements within the FIX promoter segment in vitro. These proteins include CAAT enhancer binding protein (C/EBPα),14 liver nuclear factor 1 (NF-1L),14 hepatocyte nuclear factor 4 (HNF-4),17 androgen receptor (AR),18 and D-site binding protein (DBP).19 The +8 and +13 Leyden mutations appear to disrupt the binding of C/EBPα,6,14 whereas the −20 and −26 mutations disrupt HNF-4 binding to human promoter fragments in vitro.17,18 In addition to these disruptions, the synergy between DBP and C/EBPα has been shown to compensate for the −5 mutation.19

The postpubertal increase in FIX activity in male Leyden patients has been suggested to be caused by androgens. This idea was supported by the observation that danazol, an androgen derivative, stimulates FIX activity in pubescent Leyden male patients.20 Furthermore, afflicted individuals with another form of hemophilia B, hemophilia B Brandenburg (caused by a −26 mutation) do not exhibit postpubertal increases in FIX activity.21 This mutation has been shown to disrupt an HNF-4 binding site as well as that of an overlapping androgen-responsive element (ARE).21 The identification of ARE within the FIX promoter directly links androgens with the upregulation of the FIX gene in Leyden patients. However, testosterone22 and androgen receptor mRNA23 levels reach a maximum shortly after puberty, whereas FIX levels in Leyden patients continue to increase; hence it seems that the androgen stimulus cannot be the sole factor responsible for the age-specific increase in FIX activity seen in Leyden patients. Rather, it suggests that another age-dependent regulatory protein(s) must be involved in the regulation of the FIX Leyden gene. Thus, the mechanism of age-specific recovery in Leyden patients still remains elusive.

Therefore, to investigate, in vivo, the age-specific expression of the FIX Leyden promoter, we have prepared two separate fusion genes by linking either a normal −189 to +21 bp human FIX promoter or the same promoter containing the +13 Leyden mutation to a chloramphenicol acetyltransferase (CAT) reporter gene (Fig 1A). Subsequently,
we have introduced these chimeric genes separately into transgenic mouse lines and have analyzed the age-dependent expression of the normal and the Leyden promoters. We have also analyzed the expression of endogenous mouse FIX gene during aging. We report here the demonstration of the age-related FIX Leyden pattern of expression in a transgenic mouse model.

MATERIALS AND METHODS

Transgenic animals. We chose to use the −189 to +21 bp fragment of the human FIX promoter in the preparation of our fusion gene constructs. This selection was based on the demonstration by Crossley and Brownlee14 and Salier et al.15 of essentially comparable FIX promoter activity whether roughly 2,300 or 200 bp of 5′ flanking DNA was incorporated into fusion genes, which were then expressed in HepG2 cells by transient transfections. Two chimeric genes were constructed (in pCAT-O vector16) that consisted of the human FIX promoter segment extending from bp −189 to +212 (equivalent to the shorter promoter of the earlier studies) ligated to the CAT reporter gene and SV40 polyadenylation site (Fig A and B). The first chimeric gene contained the normal promoter sequence and was constructed in our laboratory; the second contained the same promoter sequence except for the +13 mutation (transversion; A to G) present in the FIX Leyden transgene is labeled.

Materials and Methods

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C57Bl/6 fertilized eggs were microinjected with the appropriate transgene according to standard procedures,26 and the injected embryos were transferred to pseudopregnant B6D2/F1 females on the day of microinjection. Offspring were weaned at approximately 28 days and anesthetized to remove a 1-cm section of tail for DNA isolation and genomic DNA was analyzed by Southern blotting.27 DNA samples were also subjected to polymerase chain reaction (PCR) analysis using a human FIX promoter-specific 5′ primer −10TGCCTCAAGAGGCCATTTGGA91 (coding strand sequence)18 and a cat gene-specific 3′ primer +5GCGAAGTTTCTCAGGAGCTAAGGAGGATGCTGCAAC+21 (complementary strand sequence; Fig 1B). Animals whose genomic DNA tested positive by both analyses were preserved and bred with normal C57Bl/6 to establish the transgenic lines.

The copy number of the transgenes was determined by Southern blot analysis27 using genomic DNA from transgenic founder mice and several transgenic progeny in each transgenic line. Approximately 10 μg of each genomic DNA was digested with PvuII and electrophoresed in 0.8% agarose gels. Transgene copy controls were prepared based on the number of mouse cells represented in each transgenic founder line. Approximately 10 μg of genomic DNA such that 1, 5, 10, and 50 copies per cell were represented as appropriate. Transgene copy control DNA was combined with 10 μg of negative control mouse DNA and digested with PvuII. Standard procedures were followed in preparing DNA for transfer to Nytran membrane. Prehybridization and hybridization were performed using a 32P-labeled nick-translated full-length transgene as a probe. After washing, the membrane was used to expose x-ray film with intensifying screens. Hybridizing bands were also counted in a betascope (Betagen, Waltham, MA) before stripping and probing a second time with the murine thyroid-stimulating hormone β-subunit cDNA (TSHβ). Counts from the hybridizing bands detected with the TSHβ probe were used to correct for differences in loading and/or transfer when calculating the number of transgene copies.

PCR protocol. Standard PCR was performed in 25 μL reaction volume containing 50 to 100 ng of template DNA, 10 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.1% Triton X-100, 0.8 μmol/L of each primer, 0.2 mmol/L of each dNTP, and 1 U of Taq DNA polymerase (Promega, Madison, WI). The DNA was denatured for 1 minute at 95°C followed by a 35-cycle reaction (94°C for 30 seconds, 54°C for 20 seconds, and 72°C for 30 seconds)
and a final elongation at 72°C for 2 minutes. The reaction sample was resolved on a 5% polyacrylamide gel. If a particular band was to be analyzed, it was excised, eluted, and purified by column chromatography (NAC 52’s) using the manufacturer’s protocol.

RNA isolation. Total cellular RNA was isolated from fresh or frozen tissue with TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) using the manufacturer’s protocol. The RNA was rehydrated in 100 μL of diethyl pyrocarbonate (DEPC)-treated distilled water, the concentration was determined spectrophotometrically, and the integrity was determined by agarose gel electrophoresis.

Reverse transcription and PCR (RT-PCR). RT-PCR was performed using a modification of a one tube protocol. The initial annealing of the primers to the RNA was in 9 μL of distilled water containing 1 μg of total RNA and 2 μmol/L mouse FIX 3’ primer 1560GACAAATTTTCCAATGCGC1775 (complementary strand sequence) and 2 μmol/L aldolase 3’ primer 666TTATGC- CAGTATCTGCCAGCAAT770 (complementary strand sequence; Genbank accession no. M11560), followed by incubation at 65°C for 15 minutes and then at 4°C for 5 minutes. Sixteen units of avian myeloblastosis virus (AMV) reverse transcriptase (Promega) and 40 U of RNAsin (Promega) were added to the hybridization mixture; the volume was adjusted to 20 μL with buffer containing 50 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 10 mmol/L MgCl2, 10 mmol/L diethiothreitol (DTT), 0.5 mmol/L spermidine, and 0.5 mmol/L of each dNTP; and the solution was incubated at 42°C for 1 hour. The FIX 5’ primer 773AATGGATTGTAACTGCC779 (coding strand sequence) and the aldolase 5’ primer 779TACCTCT- CTTCCATGAGACATCTCA81 (coding strand sequence; Genbank accession no. M11560) were labeled in the same tube with 2 μL of T4 kinase (BRL) in the presence of 20 μCi [γ-32P]ATP at a volume of 5 μL containing 70 mmol/L Tris-HCl, pH 7.6, 10 mmol/L MgCl2, 5 mmol/L DTT, and 1 μmol/L of each primer for 1 hour at 37°C.

For the PCR reaction, the above 32P-labeled 5’ primers were added to the reverse transcription mixture and brought to a total volume of 100 μL. The final concentration of the various components of the reaction was 20 mmol/L Tris-HCl, pH 8.4, 60 mmol/L KCl, 2.5 mmol/L MgCl2, 2 mmol/L DTT, 0.2 mmol/L of each dNTP, 0.2 μmol/L of each primer, 0.1% Triton X-100, and 2 U of Taq DNA polymerase (Promega). The solution was denatured for 1 minute at 95°C and then an 18-cycle PCR reaction was performed (95°C for 30 seconds, 55°C for 20 seconds, and 72°C for 30 seconds). Identical aliquots (15 μL) of amplified products were resolved on a 5% polyacrylamide gel and dried and the incorporated radioactivity was determined by Betascope analysis (Betagen). Background was subtracted with the Betascope.

Analysis of transgene expression. Approximately 0.05 g of frozen tissue was placed in 200 μL of ice-cold Tris-HCl, pH 7.8, in a 2-mL Eppendorf tube and homogenized with two 15-second bursts by a Brinkman homogenizer (Brinkman Instruments Inc, Westbury, NY) at setting 6. The homogenate was freeze-thawed three times and cellular debris was pelleted at 12,000g for 10 minutes at 4°C. The supernatant was incubated at 65°C for 5 minutes and spun at 12,000g for 10 minutes at 4°C. The protein concentration of the resulting supernatant was determined using the Bradford assay, and the extract was stored at −80°C until use.

Twenty micrograms of crude extract were incubated for 60 minutes at 37°C in 150 μL of total volume containing 0.25 μmol/L Tris-HCl, pH 7.5, 530 μmol/L acetyl CoA, and 0.25 μCi of [3H]-labeled chloramphenicol (56 mCi/mmol; Amersham, Arlington Heights, IL). A second aliquot of acetyl CoA was added and the sample incubated for a second 60-minute period. The resulting mixture was then extracted with 1 mL of ethyl acetate, lyophilized until dry, and then dissolved in 35 μL of ethyl acetate. The samples were spotted onto a 20 × 20 cm silica gel TLC plate (Sigma, St Louis, MO) and run to the top with a 95% chloroform/5% methanol solution. The plate was dried and exposed to Kodak XAR autoradiography film (Eastman Kodak, Rochester, NY) at −80°C. Quantitative data were obtained by subsequent analysis of the radiochromatogram incorporated into the spots corresponding to the acetylated products of chloramphenicol with the Betascope.

RESULTS

To investigate whether murine FIX mRNA levels increased during aging, we measured relative FIX mRNA levels using RT-PCR in 2-, 6-, 8-, 20-, and 28-month-old mice total liver RNA. Aldolase, a medium to low abundant housekeeping mRNA, showed no significant change with age and was used as an internal standard in RT-PCR analysis. To show that the RT-PCR reactions are quantitative, we have performed the RT-PCR reactions with increasing numbers of PCR cycles and also with increasing quantities of total RNA.

**Fig 2.** Measurement of murine FIX mRNA and activity levels with age. FIX and aldolase are shown by arrows. (A) FIX. (B) Aldolase; (C) FIX. (A) Optimization of number of PCR cycles in RT-PCR reactions. One microgram total liver RNA was amplified by RT-PCR using an end-labeled primer at an increasing number of cycles as indicated. After gel electrophoresis, the radioactivity in appropriate bands was counted. The CPM were plotted against the increasing number of PCR cycles. (B) Optimization of RNA concentration in RT-PCR reactions. RT-PCR reactions were performed at 18 cycles with increasing concentrations of RNA and the bands were analyzed as above. The CPM were plotted against increasing concentration of total liver RNA. (C) Representative RT-PCR assay on total liver RNA from C57Bl/6 mice of various ages. (D) Cumulative data from four experiments. The ratio of CPM of FIX and aldolase bands (Mean Ratio) was plotted against different ages of mice. Error bars indicate the standard deviations (n = 10). The mean ratios of 6- and 8-month-old animals were significantly greater than those for the 2-month-old animals (P < .05). In 20- and 28-month-old animals, these ratios were also significantly greater than those for the 2-month-old animals (P < .001). (E) FIX activity during aging. FIX activity of 28-month-old animals was significantly greater than that for the 2-month-old animals (P < .001). Error bars indicate the standard deviations (n = 4).
The counts per minute (CPM) from the FIX and aldolase bands were plotted against the number of cycles (Fig 2A) as well as against the concentration of total RNA (Fig 2B). No nonspecific amplifications were observed under these conditions. This range was in agreement with the earlier report on RT-PCR analysis of aldolase mRNA.24
We have used the above optimal conditions in our analysis of relative FIX mRNA levels. The ratio of FIX mRNA/aldolase mRNA was measured in various ages of both male and female mice. A representative gel separation of RT-PCR products derived from liver RNA that was obtained from various ages of male mice is given in Fig 2C.

A moderate but significant increase in relative FIX mRNA level between mice (of both sexes) of 2, 6, 8, 20, and 28 months of age was shown using the RT-PCR procedure. There was no significant difference in FIX mRNA levels between male and female mice of same age (data not shown). Figure 2D shows the cumulative data on relative FIX mRNA levels from both male and female mice of various ages. Because of the low levels of endogenous FIX mRNA, it has been difficult to use nuclear run-on assays to determine if there are age-related changes in the production of newly transcribed FIX mRNA. Hence, we were unable to definitively show that the increase in FIX mRNA levels is due to an increase in FIX mRNA synthesis at the transcriptional level.

To determine if the increase in FIX mRNA levels that is observed between 2- and 28-month-old mice is physiologically relevant, we have used the one-stage clotting assay to measure FIX activity in 2- and 28-month-old mice of both sexes. Two-month-old male mice showed only 50% to 60% of the FIX activity measured in 28-month-old male mice (Fig 2E). Female mice also showed similar FIX activity levels as a function of age (data not shown).

Because aged C57BL/6 mice, like humans, show increased levels of FIX activity with age, we wished to determine if the expression of a chimeric construct driven either by the normal human FIX promoter or the mutant Leyden promoter would also manifest age-related changes in the transgenic mouse model. Five transgenic lines were generated that carried the chimeric construct consisting of the −189 to +21 region of the normal human FIX promoter linked to a CAT reporter. Two transgenic lines were generated that carried the same −189 to +21 region of the human FIX promoter but with the Leyden mutation at +13 position. Three of the founder lines bearing the human normal FIX promoter and one of the lines with the Leyden promoter did not breed or died before the generation of viable offspring. Analysis of the two remaining normal lines, designated N71 and N4III, and the +13 mutant line, designated N3II, generated the data outlined in the next paragraph. Southern and PCR analysis showed that N4III had incorporated 15 to 20 copies of the transgene into its genome, N71 had incorporated 70 to 80 copies, and N3II had incorporated 5 to 15 copies.

The transgene expression was analyzed by measuring CAT activity in various tissues. A representative CAT activity analysis from N71 animal is shown in Fig 3A. The expression was liver-specific regardless of whether the transgenic lines were expressing CAT activity driven by the human normal FIX promoter or the Leyden promoter (Fig 3B). The expression of the human FIX promoter’s activity was also evaluated throughout the life span of the mouse by analysis of CAT activity in liver extracts from mice of various ages. In the transgenic mice carrying the human normal FIX promoter, no difference in the level of CAT activity was detected in liver extracts (Fig 3B) regardless of age, sex, or founder line. In contrast, liver-specific expression of the Leyden promoter-regulated transgene was detected only in 6- and 8-month-old male mice (Fig 3B). Liver extracts from 2- and 3-month-old male mice and from 2- and 8-month-old female mice did not display CAT activity. The CAT activity in the liver extracts of 8-month-old Leyden promoter-bearing male mice was approximately 50% to 60% of that observed in equal amounts of liver extracts from mice carrying normal FIX promoter of the same age. The liver extracts from 6-month-old animals showed approximately 25% to 30% of the CAT activity observed in normal FIX promoter carrying mice. We have also analyzed 2 animals (18-month-old male and female mice) of the Leyden promoter containing line that did not breed (copy number of transgene unknown). Only the male mouse showed CAT activity (data not shown) comparable to the 8-month-old N3II male mice, but the female mouse did not show any detectable CAT activity. Because this line did not breed, we could not obtain statistically meaningful data. However, the lack of expression in the female mouse was similar to the data collected from N3II female mice.

**DISCUSSION**

Our data show that FIX mRNA levels and FIX activity increase with increasing age in mice of both sexes. In addition, our results provide the first demonstration that the age-related increase in FIX activity in mice age can be correlated with a corresponding increase in the level of FIX mRNA. These observations are consistent with the previous demonstration of an increase in FIX activity in humans with aging. The physiologic significance of this age-related increase in FIX activity is unknown. The developmental expression of murine FIX mRNA begins at day 4 of gestation but is low (<10% of that found in 2-month-old animals) until just before parturition, at which time it increases dramatically to 40% to 50% of the adult level. This increase continues through 2 months of age, when FIX mRNA levels are reported to stabilize. However, until now, neither FIX activity nor FIX mRNA levels have been examined after 2 months of age in mice. Our data complement the work of Yao et al and provide a complete description of FIX mRNA levels throughout the life span of the mouse. One clear observation, based on our FIX data and the developmental data of Yao et al, is that expression of the murine FIX gene throughout the relative life span of mouse is comparable to that of the human. This finding suggests that the murine hepatocyte may serve as a model for the human system to study the regulation of the FIX gene in vivo.

Our results provide the first demonstration that the −189 to +21 bp segment of the normal human FIX gene promoter and the homologous Leyden promoter are capable of directing the tissue-specific expression of a CAT reporter gene in transgenic mice. Because transgenic lines with different copy numbers of normal human FIX gene promoter have similar expression levels, low levels of expression of the Leyden promoter are probably not due to low copy number but may reflect the mutant promoter. Earlier studies with transgenic animals expressing an FIX transgene have shown that a 5-
Fig 3. Transgene expression in transgenic mice. (A) Representative CAT assay of cytoplasmic extracts from various tissues of an N71 line female. The positive control is a liver extract of a transgenic mouse expressing a chimeric construct in which human transferrin promoter is driving the CAT reporter gene and the negative control is a liver extract of a nontransgenic C57Bl/6 mouse. (B) Quantitative analysis of CAT expression with age in the transgenic mouse lines. Relative CAT activity is calculated with reference to 2-month-old male N71 line mice. Each error calculation is the standard deviation of activity from 4 mice. (□) Males and (■) females. N71, N4111, and N311 are the founder transgenic lines. The numbers above the founder lines indicate the age of mice in months.

kb segment of the FIX promoter could regulate the liverspecific expression of an FIX cDNA. Our data show that a much smaller segment of the FIX promoter is capable of directing liver-specific transcriptional activity in transgenic mice.

The normal FIX promoter-driven transgene did not exhibit any sex- or age-related differences in its expression pattern. Although equal expression of FIX in both male and female mice is consistent with previous clinical data, some increases in expression of the normal promoter during aging might be expected based on clinical data and on our own observations (see above). However, no significant changes in the expression of the normal FIX promoter-driven transgene were detectable with aging. On the other hand, the Leyden promoter-driven transgene was expressed in a distinct age- and sex-specific pattern that closely parallels that observed in the human Leyden phenotype. In the human Leyden male patients, FIX activity is between 10% and 20% of normal at 20 years of age and is even lower at puberty. Interestingly, however, FIX activity in these individuals shows a progressive increase with age to reach 50% to 60% of normal in the fifth decade of life. Considering the relative life span of the mouse and human, 2-month-old mice are probably equivalent to 20-year-old individuals and 8-month-old mice...
are probably equivalent to 50-year-old individuals. In the transgenic animals that carried the fusion gene with the Leyden promoter, we were unable to detect CAT activity at 2 months of age. However, 8-month-old mice presented a clearly demonstrable increase in CAT activity. This is the first demonstration that the +13 mutation in the −189 to +21 bp FIX promoter segment is capable of generating the Leyden phenotype in a male transgenic mouse model. The failure of female transgenic mice that carry the Leyden promoter to express CAT activity at any age suggests that a male-specific factor may be necessary for the expression of the Leyden phenotype. It also suggests that human females who are carriers for FIX Leyden should have 50% of normal levels throughout life, but this possibility remains to be investigated. Our data are consistent with the previous suggestion of an important role of androgens in the presentation of Leyden phenotype. On the other hand, although puberty in mice occurs at or near 1 month of age and although androgen receptor mRNA and hormone levels have stabilized in 2-month-old male mice, the Leyden promoter does not appear to be activated in transgenic mice of this age. Hence, although androgens are apparently essential for the expression of the Leyden phenotype, these steroids do not appear to be sufficient, by themselves, to produce significant promoter activity in transgenic males. From our transgenic data, it appears that an additional factor(s) whose expression or activation commences some time after puberty is also required for mediating the age-related increase in the expression of the Leyden promoter.

It has been recently shown that DBP, a liver-specific DNA-binding protein with a postpubertal expression, may be involved in the regulation of the FIX gene through a specific synergistic interaction with C/EBPα at a binding site −219/−202. It has also been shown that DBP binds weakly to the +13 mutation site and that the complex of C/EBPα and DBP binds even better to this site. It is interesting that our +13 mutant promoter does not contain the major 202−219 binding site and still shows age-dependent expression. If the age-dependent increase is in part due to DBP or a complex of C/EBPα and DBP binding to the C/EBPα site, it probably participates in the complex protein-protein interactions.

In conclusion, our data have shown that the levels of FIX mRNA in mice increase with age. Furthermore, the mouse model regulates a −189 to +21 bp FIX promoter segment from normal and Leyden FIX genes in a manner analogous to that observed in the human.

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